Proceedings of the 4th International Workshop: Microbeam Probes of Cellular Radiation ResponseKilliney Bay, Dublin, Ireland, July 17–18, 1999

EXTENDED ABSTRACTS

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The extended abstracts that follow provide a summary of the Proceedings of the 4th International Workshop: Microbeam Probes of Cellular Radiation Response, held in Killiney Bay, Dublin, on July 17–18, 1999, which was jointly organized by the Columbia University Radiological Research Accelerator Facility and the MIT Laboratory for Accelerator Beam Applications.

There is increasing interest in the use of microbeam systems, which can deliver beams of different radiations with a spatial resolution of a few micrometers or less, for radiobiological research. Single-particle microbeams can be used to address such questions as the relative sensitivities of different parts of the cell (e.g. nucleus compared to cytoplasm), and the effects of irradiation of neighboring (bystander) cells. For particle (e.g. α-particle) beams, irradiation with exactly one (or more) particle per cell can be achieved, allowing questions of risks of very low doses of ionizing radiations, such as radon, to be addressed. Several microbeams are now in operation, and others are being developed. The workshop provided a forum to assess the current state of microbeam technology and current biological applications, and to discuss future directions, both technological and biological.

Roughly 75 scientists (about equal numbers of physicists and biologists) attended the workshop, the fourth in a biannual series (*1*). A list of attendees can be obtained from David Brenner (djb3@columbia.edu). A fifth meeting is planned for the year 2001.

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The Performance of the Gray Laboratory Charged-Particle Microbeam

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The microbeam offers a unique method for selecting and irradiating individual cells with a controlled number of charged particles. For a microbeam to truly fulfill its role in modern radiobiology, it must excel in a number of technical aspects. The versatility of a modern microbeam can be assessed by considering the three most important aspects of its performance: the targeting accuracy, the particle counting efficiency, and the rate at which cellular targets can be identified and irradiated.

The targeting accuracy of any microbeam is fundamentally limited by the chosen method of ''aiming''. While focusing potentially offers the finest probes, the overall accuracy of a focused microbeam may be no better than a collimated system, once other requirements are fulfilled. The Gray Laboratory has endeavored to develop a collimated microbeam with state-of-the-art targeting accuracy for an *in vitro* system, and without undue compromise in other areas. The development of the microbeam and preliminary measurements of the collimator performance using protons have been reported elsewhere (*1, 2*). Briefly, our system uses collimated particles accelerated by a 4 MV Van de Graaff accelerator to irradiate cells attached to a thin film that forms the base of a cell dish, itself supported on a motorized stage. A computer-controlled charge-coupled device (CCD) camera and epifluorescence microscope are used to automatically find and record the position of stained cells. To irradiate the cells, the microscope objective is replaced with a photomultiplier tube that detects photons produced when a particle traverses a thin scintillator situated between the collimator and the cells. Cells are positioned and irradiated automatically at a rate of just over 1 s per cell. Recent experiments have used mainly ^{3}He ions to selectively target either the cell nucleus (*3*) or the cytoplasm. Alongside these experiments have been developments to improve the targeting accuracy and subsequent performance-related studies using CR-39 track-etch detectors.

The overall targeting accuracy of any microbeam is limited not just

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by the properties of the collimator, but also by the accuracy with which cells can be aligned relative to the beam. Our collimation system is based on a 1-mm-long borosilicate glass capillary with a 1-, 1.5- or 5-μm bore and remains essentially unchanged from that reported previously (2). To align a cell requires that we can accurately determine the position of the collimated beam. Previously, this has required visualizing the end of the capillary *in situ* (with a cell dish in place) using the CCD camera/microscope assembly. This is possible only by removing the scintillation foil (and its opaque reflective backing) that is normally fitted over the end of the collimator. The beam position is now identified by visualizing the spot of light produced within the scintillation foil, rather than by viewing the collimator, and has been achieved by installing a variable-gain image intensifier before the CCD camera. This is a much more straightforward procedure and allows for rapid and routine checking of the beam position. A further advantage of using an image intensifier is that lower stain concentrations and/or less UV exposure are required to visualize cells.

Another source of inaccuracy when positioning cells is caused by optical aberrations in the imaging system (due partly to the refracting optics and partly to the intensifier). The coordinates assigned to cells found at the edge of the microscope field-of-view may be inaccurate for this reason, and therefore the cells will not be correctly positioned over the collimator (usually located at the center of the field-of-view). High positioning accuracy is now achieved by moving each cell to the nominal irradiation position, taking a second image, and reassigning coordinates. While this step improves the targeting accuracy, it doubles the time taken to find and record the cell positions and increases the UV exposure. Typically, the cell-finding procedure using this option now takes 20–30 min for a cell dish with 700–1,000 cells.

We have used nuclear track-etch detectors extensively to assess the performance of the microbeam. CR-39 plastic has been used to quantify the extent to which particles are scattered off-axis at the sample position for different collimator/scintillator assemblies and sample collimator distances. Our findings have been compared with results from computer simulations using SRIM-2000 (4) to verify that our collimators are performing optimally. We have measured the diameters of pit clusters formed by various numbers of ions per pit (i.e. from 10 to 10,000 ions), from which we can ''unfold'' the probability of a particle being a certain distance from the mean position. Using 3.5 MeV ³He ions, and a 5-μm-diameter by 1-mm-long collimator, we find that 99% of particles are within 2.5 μm of the mean position. Similar experiments in which the sample–collimator distance is increased show that the uncertainty in the particle position increases by about 1 μm for every 24-μm increase in the distance of the sample from the end of the collimator assembly. Normally, we would irradiate cells with the collimator/scintillator assembly just touching the base of the cell dish.

To examine the positioning accuracy of the microbeam fully requires that we involve the facility's automated cell-finding and positioning routines. To do this, a microarray of pits is formed on the track-etch detector using particles from the microbeam. After etching, these pits can be visualized by the automated cell-finding routine and serve as targets during a second exposure of the detector. Each pit is formed by targeting ~20 ³He ions to each location. When etched, this will appear as a single pit 5–6 μm in diameter. By using more than one particle, the position of the center of the etched pit will not be influenced by scatter or other properties of the collimator assembly (which are evaluated separately). Note that it is actually the midpoint between adjacent pits that is targeted; otherwise, a ''direct hit'' would not be apparent when the detector is etched for the second time. From studies such as these, we have been able to ascertain that the optical ''center of gravity'' of an imaged object (frequently, the nucleus of a DNA-stained cell) can be positioned relative to the location of the particle beam with an accuracy of less than 1 μm with a 63% success rate, and an accuracy of less than 2 μm with a 93% success rate.

Track-etch plastic has again been used to evaluate the particle detection efficiency using an 18-μm-thick plastic scintillator (BC400, Bicron). By measuring the percentage of single particles delivered correctly (i.e. as a grid, with nominally one particle at each location), the particle de-

tection efficiency is estimated to be >99%. The detection efficiency of the scintillator can degrade with use, however, so it is now usual to monitor the photomultiplier tube pulse-height spectrum during all cell experiments, so that the appropriate action can be taken.

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Technical Characteristics of the Columbia University Single-Ion Microbeam

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Introduction

Microprobes for the study of radiation damage to biological systems have been known since the 1950s (1) but were not used extensively until a recent resurgence of interest (2–4) due in part to recent developments in computer-based microscopy systems that allow rapid location and accurate positioning of target cells.

A single-ion microbeam facility comprises a number of elements arranged to deliver reliably counted numbers of ions to a chosen biological target. The elements are: (1) a source of ions of the appropriate energy, (2) a means of limiting the location of the ions to an area less than the area of the target, (3) a means of locating and moving the biological targets to the beam position, (4) a means of detecting each ion as it traverses the target, and (5) a means of shutting off the beam after the arrival of the chosen number of ions. The characteristics of each of these elements determine the type of experiment that can be performed at the facility.

Van de Graaff Accelerator

The source of ions for our microbeam is a model D1 4.2 MV Van de Graaff accelerator. This machine, which was originally the injector for the Cosmotron at Brookhaven National Laboratory, was converted to a dedicated radiobiology facility in 1966 and later moved to Irvington, NY, where it presently operates. The terminal is fitted with a duo-plasmatron ion source, which can produce beams of the isotopes of hydrogen and helium. Most of the work to date on the microbeam has been performed with α particles of 6 MeV corresponding to an LET of approximately 90 keV/μm at the center of the cells. Experiments could be performed with stopping α particles or with hydrogen ions with a lowest LET of 30 keV/μm limited by transparency of the collimator system.

Collimator

The area of the beam of ions can be limited either by collimation or by focusing. For the present system, we chose to use collimation because of the simplicity of setup and operation compared to focused systems. The collimator consists of a pair of apertures laser-drilled in 12.5-μm-thick stainless steel foils separated by 300 μm (Lenox Laser, Phoenix, MD). The limiting aperture is a 5-μm-diameter hole in the first foil. The

second aperture, which is 6 μm in diameter, acts as an anti-scatter element. The relative alignment of the two apertures is fixed during manufacture as a three-layer sandwich with the spacer in between. Monte Carlo modeling of this geometry and comparison to the energy spectrum of transmitted particles indicate that about 91% of them are within the unscattered core of the beam, which has a diameter of 5 μm. Approximately 7% of the beam is contained in a halo around the core resulting from particles that scatter in the edge of the first aperture and then pass through the anti-scatter aperture. The halo has a diameter of about 8 μm at the cell irradiation position. The remaining 2% of the particles are scattered by both apertures and appear at larger distances from the target position. The beam characteristics are appropriate for the originally intended targets for the microbeam, namely the nuclei of mammalian cells in culture.

Imaging and Control Program

The most important factor in determining the throughput of a microprobe system for irradiating cells is the ability of the microscopic video analysis system to recognize the targets and move them into position. A program written in Visual Basic under the Windows NT operating system controls the video analysis system and microscope stage motion. Cells are grown attached to a thin polypropylene foil treated with Cel–Tak. Polypropylene was chosen because it is nonfluorescent. The stock cell suspension is diluted so that a chosen number of cells will be contained in a 2-μl drop of medium. The cells are stained by exposure to a 50-n*M* solution of the vital DNA stain Hoechst 33342 for 30 min prior to analysis. This low stain concentration requires the use of a channel-plate image-intensified CCD camera (GenSysII and CCD-72, Dage-MTI, Michigan City, IN) to obtain a high-contrast image. A narrow-band epifluorescence cube (XF-06, Omega Optical, Brattleboro, VT) selects the 366-nm line from a mercury arc lamp for the observation. The video signal is captured by a Matrox Genesis image processing board using the Matrox Imaging Library (Matrox Electronic Systems, Dorval, Canada). Each image is a 10-frame average that has been smoothed with a mean intensity filter and corrected for nonuniformity of illumination. A threshold in intensity is set to separate the cells from the background. For normal irradiation of nuclei, the centroid of each cell is found relative to the position of the exit aperture (located by laser light shining through the collimator system). Each cell is then positioned over the exit aperture by the stepping motors driving the microscope stage (Daedal, Inc., Harrison City, PA). The computer maintains a list of centroids of cells that have been irradiated to prevent an accidental second irradiation. The combined precision of the video analysis and stage positioning is estimated to be about 2 μm. The total time required to irradiate a single dish of 2,000 cells is approximately 10 min, corresponding to a throughput of 12,000 cells/h.

Ion Detection and Beam Shutter

To shut off the beam after delivering a certain number of ions, a reliable counter must be used. Our main counter, which is used when the ion beam has a sufficient residual range after passing through the target cells, is a P10 gas-filled pulsed ion counter mounted on the high-power objective of the observation microscope. Because the counter is above the cell culture, it is necessary to aspirate off all but a thin layer of medium for the duration of the radiation exposure. Humidified air with 5% CO_2 is passed through a passage in the counter body over the culture to prevent dehydration. An entrance window of 2.5-μm-thick optically clear mica separates the gas in the counter from the gas over the cell culture. The path length for the particles is 8 mm. When the counter is operated at 300 V, it is working without gas gain and provides a very stable signal well separated from the noise. The signal from the detector preamplifier is shaped by a standard NIM amplifier, which feeds an SCA and computer-controlled scaler. The gate period output of the scaler is fed to a high-voltage amplifier (Technisches Büro S. Fischer, Ober-Ramstadt, Germany) connected to electrostatic deflection plates to turn on the beam until the chosen number of particles has arrived. The rise and fall time of the deflection voltage is such that 4 times out of 10,000 there

will be an extra particle delivered to the sample. There is a background signal of about 7 counts per day due to α-particle activity in the glass elements of the microscope objective or in the body of the counter.

A second counter is available for studies with stopping particles or particles with a small residual range. It is a Schottky barrier detector constructed from a 2-μm-thick silicon wafer. It provides a signal with excellent resolution and 100% efficiency but suffers from light sensitivity and radiation damage, so it will be used only for experiments where the gas counter is inappropriate.

Alternative Irradiation Protocols

In addition to the straightforward standard protocol in which we deliver a counted number of ions to the center of each cell nucleus present in the dish (*5, 6*), we are developing and using several other irradiation protocols. The first of these new protocols was developed to irradiate the cytoplasm of each cell. The image analysis system finds the long axis of each cell and then the computer system delivers particles at two target positions 8 μm away from each end of the cell. In these experiments, the computer generated exclusion zones around each nucleus to ensure that the target positions from one nucleus is not accidentally within the nucleus of a nearby cell. Wu *et al.* (*7*) reported mutation induction by cytoplasmic irradiation using this technique.

We are using two variants of the standard protocol to study bystander effects. In the first case, all of the cells are imaged, but the computer randomly irradiates only a chosen fraction of them. Zhou *et al.* (*8*) are using this technique. The second approach is to have a mixture of cells growing in the irradiation dish, but only a fraction of them are stained with Hoechst and are therefore visible to the image analysis system. The other cells might be stained with a dye of another color so they can be distinguished during later analysis. This is the technique preferred by Geard *et al.* (*9*).

Future Developments

It is clear from the present requests for beam time and discussion with users of the Columbia microbeam facility that the main interest for future use is to study bystander effects and to irradiate subcellular components. Both of these classes of experiments require better spatial resolution and the absence of a beam halo. It is not possible to obtain a beam with those properties using a collimator system. We are therefore designing a new microbeam facility that will use a compound electrostatic lens system to obtain a beam spot of sub-micrometer precision (*10*). A prototype of the lens has been constructed and is undergoing tests on the present microbeam station. The prototype is expected to provide a beam 2 μm in diameter with essentially no halo, while the final objective is to obtain a beam with a diameter of 0.3 μm.

Another limit of the present facility is that it can only be used to provide light ions with a limited range of LET. We plan to correct this limit by installing a laser-driven ion source that will produce ions with masses up to that of iron and thereby LETs as high at 4500 keV/μm. The combination of a large variety of ions and a focusing system will require new diagnostic techniques to ensure that all the parameters of the system are set to optimum values. We are designing an electron microscope to image the impact position of each ion by focusing the secondary electrons produced.

Conclusion

The Columbia University microbeam facility has been proven capable of satisfying its original objective of studying the ability of single α particles to produce transformational and mutational events in mammalian cells irradiated through the nucleus. New studies of subcellular targeting of radiation and bystander effects require upgrade of the facility to obtain a smaller diameter, halo-free beam.

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Nanoprobe Capabilities Using 25 MeV Protons or 200 MeV Heavy Ions

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A new microbeam facility, SNAKE [Superconducting Nanoscope for Applied nuclear physics (Kernphysikalische) Experiments], at the Munich 15 MV tandem accelerator is currently under construction (*1, 2*). Focusing 25 MeV protons or heavy ions with energies up to 200 MeV to submicrometer beam spot sizes gives new opportunities in material analysis and modification, especially for biological samples. The facility will be equipped with all the tools for materials analysis and irradiation like a germanium detector for PIXE and PIGE, a channeltron for secondary electron analysis, silicon strip detectors for identification of scattered particles, and a high-precision spectrometer for energy analysis of transmitted ions. This report describes the analytical potential of the new system with special emphasis on the physical properties of the high energies and the use of heavy ions. The main new capabilities are investigation of thick samples with high lateral resolution, a wide range of applicable energy losses, high-precision thickness measurements, and three-dimensional hydrogen analysis with the least radiation damage.

The whole microbeam facility is under construction at the Munich tandem accelerator. A two-stage ion optical system with an intermediate focus will focus the ion beam to a beam spot in the submicrometer range. The main demagnification is performed by the second lens, which is a completely new development. Its superconducting design enables a magnetic gradient of 1.2 T/cm and low aberration coefficient.

One limitation of microprobes is the finite range of particles in matter and the increasing lateral straggling with depth. Using the unprecedented high energies for proton microprobes, we will be able to investigate and modify even thick samples. The projected range of 25 MeV protons in water amounts to more than 6 mm. Furthermore, the lateral straggling of the incident ions due to small-angle scattering is drastically reduced due to the high proton energies, since the FWHM of the lateral distribution depends inversely on the ion energy. Even after passing a sample through 100 μm of water, the lateral spread of 25 MeV protons is less than 400 nm. This allows us to prepare specimens that are separated from the beam transport vacuum through thin foils and investigate or irradiate them *in vivo* with submicrometer resolution.

The use of heavy ions with energies up to 200 MeV·q²/nucleon allows us to adjust the stopping power from 2 keV/μm (25 MeV protons on water) to 10^4 keV/μm (200 MeV gold ions on water) (*3*). Therefore, the damage density for radiobiological experiments can be adjusted by using different ions and ion energies.

Spatially resolved density or thickness measurements of the specimen will be performed using STIM (scanning transmission ion microscopy), where the energy loss of each impinging ion is measured. A 90° magnet is installed in the 0° direction behind the target chamber, which allows an optimal energy resolution. Using heavy ions, a thickness resolution better than 1 nm will be possible for thin probes.

The highlight of the facility will be a sensitive three-dimensional detection of hydrogen using elastic proton–proton scattering. A coincident detection of the projectile and the target nuclei provides an effective suppression of background events. Furthermore, the proton–proton scattering cross section for 25 MeV protons is enhanced by a factor of 600 over the Rutherford cross section, and large solid angles of detection in forward direction can be used. Therefore, radiation damage, which is dominantly produced via Rutherford scattering processes on electrons or on protons, is reduced to an acceptable level for microbeam applications. As a consequence, the hydrogen distribution may be analyzed even in biological materials.

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The Ultrasoft X-Ray Microbeam: A Subcellular Probe of Radiation Response

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A new microbeam facility has been developed at the Gray Laboratory in collaboration with the King's College London. This microbeam uses diffraction optic techniques to focus ultrasoft carbon K-shell X rays (278 eV) to a spot of submicrometer size which can be used to irradiate single

cells *in vitro*. Characteristic carbon X rays deposit their energy through the photoelectric effect to produce about 14 ionizations in a 7-nm range in biological tissue. Focusing the X rays in a very fine beam will allow us to produce very localized clusters of energy deposition inside the biological samples. The extent to which the ionizations are clustered can be precisely controlled and reduced to the size of critical subnuclear targets such as higher-order DNA structures. The radiobiological importance of clusters of ionizations has been highlighted both by Monte Carlo track-structure simulations (*1, 2*) and by experimental studies (*3, 4*). Damage arising from clustered ionizations is considered particularly critical for the cell, as repair is expected to occur less efficiently with increasing damage complexity. The ultrasoft X-ray microbeam represents a unique tool for studying the relationship between radiobiological effect and the size of the clusters of ionizations produced. It is possible to vary independently both the flux of photons and their focused spatial distribution. In some studies we have substituted a laser plasma microfocus X-ray source (308 eV, single 7-ps pulse) to mimic certain features of high-LET tracks. In addition, the fine spatial resolution achieved with the X-ray microbeam can be used to assess how radiosensitive sites are distributed across the cell. Contrasting data have been reported regarding the sensitivity of different regions of the cell nucleus. Using α particles, Raju *et al.* (*5*) and Lloyd *et al.* (*6*) found that the most radiosensitive sites are located in the middle of the cell nucleus, while Cole *et al.* (*7*) indicated that the DNA close to the nuclear membrane is the main target. The identification of the major radiosensitive sites inside the cell represents an important step in analyzing data and in designing new experimental approaches. Should the chromatin be considered the major radiosensitive material, cells will have to be considered systems in which the spatial distribution of such material changes dramatically during the stages of the cell cycle. The ultrasoft X-ray microprobe offers also the possibility to carry out ''single-cell experiments'' in which only preselected cells can be irradiated and the radiation effect assessed, following the fate of each irradiated cell and its unirradiated neighbors. This will provide a new and very accurate approach to investigate the bystander effect by measuring critical parameters such as the range of intracellular interactions.

Characteristic carbon X rays are produced by bombarding a graphite target with electrons accelerated up to 30 keV. The electrons are produced by a heated tungsten filament and subsequently focused onto the graphite target by a water-cooled electromagnetic lens to produce a ''point'' X-ray source (less than 4 μm in diameter). The high-energy photons (bremsstrahlung), also produced by the electron bombardment, are eliminated using shallow angle reflection on a silica mirror. Measurements indicate that a nearly monochromatic X-ray beam (>95% beam purity) is achieved for a 2° reflection angle, while the carbon K-shell component is reduced by less than 30%. The main X-ray focusing element is a zone plate, which is a circular diffraction grating, typically 200 μm in diameter, with a radially increasing line density (*8*). A specific set of masks (i.e. a 30-μm-diameter anodized spot and a 12-μm pinhole) must be accurately aligned with the zone plate to avoid background and high-order focused radiation from reaching the samples. Accurate measurements performed by scanning a knife-edge mask across the pinhole indicate that the emerging X-ray beam is focused into a spot with a radius less than 250 nm. The present X-ray flux (about 1.5×10^3 carbon K-shell X rays per second) corresponds to a dose rate of about 0.15 Gy/s averaged over a typical mammalian cell. This low dose rate together with the high stability of the X-ray source (less than 5% fluctuation of the dose rate over a period of 3 s) and the possibility to measure the radiation effect on a single-cell basis makes the microprobe particularly suitable for low-dose studies. Computerized image analysis and micropositioning techniques are also employed to perform fast and accurate experiments. The system is similar to that used for the charged-particle microbeam (*9*). It is based on a three-axis micropositioning stage (precision of 250 nm/motor step) and an epifluorescence UV microscope with intensified CCD camera coupled to a fast PC. Biological targets are stained with fluorescent dye (e.g. Hoechst 33258 for the cell nucleus) and viewed under UV illumination (UV-radiation exposure of about 30 ms per cell) using a 10× or 40× objective.

Using software developed in-house, about 100 samples can be correctly located and aligned with the probe in less than 5 min during a completely automated experiment.

A single-cell survival assay has been established in preliminary experiments using cells of the Chinese hamster V79 line. The cells are seeded on a 0.9-μm Mylar film (65% transmission for carbon K-shell X rays) and, once attached (about 4 h), their nuclei are stained with a fluorescent dye (1 μM of Hoechst 33258 for 1 h). The cell nuclei are then located by scanning the cell dish under UV illumination and recording their positions. These coordinates are subsequently used to position the sample over the probe. The desired dose is then delivered by exposing the samples to the X-ray beam for a predetermined period (based on cell morphology and dose rate). After irradiation, the cells are incubated for 3 days, then individually revisited (again using the recorded coordinates), and the presence of surviving colonies (≥ 50 cells) is assessed. Measurements performed in the dose range (0.25 Gy) delineate a linear-quadratic survival curve, in good agreement with data obtained using conventional soft X-ray techniques. In particular, the low survival value ($1.2 \pm 1.1\%$) measured after 2.5 Gy indicates a correct dose delivery, while the small effect detected after 80 mGy underlines the possibility to perform precise low-dose experiments. The microprobe data do not show the hypersensitive response at low dose that has been detected previously using the charged-particle microbeam (3.2 and 1.0 MeV protons) and 240 kVp X rays (*10*). However, this is not in contradiction with the induced radioresistance hypothesis considering the high biological effectiveness of the carbon K-shell X rays [the hypersensitivity response has been found to be reduced by increasing the LET (*11*; Schettino *et al.*, unpublished results)] and the localized irradiation technique. The complex lesions produced by the carbon K-shell photons and the localization of the dose deposited could minimize the effect of the repair mechanisms triggered at low dose.

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Microbeam Irradiation Patterns to Simulate Dose

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Underlying the argument for the use of a linear extrapolation to estimate the risk resulting from low doses of radiation is the assumption that the effect is the result of an individual cell responding to damage done by energy deposited in that cell. Single-particle microbeam irradiation has shown that at least some biological end points can be produced by passage of a single α particle through a cell. There is adequate evidence that the health effect of greatest concern, cancer, is the result of the abnormal growth of a single cell. It is also known that, under normal circumstances, cells are influenced in many ways by their local environment. Cells are stimulated to divide, differentiate or self-destruct by signals from neighboring cells and the extracellular matrix. There is also evidence that some members of an irradiated population alter their levels of repair-related proteins and change their DNA metabolism even though they cannot have been hit directly by an ionizing particle. This strongly suggests that cells do not act autonomously, and it may be that interactions between hit and unhit cells influence the probability of an adverse effect in a way that depends on the number of hit cells in the population, that is in a dose-dependent way. Several characteristics of the radiation and the target system are likely to influence the magnitude and frequency of modifications to the response of individual cells. The obvious properties of the radiation are the stopping power, mass and velocity of the charged particle, the spatial distribution of the charged-particle tracks, and the temporal distribution of events. For relatively high-LET radiations (protons and α particles), the spatial distribution of events at a ''low'' dose is relatively sparse, typically an average of many cell diameters between charged-particle tracks. If the dose rate is low, adjacent tracks may be separated in time by many cell divisions. The relevant characteristics of the target system include everything that influences the amount of energy deposited, conditions (such as oxygen concentration) that influence the consequences of energy deposition, and factors (such as cell contact and extracellular matrix structure) which influence the communication of information and materials between cells.

Microbeam irradiation provides the means to vary the spatial and temporal distribution of the irradiation in a controlled fashion. There are many different ways to deliver the same average number of charged particles per cell. The geometry conventionally used for track-segment irradiation of cells in culture, charged-particle tracks perpendicular to the plane of a thin substrate supporting a monolayer of (flattened) cells, is not a realistic simulation of the energy deposition around cells irradiated in a tissue. In the tissue, typically several adjacent cells are traversed by the charged particle, potentially resulting in communication of damage-related signals between them and possibly in strengthening the signal transmitted to unirradiated adjacent cells. To select among the alternative irradiation patterns, we need some plan for investigating the mechanisms that may be important for health effects. The best approach is to start with models of some proposed mechanisms and devise experiments that can prove one or more of the alternatives to be wrong.

It is very early in the development of understanding of the effects of neighboring cells on radiation response, but a few models can be proposed on purely logical grounds. Communication may be through direct contact between cells or through release of compounds into the medium. To simplify, we will consider direct cell-to-cell communication only. The signal strength, that is the probability of a signal producing a specific observable change in an unirradiated cell, can be assumed to be proportional to the amount of energy deposited in the irradiated neighboring cell. An alternative model is that the signal strength produced by an irradiated cell is constant for any energy deposition above a threshold level, but the strength of the signal at an unirradiated cell is proportional to the number of cells surrounding it which have received more than the threshold level.

Both of these models could result in nonlinear effects at low doses, but the dependence on dose and LET they would produce would probably be quite different. Another factor that is important in the response is the distance (*d*) over which the signal acts. The simplest model is that the signal decreases as $1/d^2$ as it propagates through adjacent cells. However, the possible extremes are that it can be transferred to neighboring cells only through direct contact with the irradiated cell, or that it is amplified by each cell receiving it and is passed on without attenuation until it reaches a boundary it cannot pass (for example, edge of cell clone, change of cell type in a tissue).

These models suggest a number of experiments, involving different energy deposition patterns, which can be used to distinguish between the alternatives. These experiments would determine if cell communication is significant in determining the dose–response relationship at low doses. First, it will be necessary to determine how the probability of an effect of an energy deposition in a target cell depends on the spatial distribution of the initiating energy. If a confluent monolayer of cells, *t* micrometers thick, is used as a two-dimensional model of cells in a tissue, a given dose can be delivered by *n* particles, with range *r*, per cm² distributed as one per site with the sites uniformly spaced $n^{-1/2}$ cm apart, as *p* tracks per location with the spacing between locations increased to $(n/p)^{-1/2}$, or by *r/t* tracks spaced *t* apart with the pattern repeated at a spacing of $(nt/r)^{-1/2}$. This last distribution simulates the distribution of energy that would occur in a three-dimensional tissue. If the probability of producing a specific bystander effect is proportional to the total amount of energy deposited, the three patterns should produce the same frequency of effects. If there is a threshold value of energy deposition in a cell that is sufficient to trigger communication to neighboring cells, the number of affected cells should decrease with increasing *p* because the total number of cells exceeding the threshold energy deposition decreases. If the probability of effect is related to the number of cells exceeding the threshold level, the result will depend on the range of the interaction. We expect that use of models of the effects of cell communication will allow us to design more efficient experiments to test the linear, no-threshold model.

Track Theory Predictions for Single-Hit Cell Survival

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The track theory of cell survival after heavy-ion irradiation is based on the probability of survival after a single-particle transit, called ''ion-kill'' (not track core), joined to the cumulative effect of δ rays from adjacent ions in a beam, called ''gamma-kill'' (not penumbra). The model offers a set of equations containing four parameters, E_0 and *m* for the single-hit multitarget statistical model for gamma-kill, and additionally σ_0 and κ for ion-kill. A single set of parameters is used to fit a family of survival curves obtained with ions of different LET simultaneously. With these parameters, the model then predicts cell survival for arbitrary radiation fields (as in the spread-out Bragg peak, neutrons, even admixed with γ rays) whose particle-energy spectrum is known. It has predicted ''Katz tails'' (also called ''Darmstadt hooks''). Parameters have been fitted to upward of 40 sets of data for cell survival and transformation (*1*). These parameters and the equations of the model are now used to predict single-hit survival. We require as input data the atomic number *Z* of the bombarding ion and its relative speed β, and the identity of the cell so as to select the appropriate parameters κ and *m*. We then calculate the probability for ion-kill from Eq. (4) of ref. (*1*) as $P = [1 - \exp(-Z^{*2}/\kappa\beta^2)]^m$, with the effective charge Z^* from Eq. (2) of the reference. Note that ion-kill is responsible for all high-LET effects: increased RBE, decreased OER, loss of repair with the consequent fibrosis in heavy-ion and

neutron therapy, presumably from repopulation (analogous to scar formation).

Some results:

1. *Experiment*: At the Gray Laboratory: Single 3 MeV protons onto Chinese hamster V79 cells. No killing observed. Number of trials unstated (private communication). *Theory*: Probability for cell killing ~0.001. *Related data*: Warters *et al.* found that some 500 tritium β-particle decays in the nucleus of a CHO cell are required for observable cell killing (*2*).

2. *Experiment*: At Columbia (*3*): Single α particles (110 keV/μm) onto unidentified cells; 60–85% survival. *Theory*: For Chinese hamster cells, $\kappa = 1400$, $m = 3$, probable surviving fraction 33%. For T-1 kidney cells, $\kappa = 1900$, $m = 2.5$, probable surviving fraction 42%. Our calculations are made for α particles at 116 keV/μm, $Z^{*2}/\beta^2 = 2{,}290$.

3. *Experiment*: At Columbia University:[2] Single α particles (110 keV/μm) onto C3H 10T½ cells. Observed probability of oncogenic transformation 0.0001. *Theory*: At 116 keV/μm, probability for transformation 0.00021. Here $\kappa = 750$, $m = 2$. There is a geometric factor here, for α particles are directed through the nucleus rather than through the genome. Calculating for the nucleus, 91% of the cells are mutated. But if we take the geometric factor to be the ratio of s_0 for cell killing and for transformation, we find it to be 2.3×10^{-4}. Thus we expect that the fraction of cells undergoing an oncogenic transformation will be 2.1×10^{-4}.

4. *Experiment*: At Naples:[3] Single 4.3 MeV α particles onto Chinese hamster V79 cells. Probability of surviving 1 nuclear traversal 67 ± 10%. *Theory*: Probability for surviving 58%. Calculated for 1.167 MeV/nucleon for which $Z^{*2}/\beta^2 = 1{,}538$. Values of κ and m are as quoted above for these cells.

Some additional comments: We note that since single-particle transits are deterministic, while dose is a statistical concept, it is inappropriate to refer the effect produced by a single-particle transit to the ''dose'' it deposits, just as if one referred the kinetic energy of a single electron to its ''temperature'' (*4*). So also cross section (*5*) is a statistical concept. Thus it is inappropriate to apply the term cross section to the fraction of successes in targeted trials, as in microbeam experiments.

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Evaluation of the Risks Associated with Single-Particle Effects Using a Charged-Particle Microbeam

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We are developing *in vitro* models relevant to radiation risk using a microbeam capable of delivering individual charged particles to individual cells *in situ*. The system will allow us to critically determine the response of human cells to the single-particle traversals typically encountered in environmental exposures such as with radon, avoiding the confounding effect of the Poisson distribution of particle traversals inherent in conventional exposure systems. After environmental radon exposure, for example, virtually no cell receives more than one charged-particle traversal in its lifetime. Thus use of the microbeam will aim to produce data for direct input into the analysis of human health risks of environmental and occupational exposures involving charged particles.

The Gray Laboratory charged-particle microbeam delivers micro-collimated (1–5 μm) radiation using a 4 MV Van de Graaff accelerator (*1*). A computer-controlled microscope system is used to record the coordinates of and to irradiate individual cells. The cells can be stained with different fluorochromes to deliver individual particles to specified cellular locations, for example, Hoechst 33258 (nucleus) or rhodamine 123 (cytoplasm). Currently about 90–95% of the particles are delivered with a positional accuracy of ±2 μm and a detection efficiency of >99% (*2*).

The versatility of the microbeam allows for the study of acute irradiation damage, delayed instability and the bystander effect after single-cell irradiation. Previously, we have determined the induction of micronuclei in Chinese hamster V79 cells exposed to individual protons (*3*). We are currently studying radiation effects in a normal human fibroblast cell strain (AG-1522) by assessing the yields of both lethal and nonlethal chromosome damage (using FISH technology) and the yields of acentric chromosome fragments as micronuclei. Cells were irradiated by microbeam $^3He^{2+}$ particle traversals (used as surrogates for α particles, with an energy 3.5 MeV and an LET of 95 keV/μm) and by conventional ''broadfield'' α particles (^{238}Pu, 3.9 MeV, 110 keV/μm) for comparison.

We have measured chromosome aberrations produced by average numbers of α particles in chromosome 1 using FISH. Chromosome exchanges were scored by the Savage and Simpson nomenclature system (*4*). By this method, we found that around 20–30% of the total exchanges produced after 0.5–0.75 Gy α particles are complex, containing three or more breaks in two or more chromosomes. These results compared well with a previous study of chromosome 1 aberrations in human fibroblasts after α-particle irradiation (*5*). Multicolor painting of chromosomes 1, 4 and 8 showed 20–40% of complex exchanges at these radiation doses. This multicolor FISH approach is currently being developed to determine damage to chromosomes 1, 4 and 8 after microbeam $^3He^{2+}$-particle irradiation. Metaphase spreads will be prepared from irradiated cells and multicolor painted *in situ* on the microbeam dish, potentially allowing us to revisit the targeted cells to assess the quality of damage resulting from a single $^3He^{2+}$-particle traversal through the cell nucleus.

We are studying acute effects and the production of chromosomal instability at these low doses of α particles using the daughter cell micronucleus assay. Micronuclei were scored 72 h after conventional or microbeam irradiation in acridine orange-stained daughter cells. An individual $^3He^{2+}$-particle traversal induced micronuclei in 8% of the cells it traversed. At higher numbers of particles, the yield reaches a peak and then decreases, due to cell cycle delay. This effect was also seen after broadfield α-particle irradiation (*6*). Comparison between the two irradiation systems showed that an exact number of α particles (delivered by the microbeam) induces more cell damage as micronuclei than an average number of particles (delivered by conventional irradiation). At delayed times, up to 30 days later, the yield of micronuclei remains elevated due to the *de novo* production of instability. This instability is more pronounced for high-LET α particles in comparison to X rays.

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Single Alpha-Particle Traversals and Tumor Promoters

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In most homes, radon gas is present in such low concentrations that relevant bronchial cells are very rarely traversed by more than one α particle. However, cancer risk estimates for radon exposure are derived to a significant degree by extrapolation of epidemiological data from uranium miners whose bronchial cells were frequently exposed to multiple α-particle traversals. Recently, we reported results from a series of experiments in which oncogenic transformation was assessed after predefined exact numbers of α particles—delivered through the single-particle microbeam—traversed cell nuclei (*1*). Using positive controls to ensure that the dosimetry and biological controls were comparable, the measured oncogenicity from exactly one α particle was significantly lower than for a Poisson-distributed mean of one α particle, implying that cells traversed by multiple α particles contribute most of the cancer risk. Therefore, extrapolation from high-level radon risks could overestimate low-level (involving only single α particles) radon risks.

Of course, several caveats are required before such a conclusion could be applied to an epidemiological situation:

1. Our published studies so far refer only to cells that have not been damaged by tobacco, and it is likely to be the case that most (85–95%) of the lung cancer deaths that can be attributed to radon are actually the result of a synergistic interaction between α-particle damage and tobacco damage. So a direct interpretation of our results would be that the radon risk estimates for nonsmokers exposed to low levels of radon may be somewhat overestimated.

2. Our studies are in cells that are physically quite flat (in the direction of the α-particle beam), so the path length of α particles through these cells is probably shorter than that through target cells in the lung.

3. Our studies are in an *in vitro* rodent cell system, and thus potentially are not directly applicable to the appropriate human bronchial cells that are at risk. On the other hand, we are looking only at relative effects (e.g. the effects of one α particle compared to two α particles), not absolute effects, so there is no *a priori* reason why this *in vitro*

rodent cell system would produce misleading results regarding these relative effects.

It is important to recognize that homeowners are exposed to many potential carcinogens and promoters of tumorigenesis. Carcinogenesis is a multistep event that, in most cancer models, begins with exposure to a carcinogen during the initiation stage, followed by the promotion stage where tumor promoters are believed to have an impact on the expression of the initiated event (*2*). It has been known for some years that the tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA) significantly increases the frequency of X-ray- and neutron-induced oncogenic transformation (*3*). To explore the situation for a single α-particle traversal through a cell nucleus, mouse C3H 10T½ mouse cells were exposed *in vitro* to single α-particle traversals through cell nuclei—achieved with the single-particle microbeam—followed by treatment with TPA. The frequency of oncogenic transformation induced by the combination of a single α particle and a tumor promoter was compared to cells exposed to single α particles without TPA.

When compared to cells exposed to α particles alone, a synergistic increase in oncogenic transformation frequencies occurred with cells treated to the combination of single α particles and TPA. We compared these results with measurements of the TPA-enhanced transformation frequency of X rays—at an X-ray dose that produced the same transformation frequency (without TPA) as a single α particle (without TPA). Our preliminary results yield no significant difference in the TPA-induced enhancement between single α particles and X rays, suggesting that, in this case at least, RBEs of relevance to the radon problem are not very sensitive to changes in the tumor promoter environment.

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Studies of Targeted Effects on Human Lymphocytes Using a Charged-Particle Microbeam

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Evidence is rapidly accumulating that the progeny of normal cells exposed to ionizing radiation exhibit delayed responses including a high incidence of *de novo* chromosome aberrations, gene mutation and enhanced death rate. These delayed responses, most effectively demonstrated as nonclonal mutations/aberrations in the clonal descendants of irradiated cells, may be explained as manifestations of transmissible genomic instability. However, most of these heritable effects occur at a much higher frequency than would be expected from Poisson statistics of particle hits to surviving cell nuclei. This suggests that an area larger than the irradiated cell nucleus and dimensions is affected. The clearest indications

have come from studies using α particles at low doses where only a certain fraction of the cells or their nuclei are traversed by particles. Reported effects include changes in gene expression, enhanced frequency of sister chromatid exchanges, and chromosomal aberrations; and increased TP53 protein levels. Recently, similar observations generally termed bystander effects have also been made by a number of researchers. All these observations highlight the significance of non-nuclear targets, local microenvironment, and neighboring cells in modulating the mechanisms of genetic instability.

Given this background, it is clear that experiments need to be designed to identify specific targets for these effects and follow the response of cells to precisely delivered doses of ionizing radiation.

Several groups have developed charged-particle microbeams, in which cells are individually irradiated by a predefined exact number of charged particles, thus allowing the effects of exactly one or more particle traversals to be assessed. To date, the cell systems used in these studies have been established cell lines and primary human fibroblast cells. In these systems, cells are attached to the microbeam dishes to enable them to be irradiated. However, at present, there are no similar studies on the use of one of the most widely studied and important primary human cells, peripheral blood lymphocytes. The reason is that these suspension cells are not adherent to the microbeam dishes. We are reporting on the development of a robust method for microbeam irradiation of human G_0 lymphocytes from normal healthy donors by ^{3}H ions of LET 105 keV/μm. The culture method is used to assess the transmission of chromosomal instability in T lymphocytes through subsequent cell division. Furthermore, colonies derived from irradiated cells can be examined by both cytogenetic and molecular techniques. We will particularly concentrate on those abnormalities attributable to instability-derived aberrations and will compare them with those aberrations attributable to the immediate effects of the radiation.

Responses of XRCC5 Protein after Heavy-Ion Traversal of Cells

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Localized energy deposition by single energetic particles introduces a significantly large insult into a very small cell volume, but leaves most cellular constituents initially unperturbed and able to respond to the damage. Experimental quantification and localization of XRCC5 protein (formerly known as KU86) was accomplished in human cells as a function of exposure to very high-energy (1 GeV/nucleon) iron ions. Two cell types were used: the HeLa human cervical carcinoma cell line and the NFF human neonatal foreskin fibroblast cell strain. The doses of iron ions were calculated to give very low particle fluences to the cell nuclear cross section, based on a track-averaged LET of 120 keV/μm and appropriate cell nuclear cross-sectional measurements. Three nominal fluences, plus the sham control, were used: 0, 1, 2 or 4 particles per cell nuclear area. Cobalt-60 γ rays were used in parallel experiments for a comparison to low-LET radiation. After irradiation, cells are incubated for varying lengths of time, then fixed and stored at 4°C. In subsequent *in situ* assays, the XRCC5 DNA repair protein was immunocytochemically labeled. The cells were then viewed by confocal laser scanning microscopy, to locate and quantify the fluoresceinated probes within the cell.

Results so far have shown that irradiated HeLa cells show a distinct pattern of nuclear protein relocalization that is a function of the track structure of the radiation, the dose/fluence of the radiation, and the time after irradiation. In contrast, the human NFF fibroblasts show a more spread-out distribution of repair proteins in the nucleus. Measurements show that the DNA repair enzyme XRCC5 relocates to the sites of damage induced by the high-energy iron ions. There is also an apparent upregulation of the protein within the cell nucleus, as seen with *in situ* fluorescence labeling techniques, that is not fully borne out in Western

blot experiments. These show that an estimated 70% of the additional protein appearing in irradiated cells is already present in the cells.

We hypothesize that the radiation is also triggering a conformational change in the XRCC5 protein that is uncovering the antibody epitope. Using this localized damaging agent as a means to define a starting point (in time and space) for cell signaling, we are beginning to address questions on the timing and localization of protein responses to cell damage.

Use of a Collimated Heavy-Ion Microbeam for Irradiating Cells Individually

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In recent years there has been an increasing interest in the use of microbeams in radiobiological research (*1–3*). The use of a heavy-ion microbeam provides a unique way to control the number of ions traversing individual cells and localization of dose within the cell. Heavy ions transfer their energy to biological organisms through high-density ionization and excitation along the ion track even for uniform irradiation. This characteristic microscopically nonuniform dose delivery is expected to induce complex DNA damage and unique mutagenesis, and this radiation effect is in contrast to relatively uniform dose delivery in γ rays or electron-beam irradiation. Although the characteristic biological effects of heavy ions are supposed to link to the induction of high-LET-specific DNA lesions, i.e. clustered damage (*4*), other pathways of radiation effects are still interesting to consider. A heavy-ion microbeam can be used to look for pathways other than DNA damage, for example damage to the cell membrane or cytoplasm.

To investigate the distinct biological effects of heavy ions, especially to determine the effects of occupational and environmental exposure of very low fluences of heavy ions, one approach is to select cells with the desired exposures from a randomly irradiated population. Using conventional track-segment irradiation methods and ion-track recording techniques, the position of the target cells and the ion tracks can be measured together. However, this conventional approach is not practical because it is necessary to observe responses of many cells that do not contribute to the aim of the irradiation experiment before the measurement of the ion tracks. The alternative is to control each ion hit so that irradiation is not a Poisson random process. A heavy-ion microbeam can be used to irradiate selectively individual cells that can be analyzed afterward to determine what changes have occurred in that cell and in its unirradiated neighboring cells. There have been several reports that the effects of heavy ions may be transmitted from irradiated cells to neighboring unirradiated cells, i.e. bystander effects (*5*). The use of a heavy-ion microbeam will be a powerful experimental technique to study bystander responses.

Therefore, we are making an attempt to deliver a single or precise numbers of heavy ions with a spatial resolution of a few micrometers using our heavy-ion microbeam apparatus at JAERI-Takasaki (*6*). The microbeam apparatus was designed to develop a novel cell surgery technique using local irradiation of living organisms with targeted heavy-ion beams. The apparatus has been installed under a vertical beam line of the AVF cyclotron, which provides 12.5 MeV/nucleon $^4He^{2+}$, 18.3 MeV/nucleon $^{12}C^{5+}$, 17.5 MeV/nucleon $^{20}Ne^{8+}$, and 11.0 MeV/nucleon $^{40}Ar^{13+}$ ions. This heavy-ion microbeam apparatus has been applied for developmental biology as we reported at the 3rd International Workshop: Microbeam Probes of Cellular Radiation Response held in 1997. So far, fertilized eggs of the silkworm, *Bombyx mori*, have been used to analyze the fate of dividing nuclei or cells during the early embryogenesis, based

on relationships between the sites of local irradiation and localized defects induced on the resultant embryos.

Beam Collimation

Until now, neon- and argon-ion beams have been collimated to about 5–10 μm in diameter using a set of apertures. The minimum beam size is restricted by techniques for fabrication of a fine electrode for electrical discharge to drill a microaperture on a tantalum disk, and the thickness of the disk (hole depth) is limited to about 5–10 times the hole diameter by the discharge-machining technique. Accordingly, to collimate 13.0 MeV/nucleon ^{20}Ne^{7+} ions that can penetrate about 70-μm-thick tantalum, two tantalum disks 50 μm thick with nontapered 10-μm holes were stacked with their holes aligned precisely.

To characterize the collimated ion beams passing through the microaperture, the fluence and energy of the ions were measured by detecting scintillator light pulses in the atmosphere. The collimated ions were detected by a photomultiplier tube (PMT) assembly, which is capped with a plastic scintillator and mounted on the microscope turret; then the pulse-height spectrum of ions was viewed on a multichannel analyzer (MCA). The targeting accuracy of the collimator was evaluated by exposing a CR-39 film to the beams at the sample position. The exposed CR-39 film was developed by etching in 6 *M* NaOH at 60°C for about 0.5 to 1 h, depending on the LET of the ions. Thereby, the passages of the ions were visualized as a micrometer-size pit on the surface of CR-39, and the spatial distribution of etched ion tracks was observed by scanning electron microscopy. Using various sizes of microapertures (10 to 250 μm in diameter), more than 95% of the collimated ions were usually within the monoenergetic peak and about 85–90% of the ions were delivered within a 5–10-μm radius at the target.

Particle Detection and Counting

Detection of the individual heavy ions that interact with the biological target is critical for the study of effects at very low doses. Using relatively high-energy heavy ions, it is easy to detect the ions with a plastic scintillator after they have passed through the biological sample to be irradiated. Since the ions can be stopped in the scintillator, the signal is large enough to eliminate the signal-to-noise ratio problems that affect most other detection systems. This approach has another advantage that the detection process does not cause the divergence of the ion beams, compared with the use of an ion chamber or a secondary-electron foil upstream of the target.

The desired irradiation point on the biological sample was aimed to the beam position (microaperture on the tantalum disk) viewed through the object lens by remote controlling the x-y-z stage of the inverted optical microscope. Then the object lens was replaced with the PMT assembly mounted on the microscope turret. The number of ions traversing the sample was counted with a constant fraction discriminator or MCA by detection of scintillator light pulses. Every irradiation was stopped by the action of a beam shutter, which was controlled by the preset counter module or by preset timer. The particle fluence can also be determined by electron microscopic counting of etched ion tracks on CR-39 that had been exposed to the ion beams at the sample position. Sometimes the number of detected ion tracks on the exposed CR-39 did not agree with the number of ions counted by MCA using PMT signals. The difference is due mainly to the unexpected ion tracks caused by scattered ions with reduced energy. The probabilities of ''mis-irradiation'' with the uncounted ions on the target cells can be estimated from the spatial distribution of the scattered ions around the target point.

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The Role of Oxidative Stress and DNA Damage in Genomic Instability: Targeted Microbeam Studies in Primary Human Fibroblasts

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An important consequence of radiation exposure in cellular systems is the observation of delayed effects in the progeny of the surviving cells in the form of genomic instability (for review see refs. *1, 2*). Little is known regarding the mechanisms underlying this phenotype and in particular the triggering pathways, although delayed oxidative stress has been reported to play a role (*3*). An important question is whether direct damage to nuclear DNA is necessary for instability to be observed. By using a novel charged-particle microbeam (e.g. refs. *4, 5*), we can deliberately target the cell cytoplasm or nucleus and determine the role of initial DNA damage on delayed effects.

In primary human AGO1522B fibroblasts, we have measured the yield of micronucleated cells using daughter cell analysis after exposure to 240 kV X rays or α particles (LET = 110 keV/μm) from a ^{238}Pu source. For both radiations we observed a maximum in the production of micronuclei at up to 3 days after exposure which is dependent on dose. The level of micronucleated cells does not decrease to control values with time but remains elevated in the dividing population up to 30 days after irradiation. In general, the levels of delayed micronuclei are significantly higher for α particles than for X rays at doses chosen to give similar levels of cell killing, suggesting that there is a substantial effect of LET on the induction of genomic instability in this system.

We are currently measuring levels of oxidative activity in irradiated cells to determine whether the instability phenotype we observe is a consequence of an increased persistence of oxidative activity in the irradiated progeny and whether there is an LET dependence. These preliminary studies have monitored within cells using 2′,7′-dichlorofluorescein (DCF) as an indicator of the level of oxidative activity (mainly hydrogen peroxide). After irradiation, cells are incubated with 2′,7′-dichlorofluorescin diacetate (DCFH-DA), which is taken up by cells and metabolized by intracellular esterases to nonfluorescent 2′,7′-dichlorofluorescin (DCFH). In the presence of reactive oxygen species, the DCFH is rapidly converted to highly fluorescent DCF. Preliminary studies have shown increased oxidative activity after exposure of cells to X rays, both immediately after irradiation with high doses (5–50 Gy) and at delayed times up to 20 days later in the surviving progeny, using hydrogen peroxide-treated (100 μ*M*, 15 min, 4°C) cells as positive controls.

To determine whether direct damage to the DNA is necessary for

instability or oxidative stress to be observed, we have begun experiments targeting charged particles to discrete regions within cells. The cell coordinates have been determined by computerized imaging of the fluorescence of the cell nuclei stained with the DNA-binding dye Hoechst 33258. These coordinates were used directly in experiments where irradiation was targeted through the center of each cell nucleus. Preliminary experiments have targeted cells with $^3He^{2+}$ particles ($\sim$100 keV/μm). Delivery of five $^3He^{2+}$ particles through the center of the nucleus leads to the production of micronuclei in 7% of the daughter cells 3 days later, 3% 4 days later, and 2% 6 days later. For experiments targeting cytoplasm only, dihydrorhodamine 123 (DHR 123) is used to stain mitochondria and allow targeting outside of the cell nucleus. Irradiation of cells with five $^3He^{2+}$ particles through the cytoplasm also leads to micronucleus production. For the investigation of oxidative activity in this experimental system, cell medium containing DCFH-DA was added to the microbeam dish 1 h before the assay, and the coordinates of DCF-fluorescent objects were automatically imaged. The coordinates of all cells on the dish area were determined as soon as possible after oxidative activity measurements, and correlated to those of DCF-fluorescent objects. Monte Carlo-type calculations were performed to determine the probability of two color coordinates overlapping by chance. We have observed increased oxidative activity *in situ*, which in these experiments occurs within a similar time scale to that of micronucleus formation. In cells irradiated through the nucleus, the relative activity has been observed to increase with time postirradiation. After the irradiation of cytoplasm, such an increase has not been observed. Experiments are in progress, with the aim of determining the persistence of the elevated micronucleus formation over many cell generations and the correlation between the elevated levels of oxidative activity and induction of damage.

Recently we have reported the occurrence of a bystander effect in these fibroblasts (*6*). By using the DCFH-DA, we aim to investigate whether oxidative stress plays a role in situations where only one cell is irradiated in a population. Postirradiation measurements of oxidative stress levels *in situ* will be obtained, and the incidence of apoptosis and formation of micronuclei will be scored. Preliminary experiments show an increased oxidative activity in bystander cells. Moreover, we have observed the occurrence of apoptosis in the oxidatively stressed subpopulation of bystander cells after irradiation of one cell per dish only.

In summary, we present evidence that direct DNA damage is not a prerequisite for induction of the instability phenotype in normal human fibroblast cells. The correlation between induction of oxidative activity and apoptosis in cells irradiated through the nucleus has been observed. We also present the observation of delayed apoptosis within the subpopulation of oxidatively stressed bystander cells after irradiation of a single cell.

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Cellular Adaptive Response to Single Tracks of Low-LET Radiation and the Effect on Nonirradiated Neighboring Cells

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Investigation of the response of cells to microbeam irradiation has been performed using high-LET radiation. Here the dose to the irradiated nucleus is relatively high, even though the cell receives only a single radiation track. We report on the response of cells to single tracks of low-LET ^{60}Co γ radiation.

We have studied the adaptive response because this mechanism has previously been shown to be responsive to low doses of radiation. We have quantified the level of adaptation induced in cells by a prior exposure to a low priming dose by measuring the reduction in micronucleus formation after a large dose of radiation (4 Gy). Traditionally, experiments have used low doses of γ radiation in the range of 10 cGy or more to induce the response, but even at 10 cGy this dose corresponds to multiple tracks per cell. It has previously been shown that human skin fibroblasts (AG1522) exposed to 0.5 Gy delivered at a high (2 Gy/min) or low dose rate (0.0025 Gy/min) and subsequently irradiated with an acute 4.0-Gy test dose contained less micronuclei than cells exposed to the acute dose alone (*1*). This work demonstrated that low dose rates were more effective than acute dose rates at inducing the adaptive response in human cells.

We have also shown that the adaptive response can be initiated by much smaller doses of γ radiation (1 mGy) whereby, on average, each cell receives about one track per cell. The magnitude of the adaptive response induced by 1 mGy was similar to the level induced by higher priming doses and corresponded to a reduction in micronucleus formation of about 30% (E. J. Broome, M. Sc. Thesis, University of Ottawa, 1999). Similar results were obtained when the cells were exposed to ^{60}Co γ rays or tritium β particles. In other experiments, there was a reduction in the level of spontaneous neoplastic transformation in C3H 10T½ cells after exposures to 1 mGy (*2*). Also, exposure of human lymphocytes to doses at or below 1 mGy enhanced the expression of IL2 receptors on the lymphocyte surface, when the cells were subsequently stimulated to divide (*3*). Together, these results demonstrate that doses as low as one track per cell can alter cellular responses to radiation.

The influence of low doses corresponding to less than one track per cell is unclear. Our latest results from two experiments show that when cells were exposed to a priming dose of 0.1 mGy, which was 10-fold lower than previous experiments and corresponding to an average of much less than one track per cell, the level of reduction in micronucleus formation (after 4 Gy) was similar to that induced by higher priming doses, when every cell received one or more tracks. However, at even lower priming doses (0.05 and 0.02 mGy), there was no apparent adaptive response.

In conclusion, results indicate that nonirradiated neighboring cells can show an adaptive response in response to a signal produced by irradiated cells that were hit by a single low-LET radiation track. We are testing whether this cell-to-cell communication is dependent on cell-to-cell contact or mediated through diffusive factors.

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Epithermal Neutron RBE Determination via Proton Bombardment of V79 Cells

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Introduction

Data required for the determination of neutron relative biological effectiveness (RBE) values as a function of neutron energy are limited due to the scarcity of uncontaminated, monoenergetic neutron beams. Energetic neutrons, however, deliver dose to tissue primarily via elastic collisions with light hydrogen, creating energetic recoil protons. The energy transferred to the proton depends on the angle of scatter and ranges from zero up to the entire kinetic energy of the incident neutron. Since it is difficult to generate monoenergetic neutron beams, experimental determination of neutron RBEs as a function of energy can be approached by evaluation of the biological effect of monoenergetic protons of various energies. The RBE for neutrons of a given energy can thus be evaluated by integrating over the RBEs of protons expected in the recoil proton spectrum of that neutron energy. This is the approach under development at the MIT Laboratory for Accelerator Beam Applications. The neutron energy range of interest, a few tens of keV to 1 MeV, is that encountered in neutron capture therapies. Neutron RBE data in this energy range are particularly scarce.

Briefly, cells are plated on a thin film that also serves as the vacuum window for the accelerator beam tube. Monoenergetic protons irradiate the cells and cell survival is subsequently determined. Details of the development of this approach are provided below. Final determination of RBEs will be reported at a later date.

Accelerator

Proton irradiation is carried out using the tandem electrostatic accelerator installed at MIT's Laboratory for Accelerator Beam Applications. This accelerator, designed by Newton Scientific Incorporated, has been operational since 1996 and generates protons, deuterons and α-particle beams (*1*). A multiport, large-bore switching magnet allows permanent installation of five different experimental end stations. The beam energy resolution (at ~1.8 MeV) is less than 100 eV. Low-current proton beams down to 50 keV have been reliably achieved in vacuum. The accelerator beam current is measured with a programmable electrometer with a resolution of 0.1 fA.

Vacuum Window and Growth Surface

Because of the limited range of the low-energy protons used in these experiments, cells are plated directly onto the surface serving as vacuum window. The need for a very thin window (to minimize proton energy and range straggling) must be balanced by the need for a material which will hold vacuum (~1.5×10^{-6} Torr) over an area large enough to plate a significant number of cells. Polyimides and silicon nitride were found to have the highest strength properties over a sufficient area.

The polyimide Kapton™, with a nominal thickness of 7.5 μm was obtained from DuPont's High-Performance Film Division and tested as a vacuum window and cell growth surface. Stronger and thinner polyimides are available, but at considerable cost compared to Kapton™. These will be investigated at a later date. The 7.5-μm Kapton™ was chosen mainly

TABLE 1
Energy Straggling through 7.5-μm Kapton™ Window

Energy to window (keV)	Energy to cells (keV)	Straggle (keV)	Percentage straggle
604	10	9	90.00
614	25	14	56.00
625	47	16	34.04
635	70	17	24.29
645	94	17	18.09
655	117	17	14.53
675	163	17	10.43
695	205	16	7.80
795	390	18	4.62

to test the ability of the V79 cells to attach and form colonies on a polyimide and to perfect the technique of mounting the polyimide to form a suitable vacuum window.

The Kapton™ film is mounted on 18-8 stainless steel fender washers with a diameter of 37 mm, which is the approximate width of the accelerator beam port at the irradiation site. A commercially available glue, Spray Mount™, is used to bind the Kapton™ to the washer. The washer has an exposed area of 75 mm² (a 10-mm-diameter hole), where the V79 cells are plated prior to irradiation. The Kapton™ is capable of withstanding vacuum over a larger area, but the deflection of the window while exposed to vacuum is substantial. Each Kapton™ window is pretreated with Cell-Tak™, a cellular adhesion protein matrix solution, to ensure cellular attachment throughout irradiation.

Beam Uniformity

To ensure all cells plated in the Kapton™ window receive the same dose during the irradiation, it is necessary for the proton beam intensity to be uniform across the window. This is accomplished by defocusing the proton beam so that the portion of the beam profile hitting the 75-mm² area covered by the Kapton™ is flat. Beam uniformity is confirmed by replacing the Kapton™ with MD-55 GafChromic film from which the 65-μm protective layer was missing. (That is, the protons interact directly with the 20-μm sensitive layer.) The radiochromic film is irradiated under the same conditions (except for total dose) as used for cellular irradiation and then examined for uniformity of optical density. Profiles taken across the film show fluctuations in optical density of less than 10%.

Cell Plating

Chinese hamster V79 cells were chosen as the test cell line for the initial experiments because of their relatively short doubling time (10 to 12 h) and their ability to survive in relatively harsh conditions. In addition, their radioresponsiveness is well understood and documented. The cell plating efficiency was tested with all materials that composed the Kapton™ washers; each component of the Kapton™ washer window was studied independently. The 18-8 stainless steel portion had no discernible effect on cell survival, as expected, since this type of steel is routinely used in biological applications. The Kapton™ alone also had no discernible effect on cell survival. The Spray Mount™ adhesive alone prohibited cell colony growth after 4 days. The combined stainless steel, Kapton™, and Spray Mount™ washer reduced the plating efficiency to 70% from 85% for a 60-mm tissue culture dish. After irradiation (see below), cells are removed from the Kapton™ window using 100 μl trypsin and replated in a larger culture dish. This step results in 75% retention of plated cells.

Dose Determination and Radiation Delivery

Proton energy loss (and straggling) through the Kapton™ vacuum window for protons of various initial energies was calculated using the TRIM software (*2*). Absorbed dose to V79 cells plated on the film was then determined as a function of particle current by assuming cell thick-

ness and diameter of 0.5 and 10 μm, respectively. This was separately calculated for protons over a wide range of energies; a portion of the results are shown in Table 1. Knowing the energy deposited per particle allowed for the determination of the total beam fluence required to deliver the desired dose.

Prior to irradiation, cells are plated on the Kapton™ window and allowed to attach for 3 h. A cap filled with medium is then used to cover the cells to keep them moist during irradiation and handling. The cell assembly is then affixed to the end of the accelerator beam port by opening the manual gate valve. The air injected into the vacuum system is pumped out within 5 min. The manual gate valve, at that point, is open but prevents protons from striking the cells due to the presence of a blocking plate. The proton beam is then adjusted to the desired current and energy as measured on the blocking plate of the manual gate valve. With the manual gate valve open, the pneumatic gate valve (millisecond closing time) is used to control the irradiation of the cells to the desired proton fluence and hence dose. Dose rates of approximately 0.12 ± 0.01 Gy/s are generated based on accelerator beam currents of the order of 6 ± 0.01 pA. Final determination of RBEs acquired using this approach will be reported at a later date.

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X-Ray Fluorescence Spectroscopy Imaging and X-Ray Absorption Fine Structure Spectroscopy Using the Synchrotron Radiation Microbeam

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Introduction

We first discuss the problems related to the accumulation of and chemical states of metallic elements in a single neuron. In spite of the small amount of metallic elements in the bodies of humans and animals, the trace elements play an important role in living organisms. The excessive accumulation in neurons is considered to be directly correlated to neurodegenerative disorders. For this purpose, X-ray fluorescence spectroscopy (XRF) with the SR microbeam was performed to analyze the elemental distributions in small regions of human brain tissues. In addition to the distributions of the constituent elements, the chemical states of the metallic elements have great importance in considering the biochemical reaction in cells and malfunction of neurons. Therefore, X-ray absorption fine-structure spectroscopy (XAFS) with the SR microbeam was used to investigate the chemical state of iron in neurons.

In this study, human brain tissues of a patient diagnosed with Parkinson's disease and a control were analyzed. Parkinson's disease is one of the major neurodegenerative diseases. Neuropathological studies have revealed that the substantia nigra, which is a part of the midbrain, of patients with Parkinson's disease is damaged. The cause of this neurodegeneration is unknown, but there are indications that iron in substantia nigra neurons is related to the neuronal degeneration. The increased iron in affected neurons may be responsible for the degenerative process in Parkinson's disease. Therefore, mapping of iron and other elements in a single neuron has a great significance in trying to understand the role of metal elements in the neurodegenerative process.

We next present experimental results on the application of a focused beam from a synchrotron radiation source to measure the distribution and the chemical shift of the trace elements (iron, chromium, nickel, etc.) incorporated into a matrix of the human tissues.

In our previous studies we applied PIXE, microbeam PIXE, and SR-XRF to investigate the release of iron and showed that the materials from implants dissolve in the human body and release metallic or polymeric elements during the long periods that they are inserted in the human body. The chemical interactions between the tissues and surface of the implants and the mechanical friction of the implants are considered to be responsible for the release of metals into human tissues. Release of ions and evaluation of the toxicity of the released elements have been the subject of several *in vivo* and *in vitro* studies. It is very important to investigate the distribution and the chemical state of the trace elements to study the mechanism of dissolution of the implant material in the human body and the toxicity of the released elements.

XRF Imaging and XAFS Spectroscopy of a Single Brain Cell

Autopsy specimens of midbrain, including substantia nigra cells, were obtained from a male patient with Parkinson's disease. The specimen was fixed in 10% formalin, embedded in paraffin, and then cut into 8-μm-thick sections. Finally, the sections were mounted on a Mylar film for XRF spectroscopy.

XRF spectroscopy was carried out at the BL39XU in SPring-8, Harima, Japan. The experimental conditions for obtaining the elemental distribution were as follows. The X-ray energy was 7.2 keV. The incident SR beam was restricted by a set of x-y slits and a pinhole on the front side of the sample and the cross section of the beam was 10 μm. The sample stage was moved by x-y step pulse motors and distributions (fluorescence X-ray intensity maps) were obtained. The scanning area was 300×210 μm² and was divided into 40×28 pixels. The area of each pixel was 7.5×7.5 μm². Each measuring point of the sample was irradiated for 5 s. The beam line was equipped with a CCD camera in front of the sample holder. The image from this camera gave us visual information for the measuring points.

To obtain the relative concentration of all constituent elements in the cells, fluorescence X-ray spectra were also obtained at several points of interest in the scanning area. The cross section of the beam was 10 μm. The measuring time for each point was 100 s, and the X-ray energy was 7.2 keV.

XAFS spectroscopy of iron K-shell edge was performed using a Si(111) double-crystal monochromator. In addition, absorption spectra of several iron oxide powder samples were measured as experimental standards. The energy scan step was 0.001 keV. The measuring time for each step was 60 s for the brain cells and 1 s for the iron oxide powder samples.

The XRF imaging results clearly demonstrate that iron and titanium were accumulated in and around the neuromelanin granules of the dopaminergic neurons. XAFS spectroscopy using a microbeam enables us to analyze the chemical state of iron in the neuromelanin granules. Further investigation is needed to establish a clear relationship between the accumulation of metallic elements and the corresponding pathological effects.

SR-XRF and XANES Spectroscopy of Metal Ion Release in Human Tissues

The SR ring with the following parameters was used for the X-ray fluorescence spectroscopy. The monochromatic photon energy was 14.2

keV, the electron beam current was between 250 and 400 mA. In a typical spectrum of the SR-XRF analysis, metal elements such as titanium, chromium, iron and nickel are detected with a high sensitivity. These elements are originated from particles as a result of friction between the head and cup of the hip joint system. The detailed imaging shows evidence of the incorporation of micrometer- and nanometer-sized particles of metallic elements at a single cell level. These data show that iron was dissolved and was distributed in the tissues.

The iron K-shell edge XANES (X-ray Absorption Near Edge Structure) spectrum was also measured. This spectrum shows that the chemical state of iron changed slightly. Considering the change in the chemical state and the iron:chromium ratio calculated from the X-ray fluorescence spectrum, it is considered that dissolution of iron in the tissues occurred. Microbeam synchrotron radiation (SR-XRF and XANES) has been used for the detection of metal ions, mapping the elements in a large volume of biological tissues.

The results from SR-XRF imaging show that iron was released and distributed in the tissues. On the other hand, the XANES analysis shows that final chemical state of iron was $+3$, which may come from Fe_2O_3. Detailed mapping at the level of the cell will make it possible to further investigate the interactions of the accumulated elements in the cells and the effects on the normal functioning of the cells.

Intra- and Intercellular Responses after Cell Site-Specific Microbeam Irradiation

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A charged-particle microbeam has, in a definitive manner, the capacity to place defined numbers of radiation tracks in a controlled spatio-temporal framework, both within and between individual cells. At the Columbia microbeam facility, we have developed protocols to place exact numbers of charged particles through nuclear centroids of cells, at defined distances off the nuclear centroid, at defined positions in the cytoplasm relative to the nucleus, at defined positions in the cellular milieu (deliberately missing cells), and through defined fractions of cells in a population. Vital dye staining protocols have also been developed to allow the targeting of subcellular entities or of known cells in mixed cell populations (i.e. hit compared to nonhit or bystander cells). Cells can also be imaged offline using conventional transmission microscopy and their positional coordinates recorded before moving the entire stage and cell dish, with submicrometer precision, to kinematic mounts on the online microbeam microscope. In this way cellular staining and reflected fluorescence may be avoided if desired, with little impact on irradiation speed.

The accuracy of the current Columbia microbeam system is such that more than 90% of particles are within 3.5 µm of a designated coordinate which, together with cellular throughputs of up to 15,000 cells per hour (depending on the application), has allowed for definitive assessments of exact single-particle responses for mutation and oncogenic transformation; this obviates the uncertainties of Poisson-distributed particle numbers from broad-beam or isotopic sources.

The basic paradigm that the directly damaged cell nucleus is the preeminent responder to radiation has been brought into question with findings of cell responses to cytoplasmic irradiation only. In addition, microbeam irradiation of known small fractions (e.g. 10 or 20% of cells) in a population has produced responses in nonhit or bystander cells. Coculturing known hit and nonhit cells has allowed evaluations of responses in cells that are otherwise handled identically. A fluence-dependent bystander effect has been definitively demonstrated for reduced cell growth, for induced delay in cell cycle progression, for the induction of micronuclei, for the differential expression of TP53 and CDKN1A, for mutation, and probably for oncogenic transformation.

What have we found so far?

1. One transnuclear α particle can produce a micronucleus—further increases are dependent on fluence.
2. The nucleus is nonuniformly sensitive to α-particle damage.
3. One transnuclear α particle can initiate cell cycle delay—further delay is dependent on fluence.
4. One transnuclear α particle can initiate a TP53 response—dependent on cell site (cytoplasm/nucleus), time and fluence.
5. Cytoplasmic α-particle irradiation can initiate a TP53 response.
6. A bystander effect has been clearly demonstrated: Nonhit cells show hit-cell fluence-dependent cell cycle delay, slowed growth, enhanced micronuclei, and enhanced TP53/CDKN1A response.

Understanding Bystander Responses in Cellular Systems: Studies Using a Charged-Particle Microbeam

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Several recent studies have shown that bystander effects may be important in irradiated cell populations. These are observed when cells not directly exposed to radiation show effects when their neighbors are exposed. To date, these studies have involved extrapolation from situations where conventional low-dose α-particle exposures are used such that only a fraction of the population is subject to a particle traversal (*1, 2*). Alternatively, they have been observed when medium is transferred from an irradiated to a nonirradiated culture (*3*). In a series of experiments, we have shown the first direct and conclusive evidence for a bystander response in primary human fibroblasts, where an individual cell was exposed *in situ* to low numbers of charged particles (*4*).

Using our charged-particle microbeam (*5, 6*), it is possible to deliver precise numbers of particles to a selected cell within a population and then monitor effects on that cell and its nonexposed neighbors. The Gray Laboratory charged-particle microbeam collimates the beam down to below 5 µm in diameter (in contrast to the size of the cell nucleus in these studies, which is up to 20 µm in diameter). The exposure is controlled with the use of a scintillation detector, which counts each particle delivered to the selected cell and terminates the exposure by a fast-acting shutter when the desired counts have been obtained. We have selected a single cell within 600–800 cells and delivered between 1 and 15 $^3He^{2+}$ particles (105 keV/µm; used as surrogates for α particles) through the center of the nucleus. Three days later, the cells were fixed and stained. In comparison to control dishes, where the same number of particles were delivered but to a location outside a cell, we observed an approximately two- to threefold increase in the background frequency of damaged cells present. With this cell system, we observed both evidence of micronucleus formation and apoptosis. Importantly, this increase is independent of the number of particles delivered to the targeted cell, with the effect being observed after the traversal by a single ^3H particle. In these experiments, cells at the time of irradiation were spread over an area ~1 cm² with minimal cell–cell contact. The medium was normally changed with-

in 30 min of the irradiation. Damaged cells appeared essentially randomly over the area of the culture dish when scored 3 days later.

Taken together, the data suggest that an extracellular factor is involved as has been proposed by other studies (*3, 7*), although they do not entirely rule out any role of gap-junctional intracellular communication, if cells had been in close contact with one another (*8*). If bystander effects also occur *in vivo*, these observations may have important implications for risk estimates at low doses. Overall, current data indicate that the phenomenon must be a low-dose effect, or it would be relatively easy to sterilize any population of cells. A key question is thus the relationship of the bystander effect to the normal acute response and at what stage it becomes saturated. Other workers have also shown that the bystander effect may be related to genomic instability (*9*). In the fibroblast system used here, instability also occurs (*10*), and it will be important to determine its relationship to the bystander response in conditions of low-dose exposure.

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Studies of Bystander Mutagenic Response Using a Charged-Particle Microbeam

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It has been accepted for a long time that the deleterious effects of ionizing radiation such as mutation and carcinogenesis are due mainly to direct damage to DNA. However, recent circumstantial evidence suggests that extranuclear or extracellular targets may also be important in mediating the genotoxic effect of radiation. For example, very low doses of α particles induced sister chromatid exchanges (SCE) in both CHO and human fibroblast cultures at levels significantly higher than expected based on the number of cells that had been traversed by an α particle (*1, 2*). The additional responding cells which received no radiation exposure either were neighbors of directly hit cells or resulted from agents released from the irradiated medium (*2, 3*). Subsequent studies suggested that reactive oxygen species might contribute to the induction of SCE among the bystander cells (*4*). Enhanced expression of the *Tp53* (*TP53*) tumor suppressor gene in bystander cells has also been reported in immortalized rat lung epithelial cells and human diploid fibroblast cells irradiated with α particles (*5, 6*). While circumstantial evidence in support of a bystander effect appears to be consistent, direct proof of such extranuclear/extracellular effects are not available.

Using a precision charged-particle microbeam, our laboratory showed recently, and for the first time, that irradiation of cellular cytoplasm with either a single or an exact number of α particles results in gene mutation in the nucleus while inflicting minimal toxicity and that free radicals mediated the process (*7*). These results with the radical scavenger, dimethyl sulfoxide (DMSO), and the thiol-depleting drug buthionine *S-R*-sulfoximine (BSO) provide further support of the idea that ROS modulate the mutagenic response of cytoplasmic irradiation. More recently, Prise *et al.* reported that a single human fibroblast irradiated with five α particles from a microbeam induced a significant increase in micronuclei among neighboring cells, although no explanations of the mechanisms whereby a single irradiated cell mediated the bystander effects were provided in this study (*8*).

Since individual cells are irradiated one at a time, a sensitive mutagenic assay system is essential. The A_L hybrid cells that contain a standard set of Chinese hamster chromosomes and a single copy of human chromosome 11 fulfill this requirement. Chromosome 11 encodes cell surface markers that render A_L cells sensitive to killing by specific monoclonal antibodies in the presence of complement. Approximately 500 exponentially growing A_L cells were inoculated into each of a series of microbeam dishes constructed by drilling a $\frac{1}{4}$-inch hole in the center of 60-mm-diameter nontissue culture dishes. A 3.8-mm-thick polypropylene film was epoxied over the bottom of the hole, creating a miniwell that was then coated with Cel-Tak to enhance cell attachment. Two days after plating, the DNA of attached cells was stained by treatment with a 50 n*M* solution of Hoechst 33342 dye for 30 min. The image analysis system then located the centroid of each cell nucleus and irradiated the cells one at a time with an exact number of α particles. Twenty percent of A_L cells plated on the microbeam dishes were irradiated randomly. After irradiation, cells were maintained in the dishes for 3 days before being removed by trypsinization and replated into culture flasks. After culture for 4–5 days, the cells were trypsinized and replated to measure mutation as described (*9*).

Irradiation of 20% of randomly selected cells with 2–20 α particles each resulted in mutant fractions which were significantly higher than the expected yield assuming there were no bystander effects. Concurrent treatment of cells with DMSO has no effect on the incidence of mutations. Our studies give direct evidence that irradiated cells may induce a mutagenic response in neighboring cells not directly traversed by α particles, and that a signal transduction pathway other than oxidative stress plays a critical role in mediating the bystander effect.

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Studies of Bystander Effects in Primary Uroepithelial Cells Using a Charged-Particle Microbeam

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We have recently found direct evidence for a bystander effect in normal human fibroblasts by targeting individual cells with a microbeam (*1*). The main aim of the present work is to investigate the mechanisms involved in the bystander effect after low-dose microbeam irradiation of cells of normal human uroepithelial cell lines and primary explants. In particular, we are studying the role played by the pathway to apoptosis in bystander effects. Epithelial cells have highly developed intercellular communication systems that may be involved. Also, the ureter and bladder primary explant technique (*2*) allows us to study bystander effects and genomic instability under *in vivo*-like conditions where dividing and differentiated cells are present. Both human and pig uroepithelium have a very well defined structure *in vivo* (*3*). The basal layer next to the lamina propria consists of proliferating cells. Two or three intermediate cell layers consist of nondividing and partially differentiated cells. Finally, the superficial cell layer located close to the lumen consists entirely of fully differentiated cells. The primary explant outgrowth technique allows us to reconstruct *in vitro* the normal stratification of uroepithelium. A normal *in vivo*-like proliferative structure is observed where differentiated cells are concentrated at the center and dividing cells on the periphery of the explant outgrowth.

Human or porcine ureter samples were used to establish primary uroepithelial explants on microbeam dishes and grown for 5–7 days prior to irradiation. For microbeam irradiation, the nuclei of 10 cells on the periphery or at the center of the explant were exposed to 10 ^{3}He^{2+} particles using the Gray Laboratory charged-particle microbeam (*4*). After 3 days of incubation, the samples were fixed and stained with acridine orange. Damaged cells classified as micronucleated or apoptotic cells were count-ed throughout the entire explant outgrowth. The spatial distribution of cellular damage was assessed by scanning along an axis of the ureter explant in 0.1-mm steps. Parallel measurements of cell proliferation were also made.

Pilot experiments have shown that the fraction of damaged cells was considerably higher in irradiated samples in comparison with control. Variation in the background level of damaged cells was also observed for different samples as is typical for primary samples because of interindividual genetic variations (*5*). Analysis of the spatial distribution showed that irradiation of cells on the actively proliferating edge resulted in damaged cells that were concentrated mainly at the periphery of the explant. Importantly, the irradiation of only 10 cells in the explant led to several thousand damaged cells when scored 3 days later. The increase in the fraction of damaged cells after microbeam irradiation of 10 cells within the actively proliferating regions or preirradiations of tissue fragments are thus due to a bystander effect. This finding demonstrates that bystander effects in an epithelial system with highly developed intercellular communication under *in vivo*-like conditions could be more much more significant than in isolated human fibroblasts maintained in culture (compare to ref. *1*) after low-dose microbeam irradiation.

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The PTB Microbeam Facility

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The PTB ion accelerator facility comprises a 3.75 MV Van-de-Graaff accelerator and a cyclotron with a maximum beam energy of 20 MeV for protons and of 28 MeV for ^{4}He particles. A beamline for radiobiological experiments is just being set up and is designed to be capable of targeting subcellular structures, in particular cell nuclei, with a counted number of ionizing particles. The microbeam is suitable for all energies and kinds of particles available, i.e. protons, deuterons, and ^{3}He and ^{4}He particles.

Several microbeam facilities using collimated beams with energies of up to 4 MeV are already in operation. Here the beam is defined by a micrometer-sized collimator that is located at the end of a beamline. With this setup, beam diameters of 1 μm were realized. A further decrease in the beam size is difficult to achieve, because smaller collimators are not available and, in addition, the scattering of particles at the edge of the collimator will enlarge the beam spot. Higher energies that lead to longer ranges of the beam in the collimator material also cause an increased

amount of scattered ions. The PTB facility will therefore realize a microbeam setup by means of focusing elements.

Focused ion microprobes have well proven themselves in material science for many years. High current densities (100 pA/μm²) are used here, and the specimens are irradiated under vacuum conditions. Difficulties arise from beam aberrations that are compensated by means of special ion-optical devices, such as sextupoles, octupoles and electrostatic lenses, and current densities are increased by high-brightness ion sources. Due to the very low beam intensity needed in single-ion experiments, we are able to restrict the emittance volume of the beam, and therefore higher-order effects, i.e. chromatic and geometric aberrations, are negligible. In the case of the PTB setup, beam diagnosis and the alignment of the ion-optical components are among the main challenges. In addition, parasitic aberrations (vibration, mechanical imperfections, field impurities) must be minimized or compensated.

The beam line consists of a horizontal part (5 m), a 90° bending magnet, and a downward vertical part (4.5 m). The object aperture is located at the beginning of the horizontal part, a few centimeters behind a switching magnet which can deliver either the VdG beam or the cyclotron beam to the microbeam line. The object aperture consists of a thin metal foil (2–5 μm) with holes between 1 and 10 μm in diameter, machined by sputtering with a focused ion beam. Particles passing through the hole are separated from the particles scattered in the foil by the bending magnet and the slits behind. Placing the object aperture in the horizontal part is the only way to increase the length of the beamline, as the vertical space available is limited. Besides, the object aperture need not be long enough to stop highly penetrating particles, and particles scattered in the horizontal part are sorted out by the bending magnet. The dispersion introduced by bending can be compensated by appropriate beam optics so that, in principle, focus spots near 0.2 μm can be achieved.

Electrostatic Lens Design for the Columbia Microbeam

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Introduction

Microscopy with high-energy ions is a relatively new technique. The first nuclear microprobe (*1, 2*) with magnetic Russian quadruplet lens focusing (*3*) was built in 1970 and opened up many new investigative fields. Now an increasing number of laboratories are applying nuclear microprobes to a wide range of problems in science and technology. Microbeams are used in biology and medicine, in microelectronics and photonics, in arts and archaeology, in geology and planetary science, in environmental science, in ion lithography, and in material science.

Our objective is to construct a microprobe with electrostatic focusing to study the response of a biological cell to localized irradiation. We are planning to obtain a microbeam with a diameter of about 0.4 μm. This microbeam will deliver a predetermined number (e.g. one) of charged particles (such as α particles or protons), with micrometer accuracy, through each of a number of cells growing on a dish.

Microprobe Focusing Lenses

Focusing of ion beams of MeV energy is mostly accomplished by quadrupole lenses. The great majority of these employ a combination of magnetic quadrupole lenses (*4*). Another way to obtain a microbeam is to use solenoids as probe-forming lenses. But manufactured coils do not have a perfect rotational symmetry. Existing microprobes with solenoids do not produce a resolution of less than a few micrometers (*5*).

Two Stages of Synthesis of New Design

We have divided the synthesis of the new design in two stages. During the first stage, we create the preliminary microprobe design with a probe-forming system having the following geometry: The total length $l_t = 1.3$ m (the distance between the object slit and the target), the lens length l = 0.26 m (the sum of all lengths of lenses and spaces between lenses), and the working distance $g = 0.1$ m (the distance between the last lens and the target). The purpose of this design with rather small demagnification (-4.2) is to be a prototype of the final design with $l_t = 3.7$ m. The final design synthesized for a second stage is operating with the second mode of excitations and has a large positive demagnification ($\sim 50–80$).

As the result of our numerical investigation of different field configurations, the electrostatic Russian quadruplet (RQE) has been chosen for the probe-forming lens in the preliminary design. We have chosen an electrostatic focusing system because its focusing strength depends only on the accelerating voltage used to produce the ions. This is important for us because we intend to add heavy-ion capability to our system.

Our RQE consists of four quadrupoles, each of them formed by four cylindrical rods with the same radius r and semiaperture a and with length l_r. Geometrical and electrical data are the following: the distance s_0 of the first quadrupole to the object aperture, the separation s_i between the *i*th and $i + 1$th quadrupoles: Finally, the polarization is chosen so that in every plane $V_1 = -V_4$ and $V_2 = -V_3$.

Maximum Operating Voltage

A feature of both these designs is the method by which the relative strengths of the individual quadrupoles are selected. To obtain the maximum operating voltage, we want to operate all the lenses at approximately the same voltage ($V_1 \approx -V_2$). That is, they will all be operated near the breakdown strength of the system without having one element be the weak link. We therefore chose the lengths of the electrodes such that the proper focusing will be obtained with essentially the same voltage on each electrode.

Optimal Beam Envelope and Optimal Matching Slits

We have performed a series of analytical and numerical calculations to obtain the best design for our system. Beam focusing is understood as the result of nonlinear motion of a set of particles. As a result of this motion, we have a beam spot on the target. The set has a volume, the phase volume, or emittance. For a given brightness, the phase volume is proportional to the beam current and vice versa. The beam has an envelope surface and all beam particles are located inside this surface, i.e. inside this beam envelope. For the same phase volume (or beam current), the shape of the beam envelope can be different. We consider that the beam envelope is optimal if the spot size on the target has a minimum value for a given emittance. The beam of a given emittance is defined by a set of two matching slits: objective and aperture slits. For a given emittance *em*, the shape of the beam envelope is the function of the half-width (or radius) r_1 of the objective slit and of the distance l_{12} between two slits. The size r_2 of the second (aperture) slit is determined by the expression: $r_2 = em*l_{12}/r_1$. The parameters r_{1opt}, r_{2opt} and l_{12opt} determine the optimal beam envelope or the optimal matching slits.

Optimal Probe-Forming System

The probe-forming system consists of two systems: the matching slit system and the focusing system. Usually the focusing system has two field parameters (two excitations) and several parameters of its geometry. For this case, from two conditions of stigmatism we find the first approximation of two excitations as a function of the geometry. For each geometry, we can find the optimal matching slits. The geometry that gives the smallest spot size is the optimal geometry. For this geometry and for the optimal matching slits, we find the optimal excitations giving the minimum spot size. The optimal probe-forming system comprises the optimal excitations, optimal matching slits and optimal geometry. For each emittance, we find the parameters of the optimal probe-forming system. We consider the nonlinear motion of the beam accurate to terms of third order for systems with rotational or quadrupole symmetry and to terms of second order for systems with dipole symmetry.

Matrix Approach

The essential feature of our optimization is a matrix approach for nonlinear beam motion. In this approach, we obtain and use analytical expressions for the matrizant (or transfer matrix) and for the envelope

matrix of the third order. This matrix technique is known as the matrizant method (*6*). We use this technique for solving the equations of motion, the nonlinear differential equations in four-dimensional phase space, for example, for the field with rotational or quadrupole symmetry accurate to terms of third order. These equations are replaced by two vector linear equations (for the x and y planes) in the 12-dimensional phase moment space or by one equation in the 24-dimensional phase moment space. Writing the nonlinear equations in a linearized form allows us to construct the solution using a 12×12 (or 24×24) matrizant.

We consider the evolution of the phase moment vector, which contains the elements of the phase moments of first and third order. The envelope matrix is taken as the matrix of the second moments of the distribution of this vector over the totality of the phase coordinates. We consider the case of a small-density beam; then the beam self-field as well as particle collisions can be neglected, and the distribution function satisfies Liouville's equation. The integration is done over the object and aperture slits. We find the analytical form of the 12×12 (or 24×24) initial envelope matrix as a function of *em*, r_1 and l_{12}. This matrix is normalized by equating the first diagonal element to r_{12}^2. Thus the average radius of the beam is determined by the first diagonal element of the envelope matrix, which is a function of the position along the axis.

Two Figures of Merit

To perform an optimal synthesis, we use two different figures of merit. The first is the average radius of the beam. For a given geometry of the focusing system, we compute the first approximation of lens excitations, κ_i, which provide the stigmatic property of the system. Then we find the optimal r_1 and l_{12} that give the smallest value of the average radius in the Gaussian image plane for each emittance. The minimum spot is not located at the Gaussian plane, but we can move this spot to the Gaussian plane by changing the excitations and finding the optimal ones. In the set of *n* particles, we select two particles: the reference (or axial) particle and the particle which is the most distant from the reference particle. At the end of the optimization, we take the second figure of merit, the distance ρ between these two particles, and determine the optimal lens excitations (the second approximation) that give the minimum ρ, using 1,000 particles with randomized positions and divergences. This gives us the possibility to obtain the minimum spot without a tail.

Influence of the Energy Spread

We have investigated the influence of the energy spread $\Delta E/E$ (the energy resolution of the accelerator) on the optimal spot for the RQE. To keep the increase in the average radius of the optimal spot less than 10%, we need to have $\Delta E/E$ less than 0.0001 for *em* = 1 μm mrad, 0.0002 for *em* = 3 μm mrad, and 0.0004 for *em* = 10 μm mrad. The same results are obtained for the requirements on the stability of the power supply.

Aberration due to Misalignment

An increase in the beam spot size can also be caused by a lateral displacement of the slit system with respect to the RQE longitudinal axis. Our calculations show that to limit the increase in the average radius of the optimal spot to less than 10%, we need to have the tolerance for this displacement of less than 0.1 mm.

Construction and Fabrication

One of the main features of the RQE design being used is that part of the alignment of the electrodes is accomplished by using four 0.01-m-diameter ceramic (macor) rods 0.3 m long for the entire set of four quadrupoles. The rods are centerless and are ground to a tolerance of 6 μm for the diameter and 12 μm for camber (straightness). This design essentially eliminates misalignment of the quadrupole axes, which would induce parasitic aberrations. Evaporating a thin layer of gold onto the entire cylindrical surface in bands creates the 16 positive and negative electrodes. The insulating sections between bands is the original ceramic surface with a relief machined at the end of each section.

Rotational Misalignment of Chosen Construction

For the chosen construction, we can consider the possible small rotation of the entire set of quadrupoles. Our calculations show that for the increase in the average radius of the optimal spot to be less than 10% we need to have a tolerance for this axial rotation less than 1.2 mrad. over the whole length of the lens.

Main Parameters of the Prototype Lens System

As a result of our optimization we have obtained the following optimal parameters: $r = a = 5$ mm, $l_1 = l_4 = 3$ cm, $l_2 = l_3 = 6.5$ cm, $s_0 = 94$ cm, $s_1 = s_3 = 2$ cm, $s_2 = 3$ cm, $V_1 \approx -V_2 \approx 15$ kV (for 3 MeV protons).

For the condition of satisfying all requirements for the energy spread and misalignments with optimal r_{1opt}, r_{2opt} and l_{12opt} for every emittance, we obtain the minimum spot size ρ. We have found the following values of ρ, r_{1opt}, r_{2opt} in μm and l_{12opt} in mm for three emittances.

For *em* = 1 μm mrad: ρ = 0.508, r_{1opt} = 1.859, r_{2opt} = 5.133, l_{12opt} = 9.54.

For *em* = 3 μm mrad: ρ = 1.18, r_{1opt} = 4.47, r_{2opt} = 10.215, l_{12opt} = 15.1.

For *em* = 10 μm mrad: ρ = 2.88, r_{1opt} = 10.40, r_{2opt} = 24.83, l_{12opt} = 27.4.

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Feasibility Study of a Microbeam at the National Institute of Radiological Sciences

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Radiation biophysics and microdosimetry focus on radiation mechanisms, but a full understanding of all the relevant processes has not been obtained. There is a vast variety of events in the physical, chemical and biological processes from the time of irradiation to the production of biological changes.

Analysis of RBE–LET relationship for biological end points like survival, mutation and transformation in cultured mammalian cells is still a primary method for studying these biophysical processes. The HIMAC accelerator in our institute has been used to make phenomenological studies (such as RBE measurements) in radiation biology. Biological processes such as repair have been studied phenomenologically through the dose-rate effect or by fractionation experiments. The Human Genome Project has accelerated biological sciences as a whole, using the methodology of molecular biology, where mechanisms are potentially explained at the level of the molecules involved. In such an approach, we need to know the species and their (biological) function in every process.

In radiation biophysics, three approaches have been made along these lines: The first is computer track simulation, which has allowed us to model physical and chemical events in water, and to quantify how such events occur in a biomolecular milieu like DNA. This approach has recently been expanded to simulate repair processes by using simulations of molecular dynamics. Second, the molecular biology approach in radiation biology has revealed several proteins that are involved in the repair processes. In this context, however, quantitative relationships between phenomenological data like cell survival and molecular processes have not been established. A promising approach to fill this gap may be studies using a microbeam. This is the third approach, which may enable us to see, for example, a deletion at the chromosomal level by a single particle traversal of DNA, and may suggest possible molecular processes.

Motivated by these considerations, we have begun feasibility studies to install a microbeam port in our tandem accelerator (5.1 MeV ^{4}He^{2+}). We are interested in a lens-focusing and scanning system developed by Oxford Microbeam Ltd. for micro PIXE, which achieves irradiation of cells with a position resolution of 2 μm. We think it may meet our two requirements, i.e. precise positioning and faster irradiation. Molecular biology studies require the ability to focus helium ions precisely to a target such as cell nucleus, a chromosome, cytoplasm or a cell organelle, to investigate DNA breaks, induced repair enzymes, cell signal systems, and so on. In addition, for the problem of risk estimation, mutation and transformation rates should be measured at low dose, which requires irradiation of a large sample of cells of 10^5−10^6 to get results with good statistics.

Development of a Microbeam Single-Ion-Hit Technique for Biomedical Applications

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A single-ion-hit technique may be employed to introduce a radiation effect to a specific cross-sectional area of a sample with high-LET single ions. This kind of technique has been developed at several facilities to hit individual biological cells in an air environment for biomedical applications. At JAERI Takasaki, a heavy-ion microbeam system connected to a 3 MV tandem accelerator, we have established a single-ion-hit technique which can inject heavy ions, for example 15 MeV nickel, one by one into specific positions with an accuracy of 2 μm or less.

This technique will be introduced into the existing microbeam system, which has been designed to develop a novel cell surgery technique, using local irradiation of living organisms with a targeted heavy-ion beam. The apparatus has been installed under a vertical beam line of the AVF cyclotron, which provides several different heavy-ion beams. Specific characteristics, including minimum beam spot sizes and ranges in water of available ion beams, are listed in Table 1. The beams have been collimated to about 10 μm in diameter; more than 95% of the collimated ions were within the monoenergetic peak and delivered to the designated target with 85–90% of targeting accuracy.

We also have plans to construct a focusing microbeam system on another beam line of the same cyclotron to obtain higher positioning accuracy to hit local cross-sectional areas of cells such as nuclei, mitochondria, chloroplasts, cell shells and so on. To get positional information about the cells to be hit in a reasonably short period, an automated beam-positioning technique is required. Therefore, we shall introduce an automated cell recognition system, consisting of an optical microscope, sample stages and a computer system.

TABLE 1
Microbeams Presently Available for Local Irradiation of Biological Systems

Ions	Specific energy (MeV/μm)	Minimum beam diameter (μm)	Projectile range in water (mm)
^{4}He^{2+}	12.5	40	1.8
^{12}C^{5+}	18.3	20	1.2
^{20}Ne^{8+}	17.5	10	0.7
^{40}Ar^{13+}	11.0	10	0.24