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Pacific Northwest Laboratory

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MICROBEAM STUDIES OF THE SENSITIVITY OF STRUCTURES WITHIN LIVING CELLS

L. A. Braby*

Battelle, Pacific Northwest Laboratory
Richland WA. 99352

(Received for publication May 4, 1991, and in revised form December 30, 1991)

ABSTRACT

Determining the biological effects of low doses of radiation with high linear energy transfer (LET) is complicated by the stochastic nature of charged-particle interactions. Populations of cells exposed to very low radiation doses contain a few cells which have been hit by a charged particle, while the majority of the cells receive no radiation damage. At somewhat higher doses, a few cells receive two or more events. Because the effects of damage produced by separate events can interact in the cell, we have had to make assumptions about the nature of these interactions in order to interpret the results of the experiments. Many of those assumptions can be tested if we can be sure of the number of charged-particle events which occur in individual cells, and correlate this number with the biological effect.

We have developed a special irradiation facility at Pacific Northwest Laboratory (PNL) to control the actual number of charged particle tracks that pass through cell nuclei. The beam from a 2 MeV tandem accelerator is collimated to approximately 5 µm. Cells, grown in special dishes with 1.5µm thick plastic bottoms, are positioned so that the desired portion of the cell aligns with the collimator. A shutter in the beam line is opened and closed after the desired number of particle tracks has been counted.

This approach can be used to investigate the effects of the interaction between irradiated and unirradiated cells in an organized system, as well as to study the effects of spatial and temporal distribution of radiation damage within single cells. We expect that this approach will lead to a better understanding of the mechanisms of high LET radiation effects.

KEY WORDS: microbeam, single particle, high linear energy transfer, cell survival.

INTRODUCTION

A new generation of charged-particle microbeam irradiation systems is being installed at several laboratories around the world. These systems have been designed to answer some fundamental questions about the hazards of low doses of ionizing radiation. The key to answering these long standing questions is the ability to detect each charged particle as it interacts with a cell, and limit the exposure of each cell to a predetermined value.

The ability of ionizing radiation to damage living systems was recognized soon after the discovery of x-rays. Since then, the nature of this damage has been studied extensively in order both to optimize the benefit in medical applications such as cancer therapy, and to minimize the effects of environmental exposures such as those produced by radon progeny captured in energy efficient houses. We probably know more about the effects of radiation than any other environmental carcinogen, and yet we still lack answers to basic questions such as the shape of the dose response relationship at low doses. The major factor limiting the investigation of low dose effects has been the stochastic nature of the physical interaction of ionizing radiation with individual cells, but this limitation can be overcome by irradiating individual cells with specific numbers of particles.

For a very long time microbeam irradiation has been used to study the function of living cells and to investigate the response to radiation. Initially UV light was used, but gradually techniques for charged-particle microbeams were added (Zirkle 1957). The goals of these experiments have evolved as has our understanding of the structure of living cells and the effects of radiation have developed. The earliest experiments used a microbeam as a tool for microsurgery to investigate the function of subcellular structures within individual cells. Later, the emphasis changed to determining the portion of the cell which is sensitive to the effects of radiation, and now the emphasis is on understanding the mechanisms which allow very small chemical changes in individual cells to produce major health effects such as cancer.
It is well known that ionizing radiations damage living material. Both beneficial applications as well as potential risks such as cancer induction were identified soon after X-rays were discovered. In order to estimate the magnitude of the effect which would be produced by a specific radiation exposure, it was necessary to define a unit for measuring the radiation. Radiations from different sources, for example alpha particles from radon and gamma rays from potassium 40, all deposit energy by producing ionizations and excitations in matter. It was observed that all types of ionizing radiation produced the same type of initial ionizations, and that the number of ionizations was proportional to the energy deposited, so the radiation dose was defined in terms of the energy deposited per unit mass. The definition of this quantity has evolved with changes in measurement systems and with improving understanding of the physical processes involved, but the current definition (ICRU 1980) is still based on the assumption that the biological effect is related to the energy deposited by the radiation. However, the relationship is not a simple one. The inactivation of dry enzymes and some other simple systems is a linear function of dose, but most plant and animal systems display a distinctly nonlinear behavior (Elkind and Whitmore 1967). At the lowest doses that can be used in experiments the effect per unit dose is relatively small, but as the dose increases, the effect per unit dose also increases. At still higher doses, the effect begins to decrease again (see high dose rate gamma ray curve in Figure 1). This curve indicates that products of successive energy transfers to the cell interact with each other to increase the effectiveness of the later events, but if the dose is high enough, additional factors which prevent expression of the effect become dominant. Plant and animal cells also show an increase in biological effect with increasing linear energy transfer (LET), i.e. the stopping power of the radiation, in spite of the fact that the same type of ionizations are produced by all radiations. However, the spatial separation of the ionizations along the track and the range of delta rays extending from the track do differ with LET and charged particle velocity, and it is now assumed that clusters of ionizations in volumes a few nanometers in diameter are responsible for much of the observable effect (Goodhead 1987). Finally, it is observed that the magnitude of the response of most biological systems depends on the dose rate at which the damage is delivered. For all endpoints which have been tested, decreasing the dose rate of low LET radiation reduces the effect. However, for malignant transformation by high LET particles, the dose rate effect is still unsettled. In some experiments the transformation seems to increase with decreasing dose rate, while in other...
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The use of microbeams for partial cell irradiation has a long history, going back as far as 1912. The early developments have been carefully reviewed (Zirkle 1957) and will only be mentioned briefly here. The early work took advantage of the damaging nature of ionizing radiation and used it in a form of microsurgery to investigate the function of subcellular structures. Later, as interest in the effects of ionizing radiation itself developed, these techniques were used to attempt to determine which parts of a cell were most sensitive to radiation damage. The techniques used in these early studies indicate the variety of approaches which can be taken, and some of the limitations inherent in use of microbeams.

Two approaches can be used to limit a beam to a very small spot. The beam can be collimated or it can be focused. These alternative approaches are illustrated in Figure 3. The lens collects a relatively large fraction of the particles from the source and focuses them to a reduced image of the source. The collimator only accepts those which

In order to assess the risk at low doses and low dose rates from high LET radiations such as alpha particles from radon and its daughters, we need to understand the effects of individual radiation events in cells, and how the damaged cells interact with undamaged cells in an organized tissue. However, the needed information can not be derived from the results of conventional experiments. In those experiments the number of tracks through a cell nucleus is a Poisson random variable. If the dose is equal to the mean for a single event in a cell nucleus (40 cGy in the example above) about one third of the cell nuclei will receive a single particle, another third will not receive an energy deposition event, and the rest of the nuclei will receive two or more events. Figure 2 shows typical mammalian cells growing as a monolayer on a plastic surface, and has been overlaid with a computer simulation of the actual dose to the individual cell nuclei. For this radiation, the maximum dose is 60 cGy so it is evident that several cells received two or more events. At lower doses, the fraction of the cells with two or more events decreases, but even larger fractions of the population receive no dose at all.

In order to interpret the results of experiments which produce these distributions of energy deposition, it is necessary to make several assumptions about the response of cells to combinations of radiation events, and the time between events. The only way to test those assumptions is to correlate the biological effect with the actual energy deposited in individual cells. The most efficient way to do this is to limit the irradiation to a portion of the cell so that the path length can be determined, and to limit the exposure to the desired number of particles by using a shutter to stop the beam at the desired number (often just one). This requires that each particle be detected as it goes through the cell. This approach is based on use of a low intensity microbeam, a technique which has been used extensively in the past, with the addition of single particle detection which has been made possible by improved electronics and detectors.

Microbeam Irradiation

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Two approaches can be used to limit a beam to a very small spot. The beam can be collimated or it can be focused. These alternative approaches are illustrated in Figure 3. The lens collects a relatively large fraction of the particles from the source and focuses them to a reduced image of the source. The collimator only accepts those which
are aligned with the target. However, at 40 cGy per alpha particle in a cell nucleus, the low particle fluences through a small collimator is sufficient for most experiments. For particles which are easily scattered, such as photons and electrons, a collimator may not be satisfactory and a focused beam is preferred. As illustrated in the figure, both approaches rely on the effective source of the radiation being at a large distance away from the target in order to minimize divergence of the beam. Neglecting the effects of scattering, the two approaches produce different spatial distributions of dose. The collimator produces a nearly cylindrical irradiated volume, while the lens produces converging and diverging cones of tracks, meeting at the focal plane of the lens. Depending on the focal length and aperture of the lens relative to the thickness of the sample being irradiated, this may produce significant variations in dose rate with depth in the sample. The effects of scatter are illustrated in Figure 4 for a collimated beam. Scatter can occur in the collimator aperture, path C, in the barrier between the vacuum system of an accelerator and the cell environment, path D, and in the cell and the substrate it is attached to, path B. The effect of scatter, in terms of the distance between the center of the beam and the actual position of individual tracks, depends on the angle of scatter and the distance between the scattering point and the target. Thus, it is evident that the vacuum window and the target should be as close together as possible and that the window should be as thin as possible. In some cases the window can be eliminated by substituting a hole 1 to 2 \( \mu m \) in diameter which also serves as the collimator. With proper design the total scatter can be reduced. Scattering from the aperture can be minimized by making the aperture just thick enough to stop the beam, using a rectangular edge profile, and polishing the inner surface to eliminate burrs which do not stop particles but slow them down and scatter them. If the vacuum window is between the source and the collimator, it causes the beam to diverge through the collimator, and the air in the collimator causes additional scatter.

Figure 4. Particles scattered in the aperture, vacuum window, and target broaden the irradiated volume produced by a collimated microbeam.

The most successful radiation for microsurgery by microbeam has been ultraviolet light. A typical system is illustrated in Figure 5 (Uretz et al. 1954). The microscope objective is used both to position the target using transmitted visible light, and to focus the ultraviolet light which is introduced by a partially aluminized mirror above the objective. This general approach has been used extensively to study biological processes such as the function and regulation of the mitotic spindle (Forer, 1965, Zirkle 1970). The resolution and versatility of photon microbeams were advanced significantly with the development of laser microbeam systems Figure 6 (Berns et al. 1969), and commercial laser systems are now used extensively for microsurgical and analytical techniques.

Although it is not practical to use a collimator for high energy x-rays, at least one system using 100 kVp x-rays was built (Buchholtz 1967). As shown in Figure 7, a long lead glass capillary was used as the final collimator. In order to achieve sufficient dose rate, the distance between the x-ray tube anode and the target was minimized. The target was nearly in contact with the end of the collimator to minimize the effects of scatter. This system was used with beam diameters as small as 25 \( \mu m \).

An electron microbeam system was also built (Pohlit 1957). This system relied on a magnetic lens to focus the beam of 30 to 150 keV electrons to a spot as small as 1 \( \mu m \) in diameter, Figure 8. Using a beam current of \( 10^{-5} \) amp at 150 kV the dose rate was approximately 2 x 10^4 Gy per second. However, scatter of electrons in the target limits the applicability of this type of system to very thin samples.
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Figure 6. Replacing a conventional UV light source with a laser reduces the UV spot size and increases the intensity. The rotating mirror allows essentially simultaneous viewing and irradiation.

Figure 7. An arrangement for collimating a high intensity low voltage X-ray beam.

Figure 8. Schematic of a focused electron microbeam system.

Positive ion microbeams generally proved to be more useful. Both radioactive sources and accelerator produced beams were used. Davis and Smith (1957) developed a system using a small 210Po source in a holder with an exit aperture 1 to 1.5 µm in diameter. The source was mounted so that it could be positioned between the target and the objective of the microscope to make each irradiation. The collimator aperture was aligned with a cross hair in the microscope optics and then this cross hair was used to position the object to be irradiated. An 8 mCi source provided about 13 particles per minute through the collimator. Although this system is relatively simple and compact, the use of a relatively strong radioactive source and the limited range of the alpha particles suggests that a system using a particle accelerator would be more convenient. Zirkle and Bloom (1953) used a 2 MV van de Graaff generator to produce proton beams and collimated them after the beam had left the accelerator vacuum system through a 5 µm thick mica window. Because of scatter in the air in the collimator, the collimator had to be thin and the target as close to it as possible. They used two metal plates, with a groove on one, clamped together to make apertures about 2.5 µm in diameter. Later, a system using a cyclotron was developed at Brookhaven National Laboratory (Baker et al. 1961). The longer range of the 11 MeV/amu particles allowed the addition of a helium ion chamber to monitor the beam intensity, Figure 9. Beams as small as 25 µm diameter were used to investigate the effects of damage to different cells within a tissue.

These early microbeam irradiation systems were very helpful in establishing the nature of the damage done to living systems, especially in showing...
that damage to the cytoplasm had a very limited effect on the survival of the cell. However, all of these systems were limited to the effects of relatively large doses. Since the interaction of a single charged particle could not be detected, the shutter of each system had to be left open long enough that the variance in the number of particles through the cell was acceptable. Otherwise, there would have been such large difference in the effect on different cells that the experiments would have been impossible to interpret. In order to investigate the effects of low doses, it is necessary to detect the individual particles in the beam.

**Single Particle Irradiation**

Recently the need to understand the effects of low doses of radiation has lead to the need to control the number of charged particle tracks interacting with individual cells. One system designed for this purpose is illustrated in Figure 10 (Kraske et al. 1990). This system was installed on a low energy beam line of the UNILAC linear accelerator where ions of many elements, ranging from carbon to uranium, with energies of 1.4 MeV/amu were available. The collimator consisted of a 30 µm thick sheet of mica which had been exposed to a low dose of more energetic ions and then chemically etched to remove material which had been damaged by the charged-particle tracks. By adjusting the etching time uniform size holes between 0.7 to 2.0 µm in diameter were produced, but these holes were distributed randomly over the surface of the mica. A capillary tube was used as a pre-collimator to limit the beam to a single etched hole. Since the accelerator beam was horizontal, the cells had to be irradiated while attached to a vertical surface. This necessitated removing the growth medium from the cells during irradiation, which may have affected the cell response, but it also made it possible for a solid state detector placed after the cells to detect the individual charged-particle events as they occurred. This system has been used to investigate the effects of single ion tracks with LET from 500 to 12,000 keV/µm on the growth and chromosome structure of mammalian cells. However, the irradiation procedure is time consuming and only a limited number of cells can be irradiated, thus limiting the statistical precision which could be obtained for the large number of biological responses observed.

In order to overcome some of the limitations imposed by a horizontal beam line, and to investigate the effects of ions which are produced by natural radioisotopes or radiotherapy equipment, several new single particle irradiation systems are being developed. The system now being tested at Pacific Northwest Laboratory is typical (Braby and Reece 1990). An electrostatic accelerator, in this case a 2 MV tandem, is used to produce hydrogen and helium ions. The beam is bent 90° so that the final beam is vertical upward, and the cells can be irradiated without disturbing their normal tissue culture conditions, Figure 11. In order to control the number of charged particles through the shutter, each particle must be detected. If low energy ions which stop in the target are to be used, they must be detected between the collimator and the cell. Several detection systems were considered, and they were all found to have limitations. A primary concern is that the detection system should not increase the beam size excessively. This probably excludes any type of ion chamber or proportional counter which would add at least one foil at a significant distance from the target, the equivalent of increasing the distance v in Figure 4. Secondary particle effects such as secondary electron emission were considered, but the number of electrons emitted per primary ion is so small that with realistic collection efficiencies, some ions would go undetected. The method being tested at PNL is a thin plastic scintillator serving as the exit window of the vacuum system. In order to produce enough light to detect each charged-particle track, this scintillator must be thicker, approximately 10 µm, than if it were only the vacuum window. However, the distance v can be held to a minimum, thus reducing the effect of scatter in this material.
Cells to be irradiated are grown in special petri dishes consisting of two stainless steel rings with an o-ring which clamps a thin polyester foil between them, like the head of a drum. The polyester film is 1.5 µm thick and is manufactured for the production of electronic capacitors. As a result, petri dishes require thorough washing before they are sterilized in a dry oven at 150°C for three hours. The initial cost of the stainless steel rings is higher than the glass rings which have traditionally been glued to the thin polyester film. However, they have the advantage of requiring much less time to replace the film, and they have precisely controlled dimensions so they can be positioned reproducible.

In order to visualize living cells without requiring optical components inside the accelerator vacuum system, a microscope fitted for phase contrast in reflected light mode is used. This system works quite well, but since the cells are essentially transparent they reflect very little light. In initial experiments the light intensity required to obtain a clear video image of the cells was so great that it softened the plastic scintillator below the cells and resulted in failure of the vacuum window. Use of an image intensifier allows imaging the cells at much lower light levels, and also makes it possible to directly view the light emitted by thick scintillators placed over the end of the beam line.

Plastic scintillators are much less efficient in converting high LET particle energy into light than they are for low LET particles. As a result, light with the microscope objective. The limited numerical aperture and the losses at optical surfaces would prevent collecting enough photons from each event to produce a signal which is clearly distinguished from the single photon noise. In order to obtain sufficient signal, a compact 2.54 cm diameter photomultiplier has been mounted to the microscope lens turret so that its photocathode is only 1.3 cm above the scintillator. This gives an effective numerical aperture of about 1.6. The signal to noise ratio is also improved by applying a thin reflecting coating to the back side of the scintillator. This reflecting coating also has the advantage of producing a more uniform background for viewing the cells to be irradiated.

In order to minimize the effects of slit edge scattering and to simplify alignment of the collimator, this system uses two sets of four adjustable knife edges to construct two apertures in series. The first aperture defines the beam size, and the second one, set slightly larger, stops the majority of the scattered particles. These knife edges are connected to compound micrometer screws with a special linkage which results in a positioning resolution of 0.2 µm per minor division on the micrometer screw. The piezoelectric shutter has a travel of 40 µm and a response time of less than 0.1 msec. The accelerator beam current is adjusted to give about 100 particles per second through the collimator.

The entire irradiation sequence is computer controlled. The microscope objective is rotated into place, and a video image of a 500 µm square field on the petri dish is presented to the operator. A track ball is used to position a cursor over an object to be irradiated, and the irradiate command is given to the computer. The computer then moves the petri dish, using a high-speed two axis positioning system, to place the point marked by the cursor over the collimator. A servo system rotates the lens turret to place the photomultiplier tube over the scintillator, turns off the microscope light, opens the beam line shutter, counts scintillation flashes, and closes the beam shutter at the specified dose. The computer then returns the microscope objective, turns on the light and waits for the operator to identify the next object to be irradiated. The irradiation sequence requires about 2 seconds. Plans call for also automating the cell recognition step using the digital image processor, with the goal of being able to locate and irradiate a cell every three seconds.

Similar single particle irradiation systems are being developed at the Gray Laboratory, London (B Michael, private communication) and at Columbia University (Geard et al.). These systems will be used to study the effects of low doses on the chromosomes of cells and to study the mechanisms of mutation and carcinogenic transformation. It is expected that the results of these experiments will eliminate much of the uncertainty in current estimates of risk from radiation exposure.
L. A. Braby

Acknowledgement

Work supported by the Office of Health and Environmental Research (OHER), U. S. Department of Energy under Contract DE-AC06-76RL01830.

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Discussion with Reviewers

C Geard: Does the computer simulation overlay in Fig. 2 actually correspond to a Poisson distribution based on 0.4 Gy per nucleus traversal? Do the values correspond to a constant thickness edge to edge or some sort of hemisphere? Of course the whole concept of dose is one which related to averages over many cells and the values given are actually specific energies.

Author: The overlay gives calculated specific energies for a dose of 0.4 Gy and a single event mean specific energy of 0.4 Gy. The number of tracks through each nucleus was determined from the Poisson distribution, and the energy deposited by each track was taken from the measured distribution for a spherical nucleus. Thus the calculation is probably more relevant to cells in a tissue than cells growing on plastic. The specific energy is, of course, the stochastic equivalent of dose.

Z Somosy: Are there possibilities to determine the local dose of irradiation on plasma or nuclear membranes by your system?

Author: In principle the radiation sensitivity of any structure which can be seen by light microscopy in the living cell can be studied by this method. However, the spatial resolution is limited by scattering, so very small structures can be selectively irradiated only if they is very little material between them an the accelerator vacuum. Furthermore, a thin structure perpendicular to the beam will receive the same dose as material adjacent to it, but if it is parallel to the beam a particle will have a long path length in it, and a relatively high dose will result.

G Legge: Could you give some figures on nuclear dimensions and energy deposited in nucleus for some chosen beam and cell type in your system as an example? The cells will surely not be spherical, if they are plated or growing on a surface. Is the nucleus still roughly spherical?

Author: In suspension, or in a tissue, mammalian cell nuclei are typically spherical with a diameter around 7 to 10 μm. When growing on a glass of plastic surface the flatten and take of a "fried egg" shape. There is frequently very little cytoplasm above or below the nucleus, and the thickness of the nucleus itself depends on the cell type and the culture conditions. They can range from nearly spherical to only about two micrometers thick and several times the diameter of the equivalent sphere. The actual thickness has been hard to determine due to shrinkage when cells are fixed for electron microscopy, but confocal microscopy has been used more recently.
G Legge: Presumably, it is an advantage to have cells spread on the surface so that there is little cytoplasm covering the nucleus. But with cells that do not spread or that round up during mitosis, when you may wish to irradiate them, is the effect of irradiation on the cytoplasm so small that it can be ignored? Are there no cytoplasmic organelles that are greatly affected by irradiation?

Author: With respect to cell lethality, the cell is generally much less sensitive to damage in the cytoplasm than in the nucleus. However, there are exceptions such as mouse oocytes. There is very little data about other cellular effects such as malignant transformation and promotion. It is possible that functions dependent on cell-cell communication may be highly sensitive to damage in the cytoplasm.

G Kraft: Do you have a figure of the spatial distribution of the particles after traversing through the collimator system? Such a figure would illustrate the quality of the system. In addition, an energy spectrum of the transmitted particle would help to estimate the scattering events in the collimators which are essential for all experiments of this type.

Author: We do not have a figure of this type yet. We are using three different approaches for documenting the size and shape of the beam. Scintillation light produced in a thick plastic scintillator and imaged by a video camera with image intensifier provides a real time signal, but the light output is so low that the image intensifier noise is a problem, and the image must be averaged over about a second. Unfortunately we do not have an output device for these averaged images. Radiographic film, which changes color on exposure to radiation, has been exposed to the beam and, in conjunction with a microscope and stage micrometer, has been used to measure beams as small as 5 µm. The particles scatter in the plastic so there is some halo around the entrance spot, and without a scanning densitometer with submicron resolution, it is impossible to determine the exact spot size. Probably the best approach is to use a material, glass or plastic, in which individual charged particle tracks can be revealed by etching away the radiation damaged material. Your group has shown some very nice pictures of this type which illustrate the positioning accuracy of your system. We have some similar measurements where a track was to be placed every 25 µm on a square grid. Measurement of the actual spacing will allow determination of the shutter performance as well as the collimator size, but measurements to date have shown a problem with the device which we use to hold the plastic, a problem which we think we have now corrected. Energy spectrum measurements are not of much help in evaluating our system because there is unavoidable scatter in the scintillator, but since it is very close to the target it has little effect on the beam size.