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OLIGODENDROCYTE RESPONSE TO IONIZING RADIATION: AN *IN VITRO* MODEL OF CELLULAR AND FUNCTIONAL RESPONSE[1]

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Running title: Oligodendrocyte irradiation *in vitro*

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Oligodendrocyte response to ionizing radiation: An *in vitro* model of cellular and functional response

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Abstract

The cellular and functional response of oligodendrocytes to single-dose ^{60}Co γ-irradiation at day-in-culture (DIC) 8 was examined in primary glial-cell cultures derived from neonatal rat brain. Oligodendrocytes were quantified by grid-counting with phase contrast microscopy, and their functional capacity was determined by quantitative radioimmunoassay of myelin basic protein (MBP) levels in membrane extract preparations. Oligodendrocyte counts at DIC 14 were 65% and 29% of age-matched control values after irradiation with 2 Gy and 5 Gy, respectively. At DIC 21, counts recovered to near-normal levels in the 2-Gy group and to 75% of normal in the 5-Gy group. Counts at DIC 14 were significantly lower at each successive 0.5-Gy dose increment through 2 Gy. MBP levels at DIC 14 were 60% and 40% of age-matched control values after irradiation with 2 Gy and 5 Gy, respectively. Corresponding MBP levels at DIC 21, however, were only 50% and 25%, respectively, of control values. The temporal patterns of cellular and functional depletion and recovery *in vitro* have parallels to patterns found *in vivo*. This experimental approach may help elucidate regulatory mechanisms of oligodendrocyte proliferation and differentiation required to respond to radiation injury.

Running title: Oligodendrocyte irradiation *in vitro*

Keyword phrases: oligodendrocyte irradiation *in vitro*, brain cell culture, model of CNS radiation injury

1. Introduction

Mammalian brain tissue *in vivo* is highly structured with a complex cellular hierarchy, and it exhibits only limited proliferative potential (McKhann 1978) [19]. The profound heterogeneity and integration of hierarchical structure and function in the brain make it very difficult to investigate biochemical and metabolic mechanisms in defined cell types in intact tissue. The problem of cellular heterogeneity is exacerbated that much more when the brain has been subject to pathologic alterations. Brain tissue isolated and maintained *in vitro* represents a useful system for evaluation of biochemical and metabolic properties of the various brain cell populations and their interactions under relatively simple and controlled conditions. Some of the important structural and functional attributes of brain cells are well maintained in culture (Levy 1991, McKhann 1978, Raff *et al.* 1978) [14,19,28]. Oligodendrocytes, for example, differentiate and mature *in vitro*, their morphologic and immunocytochemical properties correspond well to the *in vivo* situation, and they retain much of their biochemical specificity, including the synthesis of large quantities of myelin membrane.

Cell culture techniques have been widely used to evaluate radiation response in rapidly proliferating cells. Quantitative cell-survival analysis has been developed toward this end to study clonogen survival in selected transformed cell lines typically derived from malignant mammalian tissue (Elkind and Whitmore 1967, Puck and Marcus 1955) [10,26]. The radiation-response of glioma-derived cell clones, for example, has been studied extensively (Dwarkanath and Jain 1989, Gerweck *et al.* 1977) [9,11]. The metabolic properties of transformed cerebral cells growing *in vitro*, however, can not be extrapolated readily to normal cerebral cells. Transformed cells generally lack the distinguishing metabolic characteristics of normal differentiated cells, and they typically manifest a markedly different response to irradiation (Gerweck *et al.* 1977) [11]. A satisfactory *in vitro* model of brain irradiation, therefore, can not rely solely on standard cell culture techniques.

In this report, the cellular and functional response of the oligodendrocyte population to ^{60}Co γ-irradiation is examined in normal mammalian brain *in vitro*. The oligodendrocyte has been chosen for study because of its central role in delayed radiation injury

in the brain (Hopewell 1979, van der Kogel 1986) [12,33]. A new experimental approach is described for quantitative evaluation of dose-response to irradiation using primary disaggregated neuroglial cultures.

2. Materials and methods

2.1 *Cell culture procedures*

The method of preparation of primary mixed brain cell cultures was developed as a modification of the method of McCarthy and de Vellis (1980) [18]. The research protocol and euthanasia technique were approved by the Animal Welfare and Research Committee at Lawrence Berkeley Laboratory. All chemicals were obtained from Sigma Chemical Co. (St. Louis, MO) unless otherwise stated. Using aseptic technique, brains were removed from one-day-old Sprague Dawley rats after scissors decapitation. The cortices were dissected out, and the meninges were gently removed. The cortices were then placed in nutrient medium (1:1 Hams F-12 (Gibco, Grand Island, NY): Dulbecco's Modified Eagle's Medium (Gibco), with 10% fetal calf serum, 15 mM Hepes buffer, and 1.2 g/l sodium bicarbonate) and mechanically dissociated into single-cell suspensions via sequential filtration through a series of nylon monofilament and stainless steel screens. Fetal calf serum was added to the final cellular filtrate to a concentration of 20% prior to centrifugation at 800 rpm for 5 min. The cellular pellet was then resuspended in fresh medium, and the cells were plated in 25 cm^2 plastic culture flasks (Falcon, Becton-Dickinson Labware, Lincoln Park, NJ) at a concentration of about 1.5×10^6 cells per 1.0 ml medium (3.5 ml cell suspension per flask). The cultures were maintained at 37° C in a water-jacketed incubator at 5% CO_2 in humidified air. The first media change was on day-in-culture (DIC) 5; subsequent media changes took place on DIC 7, 9, 12, etc. (i.e., three times weekly).

2.2 *Irradiation*

Cultures were irradiated with ^{60}Co γ-rays at DIC 8 using a 5.5×10^7 MBq Gamma-beam Unit (Atomic Energy of Canada Limited, Ottawa, Canada). A dose rate of 0.5 Gy/min was present at 1.0 m from the point source. Cells were irradiated through 5-mm thick lucite filters, which were attached to the culture-flask surface, with flasks

held in the upright position. Cultures were maintained at 37° C during and following irradiation. In all experiments, randomly selected control cultures were sham-irradiated.

2.3 *Oligodendrocyte counting*

The oligodendrocyte population was quantified at selected DIC using phase contrast microscopy at ×400 magnification. Characteristic phase-dark cells were counted in a 1 mm × 1 mm square grid in ten randomly positioned flask regions. The highest and lowest counts were ignored, and the mean value of the remaining eight regions (termed the *oligodendrocyte count*) was calculated and used as a quantitative index of the oligodendrocyte population.

2.4 *Myelin basic protein assay*

Myelin basic protein (MBP) levels were used as a quantitative index of myelin synthesis (Cohen and Guarnieri 1976) [8]. Since quantitative MBP assay requires sacrificing the cultures, it was not possible to obtain serial data on individual cultures. These data were, therefore, accumulated by random sampling of groups of primary culture flasks at periodic intervals following the initial plating. Membrane extracts for assay were prepared by gently washing cell cultures four times with phosphate buffered saline at 4° C, and then bathing them in 0.02 M Tris-acetate buffer (4° C) at pH 7.2 containing 0.02 M NaCl, 0.1% octyl phenoxy polyethoxyethanol (Triton X-100) and 0.1% aprotinin. A plastic cell scraper was used to dislodge the cells from the flask surface and into suspension in the Tris buffer; the suspension was sonicated, incubated overnight at 4° C, and then centrifuged at 1,500 g for 25 min at 4° C. Aliquots of the supernatant were stored at -15° C for later MBP assay with a commercially available radioimmunoassay kit (Diagnostics Systems Laboratory, Webster, TX) and a Packard 5000 Gamma Counter (Packard Instrument Co., Inc., Downers Grove, IL). A curve-fitting logistic computer program was developed to calculate MBP concentrations.

2.5 *Total cellular protein assay*

Total cellular protein levels were used as a quantitative index of the astrocyte population (Levy 1991) [14]. Aliquots of the supernatant prepared as described for MBP

assay (see Section 2.4, above) were assayed for total protein content, using an AS-TRA spectrophotometric (545 nm) analyzer (Beckman Instruments, Fullerton, CA) calibrated for cerebrospinal fluid.

2.6 *Statistical evaluation*

Multiple groups within an experiment were compared statistically with each other using analysis of variance (Fisher's protected least significant difference test) (Winer 1971) [37]; significance was evaluated at the 95% level. The Kruskal-Wallis mean rank and Mann-Whitney tests were used for non-parametric analysis of groups consisting of ratios of two random variables (Winer 1971) [37]; here, the standard deviation was taken as the square root of the variance of ratios (Armitage and Berry 1987) [2].

3. Results

3.1 *Unirradiated control cultures*

A mixed neuroglial cell primary culture was obtained, which was readily accessible to morphologic and immunocytochemical evaluation; viable neurons do not survive this method of preparation (McCarthy and de Vellis 1980) [18]. The cells attach to the culture-flask surface and gradually assume a stratified arrangement. Relatively amorphous phase-light astrocytes proliferate to form a confluent bed-layer over the entire culture dish surface during the first 7 to 10 DIC. Interconnecting networks of distinct and morphologically characteristic phase-dark oligodendrocytes with multiple ramifying processes are seen above and loosely adherent to the underlying astrocyte layer. The morphologic criteria for oligodendrocyte identification correlate very well with immunocytochemical criteria (data not shown); for example, the oligodendrocytes exhibit highly specific binding to both anti-galactocerebroside and anti-MBP (Levy 1991, Raff *et al.* 1978) [14,28].

3.1.1 *Oligodendrocyte counts.* In unirradiated cultures, the oligodendrocyte population increased sharply between DIC 7 and DIC 14 and reached a plateau at about DIC 14 or shortly thereafter (Figure 1, upper panel). The mild fluctuations in oligodendrocyte counts seen in the plateau region of the curve illustrate the uncertainty inherent in random sampling of an intrinsically heterogeneous plated cell population.

3.1.2 *Myelin basic protein levels.* The pooled results of six separate experiments with comparable initial plating conditions indicated that the concentration of MBP in unirradiated control cultures increased very rapidly with DIC (Figure 1, middle panel). Mean MBP values, determined by random sampling of groups of primary culture flasks at weekly intervals, increased from 8 ng/ml at DIC 7 to 281 ng/ml at DIC 28.

3.1.3 *Total cellular protein levels.* The pattern of total cellular protein synthesis was also determined by random sampling of groups of primary culture flasks at weekly intervals. The results of six separate experiments with comparable initial plating conditions were pooled (Figure 1, lower panel). Mean total protein values increased from 148 μg/ml at DIC 7 to 612 μg/ml at DIC 28.

3.2 *Irradiated cultures: dose response*

A series of experiments was designed to evaluate the dose response of oligodendrocytes at DIC 14 and DIC 21 (i.e., 6 days and 13 days, respectively, after irradiation).

3.2.1 *Oligodendrocyte counts.* At DIC 14, mean oligodendrocyte counts following irradiation with 2 Gy and 5 Gy were found to be 65% and 29%, respectively, of unirradiated-control values (Figure 2). When analogous experiments were examined at DIC 21, it was apparent that there had been considerable recovery of oligodendrocyte counts from DIC 14; in a series of studies, mean counts at DIC 21 ranged from 83% to 110% of control values in cultures irradiated to 2 Gy and from 63% to 92% of control values in cultures irradiated to 5 Gy (Figure 3).

To evaluate the sensitivity of the cell-culture system to lower doses, oligodendrocyte response at DIC 14 was also examined in a single experiment consisting of unirradiated controls and groups of cultures irradiated to doses of 0.5 Gy, 1.0 Gy, 1.5 Gy and 2.0 Gy. Mean oligodendrocyte counts were significantly (95% level) lower at each successive 0.5-Gy dose increment (Figure 4).

3.2.2 *Myelin basic protein levels.* At DIC 14, MBP levels following irradiation with 2 Gy and 5 Gy were approximately 60% and 40%, respectively, of unirradiated-control values. With an additional 7 days in culture, absolute MBP levels in the 2-Gy group

increased about 45%, but were still only 50% of age-matched control values (Figure 5). Absolute MBP levels in the 5-Gy group were unchanged during this same interval, and were only 25% of age-matched control values at DIC 21.

3.2.3 *Total cellular protein levels.* At DIC 14, mean total cellular protein levels after 2 to 5 Gy were about 60% to 70% of age-matched control values in all cases, but statistical significance was reached in only one of three experiments performed, due to large variance of data. By DIC 21, mean total protein levels, even in 5-Gy irradiated cultures, had recovered to more than 90% of control values (Figure 6).

3.2.4 *Myelin synthesis index.* The *myelin synthesis index* (defined as MBP per oligodendrocyte) was determined in three separate experiments for each irradiated and control culture at DIC 21 (Figure 7). Irradiated oligodendrocytes had significantly lower mean MBP-synthetic functional capacity than did unirradiated oligodendrocytes. The indices at a given dose were quite consistent from one experiment to the next and appeared to be relatively independent of initial plating conditions.

4. Discussion

Various target-cell models have been proposed to provide a framework for investigating the cellular bases of delayed radiation injury and repair in the brain, to explain the associated spectrum of observed neurophysiologic perturbations, and to integrate the subcellular and tissue-level models of radiation response (Calvo *et al.* 1988, Hopewell 1979, Myers *et al.* 1986, van der Kogel 1986) [6,12,22,33]. Oligodendrocytes and cerebral endothelial cells are generally considered to be the target cells involved in mediating delayed radiation injury, although the relative contribution and interaction of these two cell populations under various conditions is not fully understood. The function of the oligodendrocyte is to synthesize and maintain the insulating membrane that ensheaths myelinated neuronal axons, thereby facilitating nerve impulse conduction in the central nervous system (McKhann 1978) [19]. The primary goal of the research described in this report has been to investigate the radiation-response of the oligodendrocyte population *in vitro*, free of the potential influence or contribution of the vascular system.

4.1 *Unirradiated cultures*

4.1.1 *Oligodendrocyte line.* The first stage in this study was to characterize the experimental system in unirradiated glial cell cultures. The most significant finding was the relationship between the period of increase in oligodendrocyte number and the temporal pattern of MBP synthesis. MBP is highly specific to the myelin membrane, and immunocytochemical measurement of MBP *in vivo* correlates very well with synthesis of mature myelin (Cohen and Guarnieri 1976) [8]. In this investigation, measured levels of MBP in culture remained relatively low throughout the period of oligodendrocyte population increase (DIC 7 to DIC 14). This period was followed by a phase of rapid oligodendrocyte differentiation, associated with a nearly exponential rate of increase in MBP levels (35-fold from DIC 7 to DIC 28) and corresponding well to rates of myelin synthesis found *in vivo* at comparable cellular age (Norton 1981) [23].

The observed sequence of events appears likely to have resulted from proliferation and differentiation of previously committed, but morphologically indistinct and unrecognized, precursor cells. An alternate hypothesis, that proliferation involves oligodendrocytes already possessing characteristic morphologic and immunocytochemical properties, has not been supported by tritiated-thymidine-incorporation studies carried out with comparably aged cultures (data not shown) (Levy 1991, Meir and Schachner 1982, Szuchet *et al.* 1980) [14,21,31].

The regulatory factors controlling population growth, differentiation and maturation in oligodendrocytes and their precursors are not well understood. Several studies suggest that soluble extra-oligodendroglial factors *in vitro* (Pettmann *et al.* 1980, Saneto and de Vellis 1985, Wood *et al.* 1980) [25,29,38] and surgical brain trauma (Ludwin 1985) [16] have stimulating and/or mitogenic effects. Conversely, reversible inhibition of oligodendrocyte-progenitor differentiation has been demonstrated *in vitro* by a monoclonal antibody against cell-surface galactolipids (Bansal and Pfeiffer 1989) [3]. Soluble oligodendrocyte-specific end-products (e.g., selected myelin components and enzymes) may also exert local inhibitory control *in vitro*; in cultures damaged by cytotoxic agents (including irradiation), inhibitor levels may drop below that required

to suppress the progenitor-cell population (Bhat and Pfeiffer 1986, Bologa *et al.* 1988, McMorris and Dubois-Dalcq 1988, Pettmann *et al.* 1980) [4,5,20,25].

The existence of pluripotential glial progenitor cells has been established *in vitro* by studies demonstrating that O-2A progenitor cells isolated from perinatal rat optic nerve differentiate, depending on the culture medium, into oligodendrocytes or type-2 astrocytes (Raff *et al.* 1983, van der Maazen *et al.* 1990) [27,36]. *In vivo*, the subependymal cell layer in rodents represents an anatomically-defined stem-cell compartment with self-renewal properties (Korr *et al.* 1973, Manley 1988, Paterson *et al.* 1973, van der Kogel 1983) [13,17,24,32]. About half of newly formed glial cells in the subependymal layer leave the growth fraction, but some nonproliferating cells re-enter the growth fraction and begin to proliferate; these findings support the contention that differentiated glial cells retain the capability to revert to a proliferative state and that an increased proportion may do so when cells are lost after a cytotoxic insult (Korr *et al.* 1973, Ludwin and Johnson 1979, Manley 1988, Schultze and Korr 1981) [13,15,17,30]. The population of "morphologically-indistinct precursors cells," postulated above to explain some of the experimental findings of this investigation, may be considered to constitute a stem-cell compartment in dissociated cell culture. These cells appear to be equivalent functionally to the "free subependymal" cells reported by Paterson *et al.* (1973) [24] as migrating from the intact subependymal layer *in vivo* to the cerebral cortex at the level of the corpus callosum prior to differentiating into mature oligodendrocytes.

4.1.2 *Astrocyte line.* Total cellular protein levels, though not specific for a given cell type, can be used as an index of cell density in general and of the astrocyte population in particular (Levy 1991) [14]. Astrocytes constitute the great majority of glial cells in primary mixed cultures at DIC 7, and preferential astrocyte proliferation thereafter results in progressively more heavily astrocyte-weighted cultures. Total protein measurements, therefore, strongly reflect the contribution of the astrocyte population.

The astrocyte population *in vitro* increases rapidly during the period from DIC 7 to DIC 14, as observed microscopically and determined indirectly by tritiated-thymidine incorporation studies (data not shown) and by changes in total cellular protein levels

(Levy 1991) [14]. Astrocyte proliferation continues until inhibited by apparently extrinsic cellular factors (e.g., contact inhibition). Although proliferation appears to cease once cellular confluence is attained, protein synthesis continues unabated at least through DIC 28. The sustained protein synthesis presumably reflects continuing metabolic function of the maturing astrocyte population rather than unrestrained proliferation. Accordingly, the total cellular protein level is viewed more appropriately as an index of composite functional activity of the astrocyte population rather than as a direct measure of cell number.

4.2 *Irradiated cultures*

Oligodendrocytes do not form discrete colonies even under optimal growth conditions. Futhermore, since oligodendrocytes typically have limited mitotic activity, some radiation-damaged cells would likely appear morphologically normal throughout the period of observation and be counted as if they were unaffected. Oligodendrocyte response, therefore, cannot be described adequately by cell-survival curves of the kind typically used to describe clonogenic cells in tissue culture (Alpen 1990, Elkind and Whitmore 1967) [1,10].

4.2.1 *Cellular response.* The initial oligodendrocyte response to irradiation and the pattern of recovery were dose-dependent. At DIC 14, when oligodendrocyte counts in control cultures had reached maximal steady-state levels, counts in irradiated cultures were significantly depressed. These counts recovered considerably by DIC 21, reaching near-control levels in the 2-Gy irradiated group and about 75% of control levels in the 5-Gy irradiated group (Figure 8). Considerable uncertainties exist regarding the extent to which committed oligodendrocyte precursor cells and/or pluripotential stem cells may be present in the culture at the time of the initial plating and at various intervals thereafter and the degree to which these cells are capable of recognizing and responding to the radiation insult. The number of viable precursor cells present in culture would be expected to decrease, in a dose-dependent fashion, following irradiation, thereby compromising the replenishment of the depleted oligodendrocyte population. With increasing dose, viable precursors would be fewer and would require more time to

restore the population to pre-irradiation numbers. If the dose were high enough, the precursor population would be sterilized completely and no recovery would be possible. An alternate hypothesis is that there is more extensive cell-cycle delay in the precursor population with increasing dose.

Astrocytes in culture are actively proliferating at DIC 8, and they are correspondingly sensitive to radiation injury (Levy 1991) [14]. The surviving astrocyte population, however, can compensate well for cells lost to irradiation, as reflected by the continuously well-maintained confluence of astrocytes on the culture flask surface and by the quick recovery of total cellular protein levels toward control values (Levy 1991) [14].

4.2.2 *Functional oligodendrocyte response.* The temporal pattern of MBP production in irradiated cultures demonstrated that the recovery of the functional capacity of the oligodendrocyte population was dose-dependent, but not well correlated with changes in the oligodendrocyte counts. It appeared initially, based on data obtained at DIC 14, that MBP levels could be considered simply as an index of the number of oligodendrocytes in culture. MBP levels at DIC 14 were about 60% of control values in cultures irradiated with 2 Gy and 40% of control values in cultures irradiated with 5 Gy. These relative levels are comparable to the fractional decrements observed in the oligodendrocyte counts in cultures that were similarly irradiated. At DIC 21, however, a different relationship between MBP levels and oligodendrocyte counts began to emerge. While oligodendrocyte counts in irradiated cultures had recovered to near-control levels, corresponding MBP levels lagged far behind (Figure 9). The complex temporal patterns of dose-response observed in the oligodendrocyte population and in the synthesis of MBP led to the hypothesis that irradiated oligodendrocytes had lower mean MBP-synthetic functional capacity than did unirradiated oligodendrocytes. This hypothesis was supported by the results from the myelin synthesis index. It was concluded that the oligodendrocyte population from DIC 14 to DIC 21 in irradiated cultures was qualitatively different than the oligodendrocyte population in comparably-aged control cultures. The irradiated cultures manifested significantly lower functional capacity.

The temporal pattern of MBP response between DIC 14 and DIC 21 following irra-

diation is proposed to be the composite result of several factors. Some committed stem cells and immature oligodendrocytes undergo reproductive cell death during the days immediately following irradiation, resulting in decreased numbers of morphologically identifiable oligodendrocytes. The surviving oligodendrocytes continue to differentiate and synthesize myelin. Committed oligodendrocyte precursors and stem cells undergo active compensatory proliferation, increasing the number of mature oligodendrocytes *in vitro* with a return toward control values over a period of 2 to 3 weeks. The newly formed oligodendrocytes lag behind the surviving (or unirradiated) oligodendrocytes in their functional capacity, but these new cells mature and differentiate over the next several weeks, ultimately achieving comparable metabolic capabilities. While the experimental data at present do not demonstrate recovery of MBP synthesis to near-normal control values, 2-Gy irradiated cultures have begun to show recovery of MBP levels by DIC 21. The projected pattern of continued functional recovery shown in Figure 9 will require experimental confirmation by extending culture analysis out to DIC 28 and beyond.

4.3 *Experimental models of delayed radiation injury*

Although using a system of dissociated brain cell cultures to evaluate delayed radiation injury requires that a complex three-dimensional tissue structure be converted into a quasi-two-dimensional cellular array, the *in vitro* model described herein has many similarities and correlates to the situation *in vivo*. Oligodendrocytes *in vitro* are identified and quantified by morphologic and/or immunocytochemical criteria comparable to those applicable in tissue section (Levy 1991) [14]. The temporal patterns of cellular depression and recovery in the oligodendrocyte population *in vitro* (i.e., gradual recovery to a seemingly predetermined level) are consistent with homeostatic regulatory controls manifested *in vivo* (Ludwin and Johnson 1979) [15]; furthermore, the culture system appears to contain progenitor cells capable of differentiating and proliferating in response to changes in the regional cellular environment. The functional response (i.e., myelin levels) manifested *in vitro* also appears analogous to patterns of demyelination and remyelination found *in vivo* (Ludwin and Johnson 1979) [15]. These

factors may correspond to the eventual tissue breakdown and loss of functional end-cells found *in vivo* that arise when the radiation-damaged pool of progenitor cells can no longer compensate effectively for damage in the stem-cell and proliferative compartments (Hopewell 1979, van der Kogel 1986) [12,33].

A variety of experimental strategies have been developed to examine and characterize the brain's response to radiation injury. In general, the methods can be categorized as emphasizing either quantitative population-specific endpoints or more qualitative functional endpoints. Quantitative *in vitro* assays have been developed to examine DNA strand breaks (but without specific functional endpoints) (Cerda and Rosander 1983) [7] or to evaluate clonogenic survival in glioma-derived cells (which have limited functional similarity to normal brain cells) (Gerweck *et al.* 1977) [11]. Other quantitative assays evaluate highly selected populations of glial cell precursors, including mitotically active cells of the subependymal plate (e.g., quantitative high-resolution autoradiography for evaluation of cell cycle kinetics) (Korr *et al.* 1973, Schultze and Korr 1981) [13,30] and O-2A progenitor clones (colony-counting assay) (van der Maazen *et al.* 1990, 1991) [35,36]. Functional *in vivo* assays, which examine endpoints at the tissue level (e.g., histopathologic changes and limb paralysis), typically obscure the unique roles of specific cell types in the pathogenesis of radiation injury and recovery; these assays integrate the response of the parenchymal and vascular components (van der Kogel 1977) [34].

The experimental system described in this report may be considered as complementary to both clonogenic colony-counting systems and histopathologic approaches. The quantitative cellular and functional responses of the oligodendrocyte population can now be assessed concurrently without the confounding influences of other interdependent cell populations, particularly that of the vascular system. The analytical methods developed here may prove useful for *in vitro* examination of a wide range of subjects, including: (1) fundamental cellular mechanisms of delayed radiation injury and repair in the brain; (2) regulatory control of proliferation and differentiation in neuroglial cells and their precursors in response to cytotoxic insult; (3) mechanisms of radiation sensitization and protection in the central nervous system; and (4) efficacy

and toxicity of chemical modifiers of radiation response.

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REFERENCES

[1] Alpen, E. L., 1990, Radiation Biophysics (Prentice Hall, Englewood Cliffs).

[2] Armitage, P. and Berry, G., 1987, Statistical Methods in Medical Research (Blackwell Scientific Publications, Oxford).

[3] Bansal, R. and Pfeiffer, S. E., 1989, Reversible inhibition of oligodendrocyte progenitor differentiation by a monoclonal antibody against surface galactolipids. *Proceedings of the National Academy of Sciences, USA*, **86**, 6181–6185.

[4] Bhat, S. and Pfeiffer, S. E., 1986, Stimulation of oligodendrocytes by extracts from astrocyte-enriched cultures. *Journal of Neuroscience Research*, **15**, 19–27.

[5] Bologa, L., Cole, R., Chiappelli, F., Saneto R. P. and de Vellis, J., 1988, Serum contains inducers and repressors of oligodendrocyte differentiation. *Journal of Neuroscience Research*, **20**, 182–188.

[6] Calvo, W., Hopewell, J. W., Reinhold, H. S. and Yeung, T. K., 1988, Time and dose-related changes in the white matter of the rat brain after single doses of x-rays. *British Journal of Radiology*, **61**, 1043–1052.

[7] Cerda, H. and Rosander, K., 1983, DNA damage in irradiated endothelial cells of the rat cerebral cortex. Protective action of cysteamine *in vivo*, *Radiation Research*, **95**, 317–326.

[8] Cohen, S. R. and Guarnieri, M., 1976, Immunochemical measurement of myelin basic protein in developing rat brain: An index of myelin synthesis. *Developmental Biology*, **49**, 294–299.

[9] Dwarkanath, B. S. and Jain, V. K., 1989, Energy-linked modifications of the radiation response in a human cerebral glioma cell line. *International Journal of Radiation Oncology, Biology and Physics*, **17**, 1033–1040.

[10] Elkind, M. M. and Whitmore, G. F., editors, 1967, *The Radiobiology of Cultured Mammalian Cells* (Gordon and Breach, New York).

[11] Gerweck, L. E., Kornblith, P. L., Burlett, P., Wang, J. and Sweigert, S., 1977, Radiation sensitivity of cultured human glioblastoma cells. *Radiology*, **125**, 231–234.

[12] Hopewell, J. W., 1979, Late radiation damage to the central nervous system: A radiobiological interpretation. *Neuropathology and Applied Neurobiology*, **5**, 329–343.

[13] Korr, H., Schultze, B. and Maurer, W, 1973, Autoradiographic investigations of glial proliferation in the brain of adult mice. I. The DNA synthesis phase of neuroglia and endothelial cells. *Journal of Comparative Neurology*, **150**, 169–176.

[14] Levy, R. P., 1991, *Oligodendroglial response to ionizing radiation: Dose and dose-rate response. Ph.D. Thesis* (University of California, Berkeley).

[15] Ludwin, S. K. and Johnson, E. S., 1979, An autoradiographic study of cellular proliferation in remyelination of the central nervous system. *American Journal of Pathology*, **95**, 683–690.

[16] Ludwin, S. K., 1985, The reaction of oligodendrocytes and astrocytes to trauma and implantation. *Laboratory Investigation*, **52**, 20–30.

[17] Manley, N. B., 1988, *Cell and tissue kinetics of the subependymal layer in mouse brain following heavy charged particle irradiation. Ph.D. Thesis* (University of California, Berkeley).

[18] McCarthy, K. D. and de Vellis, J., 1980, Preparation of separate astroglial and oligodendroglial cell cultures from rat cerebral tissue. *Journal of Cell Biology*, **85**, 890–902.

[19] McKhann, G. M., 1978, A cellular approach to neurological disease. *Johns Hopkins Medical Journal*, **143**, 48–57.

[20] McMorris, F. A. and Dubois-Dalcq, M., 1988, Insulin-like growth factor I promotes cell proliferation and oligodendroglial commitment in rat glial progenitor cells developing in vitro. *Journal of Neuroscience Research*, **21**, 199–209.

[21] Meir, D. and Schachner, M., 1982, Immunoselection of oligodendrocytes by magnetic beads. II. In vitro maintenance of immunoselected oligodendrocytes. *Journal of Neuroscience Research*, **7**, 135–145.

[22] Myers, R., Rogers, M. A. and Hornsey, S. A., 1986, A reappraisal of the roles of glial and vascular elements in the development of white matter necrosis in irradiated rat spinal cord. *British Journal of Cancer*, **53**, 221–223.

[23] Norton, W. T., 1981, Formation, structure and biochemistry of myelin. In: *Basic Neurochemistry.* Edited by: G. J. Siegel, R. W. Albers, B. W. Agranoff and R. Katzman. p. 63–92 (Little, Brown, Boston).

[24] Paterson, J. A., Privat, A., Ling, E. A. and LeBlond, C. P., 1973, Investigation of glial cells in semithin sections. III. Transformation of subependymal cells into glial cells, as shown by radioautography after ^{3}H-thymidine injection into the lateral ventricle of the brain of young rats. *Journal of Comparative Neurology*, **149**, 83–102.

[25] Pettmann, B., Delannoy, J. P., Cumageot, J., Devilliers, G. and Sensenbrenner, M., 1980, Rat brain glial cells in culture: Effects of brain extract on the development of oligodendrocyte-like cells. *Developmental Biology*, **75**, 278–287.

[26] Puck, T. T. and Marcus, P. I., 1955, A rapid method for viable cell titration and clone production with HeLa cells in tissue culture: The use of x-irradiated cells to supply conditioning factors. *Proceedings of the National Academy of Sciences, USA*, **41**, 432–437.

[27] Raff, M. C., Miller, R. H. and Noble, M., 1983, A glial progenitor cell that develops *in vitro* into an astrocyte or an oligodendrocyte depending on culture medium. *Nature*, **303**, 390–399.

[28] Raff, M. C. , Mirsky, R., Fields, K. L., Lisak R. P., Dorfman, S. H., Silberberg, D. H., Gregson, N. A., Leibowitz, S. and Kennedy, M. C., 1978, Galactocerebroside is a specific cell-surface antigenic marker for oligodendrocytes in culture. *Nature*, **274**, 813–816.

[29] Saneto, R. P. and de Vellis, J., 1985, Characterization of cultured rat oligodendrocytes proliferating in a serum-free, chemically defined medium. *Proceedings of the National Academy of Sciences, USA*, **82**, 3509–3513.

[30] Schultze, B. and Korr, H., 1981, Cell kinetic studies of different cell types in the developing and adult brain of the rat and mouse: A review. *Cell and Tissue Kinetics*, **14**, 309–325.

[31] Szuchet, S., Stefansson, K., Wollman, R. L., Dawson, G. and Arnason, B. G. W., 1980, Maintenance of isolated oligodendrocytes in long-term culture. *Brain Research*, **200**, 151–164, 1980.

[32] van der Kogel, A. J., 1983, The cellular basis of radiation-induced damage in the central nervous system. In: *Cytotoxic Insult to Tissue.* Edited by: C. S. Potten and J. H. Hendry. p. 329–352 (Churchill Livingstone, Edinburgh).

[33] van der Kogel, A. J., 1986, Radiation-induced damage in the central nervous system: An interpretation of target cell responses. *British Journal of Cancer*, **53[Suppl VII]**, 207–217.

[34] van der Kogel, A. J., 1977, Radiation tolerance of the rat spinal cord: Time-dose relationships. *Radiology*, **122**, 505–509.

[35] van der Maazen, R. W. M., Verhagen, I., Kleiboer, B. J. and van der Kogel, A. J., 1991, Radiosensitivity of glial progenitor cells of the perinatal and adult rat optic nerve studied by an in vitro clonogenic assay. *Radiotherapy and Oncology*, **20**, 258–264.

[36] van der Maazen, R. W. M., Verhagen, I. and van der Kogel, A. J., 1990, An *in vitro* clonogenic assay to assess radiation damage in rat CNS glial progenitor cells. *International Journal of Radiation Biology*, **58**, 835–844.

[37] Winer, B. J., 1971, *Statistical Principles in Experimental Design* (McGraw-Hill, New York).

[38] Wood, P., Okada, E. and Bunge, R. P., 1980, The use of networks of dissociated rat dorsal root ganglion neurons to induce myelination by oligodendrocytes in culture. *Brain Research*, **196**, 247–252.

Figure Legends

Figure 1

Cellular and metabolic changes in unirradiated primary mixed glial cultures are shown as a function of DIC. In each panel, data are represented as mean ± SD. **Upper**, serial oligodendrocyte counts increase sharply between DIC 7 and DIC 14 and reach a plateau shortly thereafter. **Middle**, MBP levels, pooled from six separate experiments with comparable initial plating conditions, increase at a nearly exponential rate (solid line) from DIC 7 to DIC 28. **Lower**, total cellular protein levels, pooled from six separate experiments with comparable initial plating conditions, increase at an exponential rate (solid line) from DIC 7 to DIC 28.

Figure 2

Oligodendrocyte counts (mean ± SD) at DIC 14, following 2 Gy and 5 Gy γ-irradiation at DIC 8. The dose-response was significant at the 95% level.

Figure 3

Oligodendrocyte counts (mean ± SD) in a representative experiment at DIC 21, following 2 Gy and 5 Gy γ-irradiation at DIC 8. Counts in irradiated groups had recovered considerably from DIC 14 (cf Figure 2), but the dose-response was still significant at the 95% level.

Figure 4

Oligodendrocyte counts (mean ± SD) at DIC 14, following γ-irradiation (0.5 Gy, 1.0 Gy, 1.5 Gy and 2.0 Gy) at DIC 8. A significant (95% level) differential response was observed at each dose increment of 0.5 Gy.

Figure 5

MBP levels (mean ± SD) in a representative experiment at DIC 21, following 2 Gy and 5 Gy γ-irradiation at DIC 8. The dose-response was significant at the 95% level.

Figure 6

Total cellular protein levels (mean ± SD) in a representative experiment at DIC 21, following 2 Gy and 5 Gy γ-irradiation at DIC 8. Only minor differences in mean protein content were found as a function of dose, although the levels in the 5-Gy group were still significantly lower (95% level) than the levels in the 2-Gy and control groups.

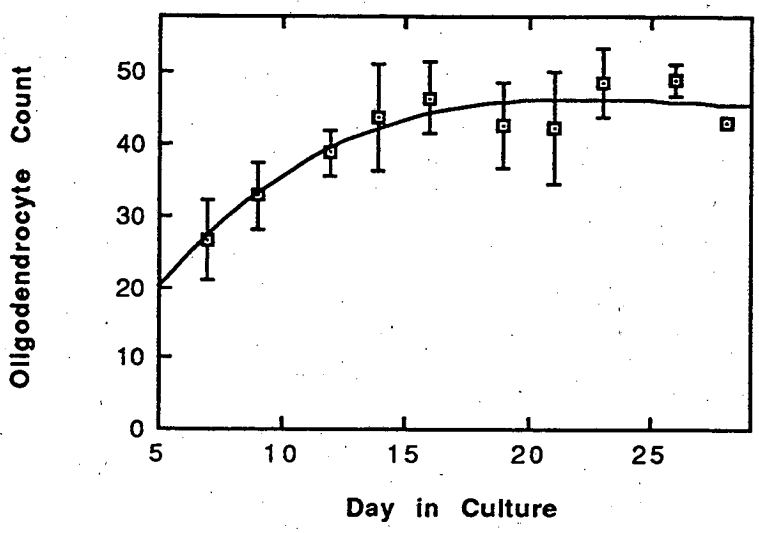
Figure 7

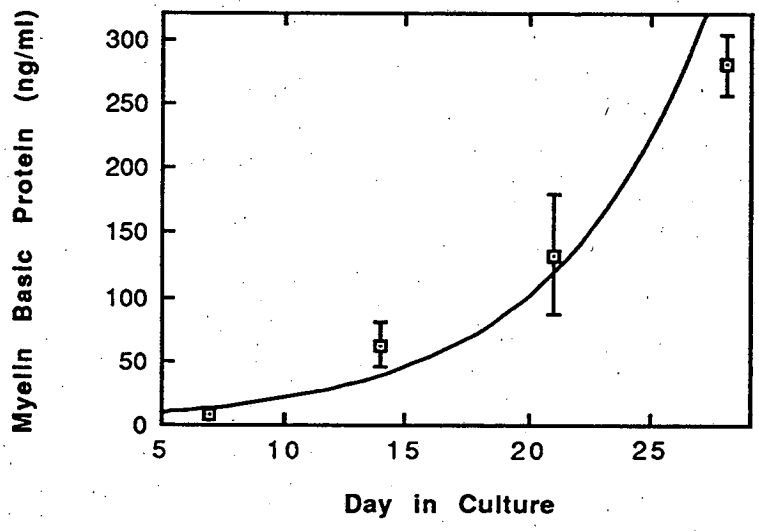
Myelin synthesis index (mean ± SD) in a representative experiment at DIC 21, following 2 Gy and 5 Gy γ-irradiation at DIC 8. The index, defined as the ratio of MBP level (ng/ml) to mean oligodendrocyte grid count, is assumed to reflect the functional maturity (i.e., mean MBP-synthetic capacity) of the oligodendrocyte population. The dose-response was significant at the 95% level.

Figure 8. Schematic representation of the response of the oligodendrocyte population as a function of DIC and radiation dose. The temporal pattern of response is shown for unirrradiated control cultures and for cultures irradiated with 2 Gy or 5 Gy γ-irradiation at DIC 8. Solid lines represent experimental data. Dashed lines represent projected patterns of response. Cultures irradiated with higher doses sustain more dramatic and longer-lasting decrements in oligodendrocyte counts than cultures irradiated with lower doses. However, even 5 Gy-irradiated cultures show significant recovery of oligodendrocyte counts by DIC 21.

Figure 9. Schematic representation of the temporal pattern of MBP levels as a function of DIC and radiation dose. The response is shown for unirrradiated control cultures and for cultures exposed to 2 Gy or 5 Gy γ-irradiation at DIC 8. Solid lines represent experimental data. Dashed lines represent projected patterns of response. Cultures irradiated with higher doses sustain greater and longer-lasting impairment in MBP production. At DIC 21, 5-Gy irradiated cultures show no recovery in MBP synthesis and 2-Gy irradiated cultures show only modest recovery, even though oligodendrocyte counts have increased considerably from their nadirs (cf Figure 8). It is projected (dashed lines) that MBP renewal will gradually recover as replacement oligodendrocytes differentiate and mature.

Figure 1


Oligodendrocyte Count
0
10
20
30
40
50
5
10
15
20
25
Day in Culture


Myelin Basic Protein (ng/ml)
0
50
100
150
200
250
300
5
10
15
20
25
Day in Culture

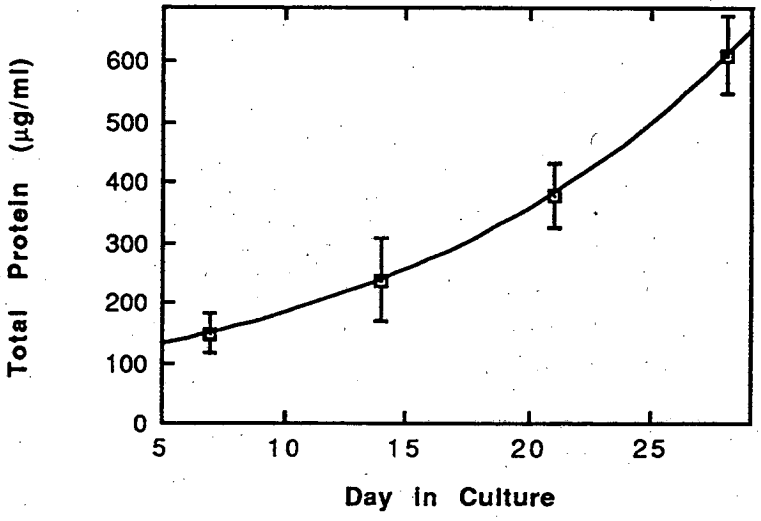

Total Protein (µg/ml)
0
100
200
300
400
500
600
5
10
15
20
25
Day in Culture

Figure 2

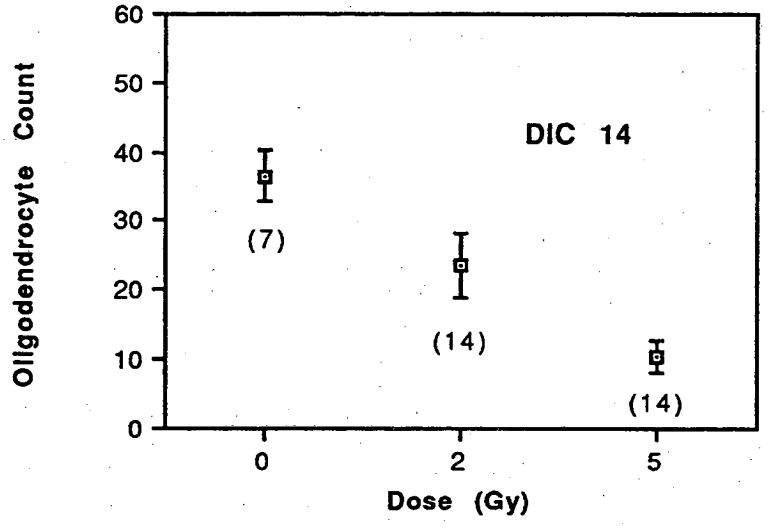


Figure 3

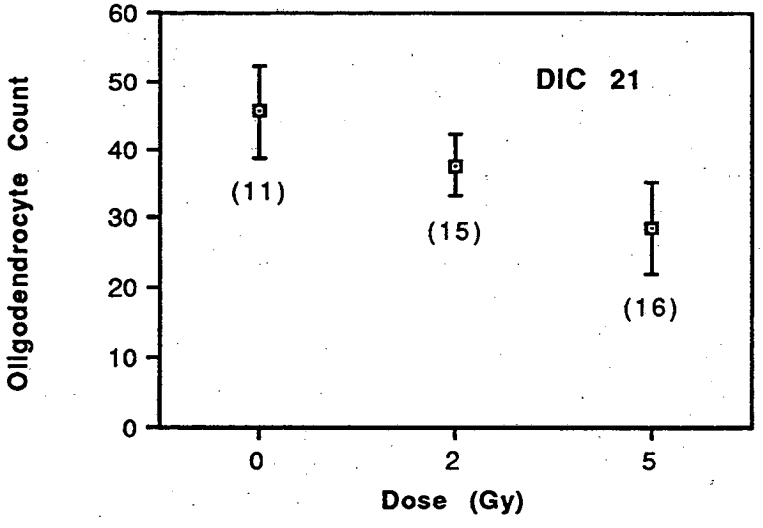


Figure 4

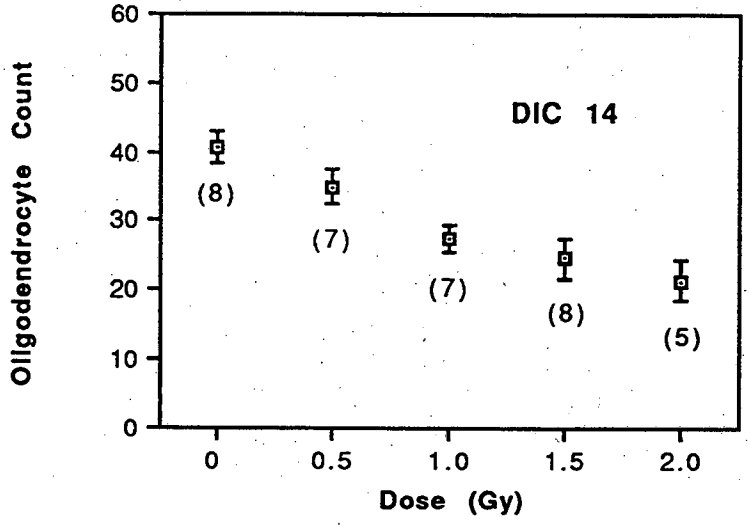


Figure 5

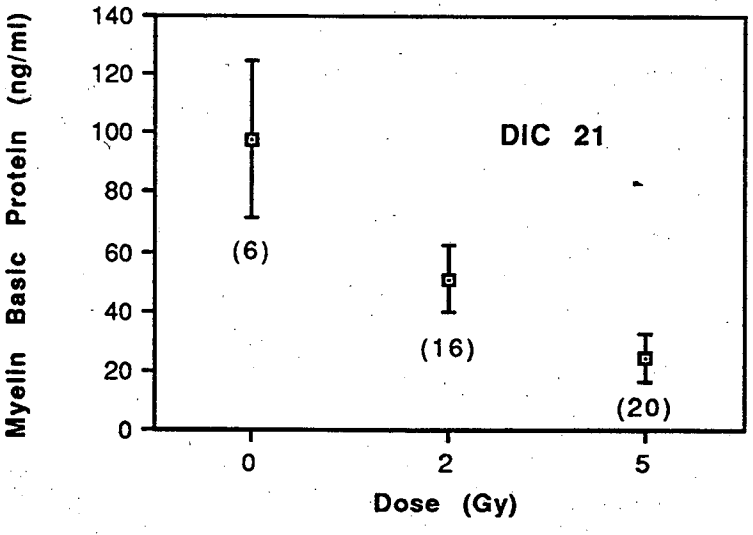


Figure 6

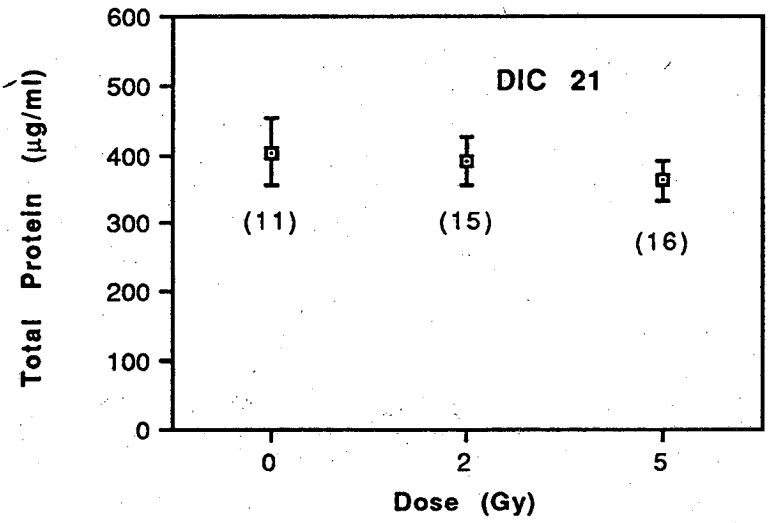


Figure 7

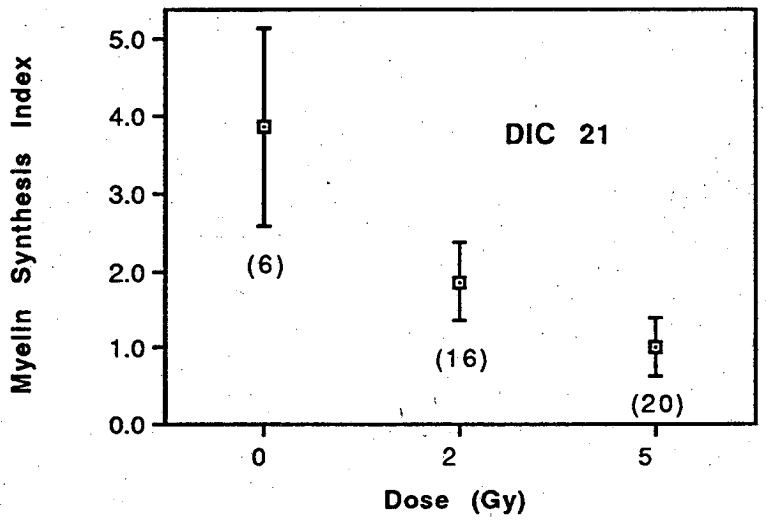


Figure 8

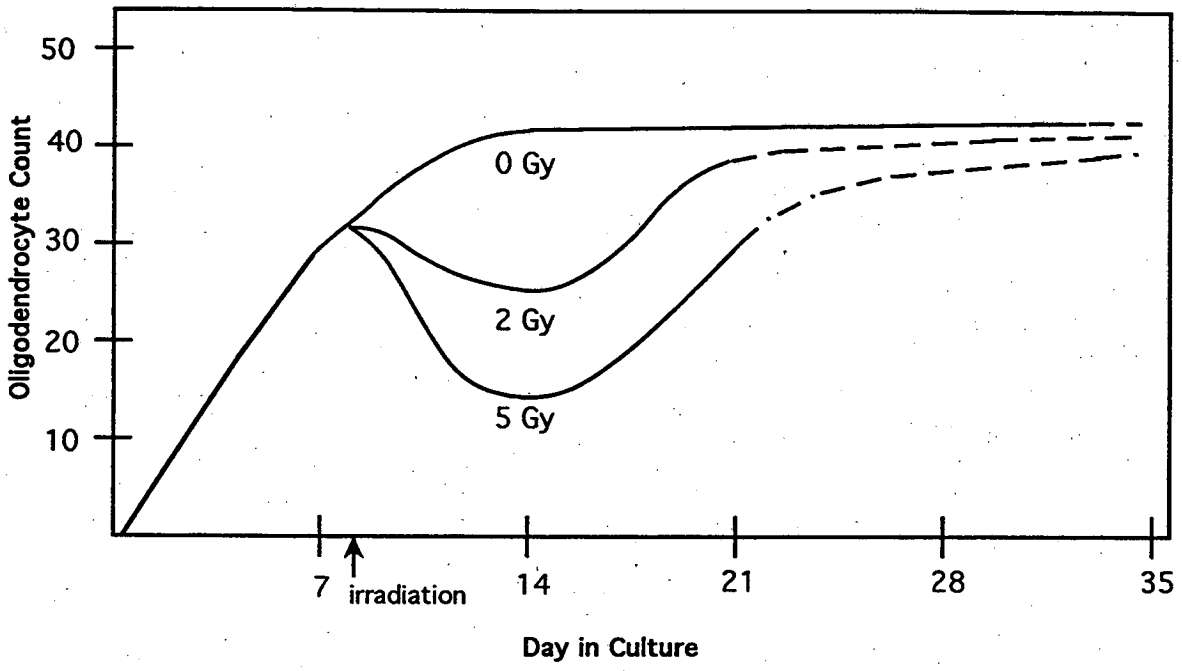
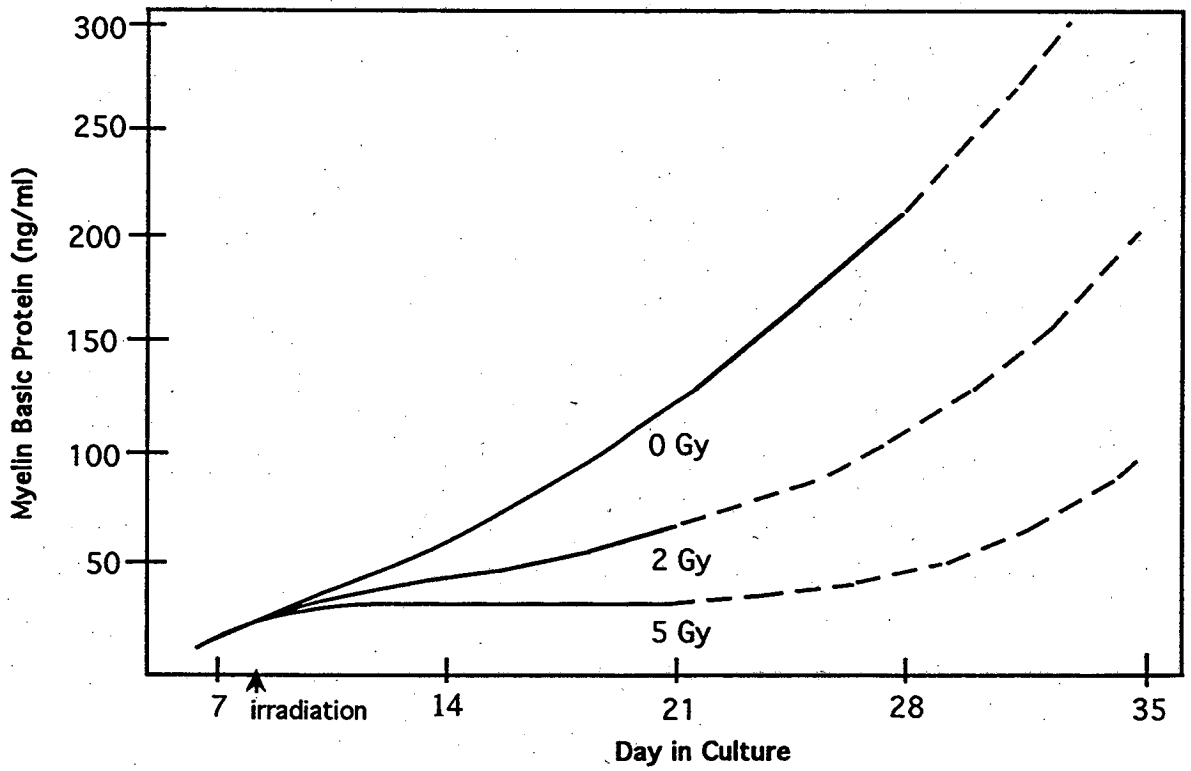


Figure 9



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