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EXTENDED ABSTRACTS

Proceedings of the 5th International Workshop: Microbeam Probes of Cellular Radiation Response

Stresa, Lago Maggiore, Italy, May 26–27, 2001

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The extended abstracts that follow provide a summary of the Proceedings of the 5th International Workshop: Microbeam Probes of Cellular Radiation Response, held in Stresa, Lago Maggiore, Italy, on May 26–27, 2001, which was organized by INFN, Laboratori Nazionali di Legnaro, Italy and Università degli Studi di Milano, Dipartimento di Fisica, Italy.

There is increasing interest in the use of microbeam systems (*1*, *2*), 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 on non-hit 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.

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Session I

Chair: D. J. Brenner

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Development of the Gray Laboratory Charged-Particle Microbeam

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In the past year, the Gray Laboratory charged-particle microbeam facility has been substantially refurbished to enhance its performance in a number of ways. Specifically, improvements have been made to the cell imaging system, the image processing, and overall experimental control, with the purpose of improving the speed and versatility of the cell-finding procedure. Other developments, such as changes to the collimator positioning system and the beam shuttering, have been designed to improve the throughput of cells during the irradiation phase. Some of these improvements have taken place in tandem with the development of our other micro-irradiation facility (the X-ray microprobe) such that where possible, the two systems resemble each other.

As before, our microbeam makes use of a purpose-built beamline from our 4 MV Van de Graaff accelerator to transport particles (protons, $^3He^{2+}$ ions or α particles) vertically upward through the floor of the laboratory to the cell irradiation apparatus, mounted on an optical table at bench height (*1*). A fine radiation beam is formed using a 1-μm-diameter bore fused silica capillary collimator, mounted at the end of the beamline (*2*). Cells to be irradiated are attached to a thin plastic membrane that forms the base of a cell dish containing cell culture medium. The dish is located on a micro-positioning stage above the collimator. During irradiation, each target is located, in turn, above the collimator and exposed to an exact, predefined number of particles.

The particles incident on the cell are counted using a photomultiplier tube mounted just above the cell dish. The photomultiplier tube detects the pulse of light (due to the passage of a particle) from a thin scintillator between the collimator exit and the cell dish. This detector arrangement is close to 100% efficient. The targeting accuracy is limited primarily by particle scattering from the vacuum window and scintillator (*3*). The effect of scattering is minimized by arranging for the collimator to be as close as possible to the cell. In fact, the collimator just touches the base of the cell dish prior to each exposure. In this way, we can achieve a targeting accuracy (for 99% of particles) of ± 2 μm when $^3He^{2+}$ ions are used (validated using track-etch methods).

A major development has been to replace the aging microscope and CCD camera with a new system based on an infinity-optical microscope and Philips intensifier-coupled CCD camera. The new microscope has been assembled using components from the Olympus BX range (specifically, a light condenser, lamp housing and four-cube filter turret are used to provide epi-fluorescent illumination of the cells). The new camera-intensifier arrangement has better resolution and is more sensitive than the system it replaces. This permits lower stain concentrations and illuminating UV exposures to be used when finding cells. The camera has been modified to permit ''live'', integrated or ''snapshot'' images to be acquired. In normal operation, the cells are located by scanning an area of the dish as a series of overlapping frames. Typically, it takes 80 frames to view a 5-mm^2 region, containing up to a few thousand cells. Each frame is a single, static image, and a shutter in the UV-excitation light path is synchronized to open only during image acquisition (typically for about 40 ms). It takes about 2 s to acquire and analyze each frame, such that all the cells in the region can be identified in about 3 min.

The computer and software for controlling the experiment have also been replaced. All key experimental operations of the microbeam are controlled using a fast PC, including stage movement (three-axis), collimator positioning (one-axis), the input and display of the microbeam source operating conditions, experiment data logging, and the CCD camera image acquisition, image processing and display. The software control and user interface have been developed using an image analysis software package (Visilog, France). The same software is implemented on both the microbeam and the X-ray microprobe facilities, such that software developments are of immediate benefit to both systems (clearly, however, some features are facility-specific). With the new software, the user is presented with a range of pull-down menus and virtual control panels for inputting and displaying information. As new experiments are devised, appropriate panels to control the irradiations can be implemented. In addition to our ''conventional'' experiments (i.e., finding and then irradiating all cells in a region through their nuclei), there is now the capability for ''point and shoot'' irradiations (useful for bystander-type experiments) and the ability to expose cells in a confluent layer to a preprogrammed pattern of radiation (e.g., a ''line'' of targeted cells across the dish). We also have software that allows us to simulate randomized particle exposures by irradiating each cell with a pseudo-random number of particles, with a Poisson distribution about a preselected average number of particles. In addition, a software module has been added to improve experiments involving irradiation of the cytoplasm. The cytoplasmic target is established through two imaging steps. First, the positions of nuclear-stained cells are established conventionally. Then, with different optical filtration, each cell is recalled and visualized using a cytoplasmic stain, such that the experimenter can select a point to irradiate within the cytoplasm of each cell.

Other aspects of the refurbishment have been undertaken to increase cell throughput during the irradiation step. We have implemented a new electrostatic deflector midway along the particle beamline that is capable of fast, sustained beam deflection such that it can serve to start and terminate the exposure of each cell, removing the need for a relatively slow mechanical shutter. A fast-switching HV power supply triggered by a signal from the particle detector instrumentation is used to apply a 3 kV d.c. deflection potential between two plates in a few microseconds.

With the electrostatic deflector implemented, it became evident that a further substantial improvement in speed could be gained by modification of the collimator positioning system. For maximum targeting accuracy, the collimator is raised a fraction of a millimeter prior to each cell exposure, such that it just touches the base of the cell dish (and is subsequently lowered to facilitate unimpeded positioning of the next cell). In the old system, this was achieved by a 12-V d.c. motor that positions the collimator through movement of a linear bearing. The contribution to the duty cycle (per cell) that this introduced was about 300 ms. In addition, the motor failed regularly because of this small, repetitive action. The new system uses the same d.c. motor for large collimator movements (i.e. prior to locating the dish on the stage) and a two-position ''actuator'' (rotary solenoid) for the small movement required before and after each cell irradiation. Using this arrangement, the contribution to the duty cycle is now 60 ms, such that cell throughputs in excess of 10,000 cells per hour are possible.

Further improvements are under way; equipment is being assembled to develop an off-line microscope that uses non-UV methods for finding cells and a new, custom-built sample stage that uses piezoelectric motors in a ''closed-loop'' mode has been constructed for faster and more accurate cell positioning. A successful trial of the stage has been completed, but substantial modification of the controlling software is required before it can be incorporated into the microbeam facility.

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The PTB Microbeam Facility

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The PTB's ion accelerator facility comprises a 3.75 MV Van de Graaff accelerator and a cyclotron with maximum beam energies of up to 20 MeV for protons, 35 MeV for ^{3}He particles, and 28 MeV for ^{4}He particles (*1*). In the summer of 2000, a beamline capable of targeting subcellular structures, in particular cell nuclei, with a counted number of ionizing particles for radiobiological experiments was completed. In contrast to existing radiobiological microbeams (*2–4*), which use collimated beams, the PTB microbeam makes use of the focusing properties of magnetic quadrupole lenses.

The beamline consists of a horizontal section 5 m long, a 9° bending magnet, a downward vertical section 4.5 m long, and two quadrupole doublets. The experimental area is placed on the concrete ground floor of the experimental hall. The object aperture is located at the beginning of the horizontal section, at the first floor, approximately 20 cm behind a switching magnet that can deliver either the Van de Graaff or the cyclotron beam to the microbeam line. The object aperture is a metal foil 2 to 5 μm thick, with pinholes between 1 and 20 μm in diameter, which are machined by focused ion-beam sputtering (*5*). The energy-selecting properties of the bending magnet and the corresponding slits behind it ensure that particles passing through the hole are separated from the particles penetrating the foil. Arrangement of the object aperture in the horizontal section is the only manner of increasing the length of the beamline, because the vertical space available is limited. The dispersion introduced by the 90° bending magnet can be compensated by appropriate beam optics allowing, in principle, a beam spot diameter smaller than 1 μm to be realized. This configuration allows the beam to be rarefied to a few particles per second without distortion of the spatial resolution; however, demagnification is limited to a value of 7 in the radial coordinate.

Tests with the completed beamline have been running since August 2000. The main components have been working satisfactorily. A spatial resolution of 1 μm was achieved in vacuum. Difficulties arose mainly from mechanical misalignments of the magnets, which led to aberrations larger than those calculated. The correct steering of the incoming beam on the object foil turned out to be another critical parameter. On the other hand, long-term stability is not a problem: A focused beam spot can be reproduced within a few micrometers without any readjustment after a 12-h overnight shutdown.

In single-particle experiments, a spatial resolution between 2 and 5 μm (FWHM) was achieved, depending on the particle energy. The resolution is compromised by small-angle scattering in the vacuum window and in the scintillating foil. An improved construction of the vacuum window is supposed to reduce this effect. The particle rate can easily be reduced to 10 ions per second by narrowing the width of the aperture slits. Single-particle detection is operational with an efficiency of about 98%. In the future, we will systematically investigate the optimization of the scintillator thickness for different particles at various energies. A fast shutter switches the beam off within 10 μs, leading to a beam suppression of almost 100%. Contrary to our expectations, we found a fraction of scattered particles of 1% to 5% outside the nominal beam spot in experiments with the CR-39 track detector.

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Design of the Munich Microprobe Facility for Single-Ion Irradiation of Cells

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The ion microprobe SNAKE [Superconducting Nanoscope for Applied Nuclear (Kernphysikalische) Experiments] at the Munich 15 MV tandem accelerator has now gone into operation after the first tests, which were performed in 1999. At low ion currents, typically used for biological experiments, it is able to focus 25 MeV protons or heavy ions with energies up to 200 MeV into a submicrometer beam spot (*1*). Using 90 MeV ^{32}S ions, a spot size of 300 nm has been obtained recently. Therefore, the microprobe SNAKE represents a promising basis for the selective irradiation of specific parts of living cells with single, counted ions.

The wide range of ion species allows an adjustment of the stopping power from 2 keV/μm (for 25 MeV protons in water) to 2 MeV/μm (for 90 MeV ^{32}S ions in water) (*2*). Thus the energy density deposited at a specific irradiation point can be varied precisely over three orders of magnitude. An advantage of the high ion energies is the large range in biological materials and the high lateral accuracy due to drastically reduced small-angle scattering. This facilitates the separation of the actual irradiation location from the beam transport vacuum by thin foils, so that cells can be irradiated under living conditions with the horizontal beamline without losing submicrometer resolution. For example, 25 MeV protons show a lateral spread of less than 60 nm (FWHM) after traversing 10 μm of plastic foil (e.g. scintillator) and subsequently 20 μm of water (*3*). Using 170 MeV ^{32}S ions passing 10 μm of plastic, the lateral enlargement of the beam spot amounts to no more than 30 nm, while a residual ion range of about 76 μm in water is preserved. Therefore, cells are easily accessible for irradiation under living conditions, even if they are part of a cellular tissue or are located in a culture medium.

The whole microbeam setup is located at the Munich tandem accelerator. The single-ion preparation for the irradiation of cells is done by

an electrostatic chopper system, which is driven by a high-voltage MOSFET switch (V_{max} = 8 kV). Using 70 MeV ^{12}C ions, this facility showed good performance in preparing single particles. The positioning of the cells with micrometer resolution can be performed by the existing four-axis manipulator. A vacuum exit window and a particle detector matching the demands of the irradiation experiments are under development. After that, the installation of an optical microscopy system will be realized in cooperation with biologists from the LMU München.

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A Focused Heavy-Ion Beam System for High-LET Studies of Cells

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Microbeams of hydrogen and helium ions have become important tools for radiobiology. State-of-the-art systems can deliver defined numbers of ions onto cell targets with sub-micrometer resolution. Overcoming the limited LET range available at light-ion (proton and helium ion) microbeam facilities has been recognized as an important technical advancement (*1*).

We describe here the first steps in the development of a compact, vertical heavy-ion microbeam system at LBNL. The central thrust of our system lies in the use of an Electron Beam Ion Trap/Source (EBIT/S) to produce highly charged heavy ions coupled with a conventional electrostatic accelerator platform. Available ion species from the EBIT/S at LBNL range from protons, Fe^{26+} and Xe^{52+} up to Au^{69+} (*2*, *3*). Ion intensities are typically $\sim 10^6$ ions/s, and ion extraction from a confined electron beam results in high brightness values favorable for ion beam focusing. Preliminary focusing studies with 0.3 MeV beams of Ar^{18+} have shown promising results (*4*, *5*). Recently, beams of highly charged ^{31}P ions at 0.1 MeV were focused and collimated to a 5-μm spot. The high charge states are advantageous both for electrostatic focusing and for achieving the desired kinetic energy in a compact setup. The kinetic energy of projectiles is determined by the high-voltage platform. A 2.5 MV terminal voltage yields heavy-ion beams with energies of about 1 MeV/nucleon, which have a range in water of about 20 μm (estimated using SRIM 2000, http://www.research.ibm.com/ionbeams/) (*6*). There are tradeoffs between key design parameters such as facility size, beam energy (i.e. accessible terminal voltages), spatial resolution (beam spot size and contributions to straggling from vacuum windows and cell substrates), and versatility.

Characteristics of ion beams extracted from the EBIT/S at LBNL, its brightness and emittance values, as well as the ease of operation and implementation into a high-voltage terminal have been examined and compared to alternative sources for high-charge-state heavy ions, such as Electron Cyclotron Resonance Sources and Laser Plasma Sources. The electrostatic focusing and collimation scheme for a spot size of 300 to 500 nm is being simulated based on beam parameters with the trajectory code IGUN (*7*, *8*).

The facility will enable studies of low-dose, high-LET (10^2–10^4 keV/μm) radiation effects on cells and will extend studies of issues such as bystander effects and genomic instability to high-LET regions that are relevant for space technology and heavy-ion-based cancer treatment.

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Session II

Chair: B. D. Michael

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Laser Ion Source Design for the Columbia University Microbeam

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A design is given for a laser ion source for the 4 MV Van de Graaff accelerator at the Columbia University Radiological Research Accelerator Facility (RARAF). The source has been designed with application in mind for the RARAF single-particle single-cell microbeam, though it will be also used for broad-beam irradiations. The laser ion source allows heavy-ion production with high charge states, so that their energies will be high enough to provide sufficient range—at least 20 μm—for irradiating cells on a thin surface.

Laser ion sources have been developed and are used at several particle accelerator laboratories (*1*). These ion sources share a common mechanism of ion production through laser ablation of a solid target. Standard

ion sources that rely on a reservoir of gas-phase atoms for ion production are limited to gaseous elemental species. On the other hand, a multitude of elemental species becomes available when using a solid target as a source for ion generation.

The laser ion source is based on plasma generation by a pulsed laser beam focused by a mirror system (or lens) on a solid movable target. The focused laser light is used to evaporate particles from that target. The electrons of the plasma, which is generated during the evaporation process, are heated by the laser radiation to temperatures up to several hundreds of eV. Electron-ion collisions produce high charge states. The temperature of the plasma and the consequent final ion charge-state distribution depend strongly on the laser power density on the target.

Many of the properties of the laser-produced plasma plumes are potentially useful for an ion source for radiobiological and microbeam studies: Among these are:

1. A copious supply of ions per pulse.
2. High charge states of ionization.
3. Short plasma generation times, which are useful for time-of-flight measurements.
4. Directional plasma plumes that can be oriented along the extraction axis (to provide low emittance).
5. Versatility in producing a variety of ions since *any* solid material is a potential plasma source. Beams (and thus LETs) can be changed rapidly, by inserting different target materials.
6. Simplicity in design and construction since, in principle, only the solid target need be at accelerator terminal potential because it is optically connected to the laser at ground potential.
7. The possibility of extraction of ions directly from the plasma plume without the application of an additional extractor potential because of its directed expansion velocity, thus making the laser ion source unique in producing slow multicharged ions.
8. The absence of a carrier gas since the source operates most efficiently in a high vacuum.
9. Continuing improvements in laser technology, including increased repetition rates and power output, make this technique a relatively inexpensive method of producing multicharged ions.
10. For single-particle microbeam applications, where the ion yield does not have to be high: Very high charge states can be used, even when the yield of such states is comparatively low.

An additional plasma formation detail that is important in extracting the ions from the source and delivering them to the acceleration stage is the spatial distribution of a charge state. Hughes and colleagues at the University of Arkansas reported a radial distribution in an extracted laser-plasma ion beam where the fastest ions, which are also those with the highest charge states, reside on the outside of the beam (*2*); space-charge-repulsion effects were used to explain the trend. The Toledo Heavy Ion Accelerator (THIA) group reported that charge state dependence on the angular spread of the laser-plasma ions was a cone of expansion that was 20 for high charge states and 45° for low charge states (D. E. George, http://www.physics.utoledo.edu/~scheng/laser.html). A common trait among contemporary laser ion sources follows the directional nature of the plasma plume; ion extraction is preferred along the direction normal to the target.

Ion pulse duration is a factor if the proposed source requires that ions within one pulse be gated out after one has arrived at the final biological target. If the ion pulse occurs too fast, it may be difficult to single out one ion from subsequent ions. On the other hand, proper gating is more certain for a longer ion pulse with the same number of particles. The ion pulse duration can be extended if the drift length prior to extraction is lengthened (*3*). The ion current would follow the inverse square law in this case.

The laser ion source being implemented at RARAF is based on the laser-operated ion source (LOIS) used at the University of Arkansas (*2*) and consists of four main components: laser generator, optical focusing system, electrostatic analyzer, and source vacuum chamber. Equipment from LOIS, including a Holobeam 5050Q Nd:YAG laser, was used to build a prototype and for proof of principle. The vacuum chamber contains the target, mounted on a target-positioning mechanism.

The laser will be mounted in front of the Van de Graaff accelerator, parallel to and alongside the charged-particle beamline. The light beam from the laser will be expanded and directed by mirrors through an existing window in the base of the accelerator. Inside the accelerator, the light will pass through one of the insulating support tubes to the terminal where it will be directed toward the window of the ion source. The attenuation of the 1.06-μm light in the insulating gas mixture is negligible. To reduce distortion of the light beam by convection currents in the 10 atm of N_2-CO_2 insulating gas mixture in the accelerator, the insulating support tube will be fitted at each end with transparent caps to restrict the motion of the gas inside the tube.

The laser beam enters through a window in the source onto a focusing mirror to produce a ~20-μm-diameter spot on the target material. Between the mirror and the target is a glass plate to protect the mirror from becoming covered by material ejected from the target. This plate can be replaced cheaply when necessary. The next laser pulse evaporates the material that condenses on the plate, so the glass plate is continually being cleaned. After a specified number of laser pulses, a motor moves the target material a short distance so that the laser strikes a fresh surface.

The created plasma enters an electrostatic analyzer consisting of a pair of cylindrical plates that bend the ions through 180°. The analyzer will be tuned to reject the lowest charge states, which constitute the majority of the ions. This will reduce considerably the beam load on the accelerator vacuum system.

We expect that the laser ion source will enable us to use ions of sufficient range from hydrogen to around iron, with an LET range from about 10 to 4,500 keV/m. This will allow biological experiments on the microbeam with fairly low-, intermediate-, high- and very high-LET radiations, under identical experimental conditions, and should represent a fruitful upgrade of the current microbeam system.

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Status of the Second Columbia University Microbeam Facility

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A single-ion microbeam facility has been in use at the Columbia University Radiological Research Accelerator Facility for several years. The system was designed to deliver defined numbers of helium or hydrogen ions produced by a Van de Graaff accelerator, covering a range of LETs from 30 to 220 keV/μm, into an area smaller than the nuclei of human cells growing in culture on thin plastic films. A pair of laser-drilled apertures that form the beamline exit collimates the beam. An integrated computer control program locates the cells and positions them for irradiation.

It has become apparent from the usage requests for the facility that there is a great interest in experiments that require a more precise definition of beam position than is possible with our collimated system, particularly with regard to the presence of a halo of scattered particles around the main core of the beam. We have therefore begun a major upgrade of the Columbia University facility. We are replacing the collimators with an electrostatic quadrupole focusing system.

Our present microbeam station has a complicated beamline—three bending magnets and three quadrupole focusing elements—making beam tuning difficult. A second failing of our present beamline for application to focused systems is that there is a limited distance from the last magnet to the target position, limiting the demagnification of a focusing element placed near the target. The prototype lens installed in the old system has a demagnification of only 4.2 using a length of 1.3 m from the object aperture to the focal point. We have therefore installed a new double focusing 90° magnet immediately after the accelerator to direct the beam upward through a 2-foot-thick concrete ceiling into a newly constructed microbeam laboratory. The distance from object slits to focal point in the new beam line is 3.7 m. The focusing system that will be installed is a pair of quadrupole triplets, which is expected to have a demagnification of 62. The system will have the so-called Russian symmetry; namely, the lens strengths will be ABCCBA and the orientation will be XYXYXY. This symmetry guarantees that the demagnification will be the same in both X and Y planes. We have added the additional constraint that the aberrations of the system will also be the same in both planes, resulting in a system with a large solid angle for a given final beam spot size. The final spot is predicted to be 0.3 μm.

To finely adjust the voltages of the lens for best focusing, it will be necessary to visualize the beam shape during the process. We are designing an electron microscope to focus secondary electrons emitted from a metal surface that can be placed at the focal spot. The microscope, dubbed SEIM for secondary electron ion microscope, will be of a new folded design that will be more compact than usual designs and will also reject high-energy electrons that are the usual limit on the resolution of an emission-type electron microscope.

Another upgrade to the system that has been requested by our users is the ability to provide particles with higher LET than are available from the light ions we use. We are planning to install a laser driven ion source in our Van de Graaff. Details of that upgrade are presented by Bigelow *et al.* in these proceedings.

A Focused Microbeam for Targeting Cells with Counted Multiple Particles

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Introduction

Experiments at the single-cell level remain one of the only possible ways to gather evidence of the response of an individual cell to a very small radiation dose, including extreme situations like exposure to a single ionizing particle. A single-event facility is currently under development at CENBG for applications in this field (*1, 2*). The aim is to target individual living cells with an exact number of ions, with the beam being delivered ion by ion to specific cell compartments (nucleus, cytoplasm, etc.).

The facility takes advantage of the focusing properties of the existing horizontal microbeam line at CENBG, which is able to focus a proton or α-particle beam, produced by a 4 MV Van de Graaff accelerator, down to a diameter below 1 μm in vacuum when very low beam fluence is used. The facility has been designed to constitute a versatile system working on demand either in external-beam mode or classical analysis mode under vacuum. The cover of the analysis chamber is indeed removable and can be replaced by an in-air target chamber. In this way, it was not necessary to construct a new line. Moreover, it is important to note that the beamline may also be employed for carrying out classical microbeam analyses in air. Imaging experiments have recently been performed on living human cells cultured on thin formvar films (*3*).

The technical challenge is to deliver the beam ion by ion, in air, while keeping a spatial resolution of the order of a few micrometers to be able to hit subcellular targets. Since the beam is positioned on individual cells by means of fast electrostatic deflection plates, this setup should allow cell targeting with a higher frequency than the collimated beams usually used for such applications.

Beam Control

For single-event control, the beam is deflected by fast-response electrostatic plates triggered by the path of each ion through a transmission detector. For this purpose, two detectors have been designed. The first is based on a thin plastic scintillator foil optically coupled to two photomultiplier tubes working in coincidence. However, because of the limited range of α particles, it can be used only for irradiation in MeV proton mode. For use in α-particle mode, a low-pressure transmission gas detector equipped with Si_3N_4 windows has been designed. The efficiencies of these detectors have been measured for MeV protons and α particles at levels higher than 99%. Beam positioning/targeting software has been developed and was tested in vacuum using plastic track detectors.

The spatial distribution of the beam after passing through the different components of the beamline has been evaluated by means of Monte Carlo simulations (*4*). These results were compared to experimental data obtained from direct measurement of beam straggling using a collimated detector placed at 0° at the end of the beamline (*5*).

Cell Targeting

The equipment for the irradiation stage is currently under development. In this design, the aim is to target adherent cells cultured on thin polymer foils stretched on dedicated culture flasks. During the irradiation, cells will be kept in their medium, in a wet chamber positioned by a high-precision x-y-z stage. Each cell of the monolayer will be online, located by a fast optical recognition system coupled with a fluorescence microscope and a computer-controlled high-sensitivity CCD camera. The whole automatic procedure is expected to be fast enough to allow a targeting rate of 10 cells per second.

The automation of the single-ion single-cell irradiation setup requires the development of a cell recognition system. To achieve high contrast, a fluorescence microscopy technique was used to locate the cells. For this, different stains were tested on CHO-K1 cells. Delimitation of cell nuclei was performed successfully using software developed in-house, based on the Image-Pro Plus graphic library (Media Cybernetics).

Conclusion

A single-ion beamline dedicated to the irradiation of targeted cultured cells with counted particles is being developed at CENBG. In its first version, the setup is currently capable of delivering either protons or α particles in air, in steady beam mode, through a gas detector. In this configuration, the cells would be mechanically positioned in the beam. Further developments in transmission detectors are planned to take advantage of the fast scanning system of the microbeam. Moreover, an algorithm allowing identification of cell nuclei has been tested on stained cells and will be inserted in the irradiation setup to automate the irradiation procedure.

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Light-Ion Microcollimated Beam Facility for Single-Ion, Single Mammalian Cell Irradiation Studies at LNL-INFN

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Introduction

The aim of this project is to set up a microbeam facility that can deliver targeted and counted particles to individual cells, with a spatial resolution of the order of 1 μm, using a semi-automatic cell recognition and automatic micropositioning system. The conventional light-ion (protons, deuterons, helium-3 and helium-4) broad-beam radiobiological facility of the LNL CN 7 MV Van de Graaff accelerator (*1*) is under development to include the added possible use of the microcollimator and micropositioning apparatuses. In the next paragraphs we describe modifications to the original facility for single-cell irradiation experiments.

Beam Deflection and Particle Detection

A fast beam switch-off is an essential element for experiments which require a high degree of reproducibility in the administration of the counted particle fluence. The response time of the facility's original electropneumatic shutter (100 μs) was too long to ensure that particles that followed the last one to be delivered were stopped. Thus an electrostatic deflector with a response time of less than 150 ns (*2*) has been installed just after the accelerator's switching magnet. When a preset number of particles have hit the biological target, a silicon surface barrier detector placed behind the cell sample will provide a signal to drive the electrostatic deflector.

Support for Microcollimator

An appropriate flange at the end of the beam pipe allows the extraction in air of the ion beam through an aluminized Mylar foil that is 10 μm thick and 5 mm in diameter. The microcollimator positioning and alignment in air are achieved with a tilting support that is fixed in parallel to this flange at a distance of 3 mm by means of three tongs and a ballpoint piston (which allows the tilting) and is driven by two remotely controlled stepper motors; the motor eccentric produces, by two pistons, a 3-mm maximum (forward and back) shift of this support from parallel/zero initial position.

Microcollimator Device

The microcollimator is obtained by the overlapping of two orthogonal sets of slits. The choice of collimator thickness must satisfy these conditions: Its lower limit for penetration must be greater than the range of a 6 MeV proton (or a 14 MeV α particle) to stop all unwanted particles from passing through the collimator; the upper limit for penetration is set by the fact that the thicker the collimator, the greater the chance of internal multiscattering. The actual collimator system is comprised of 800-μm titanium slits, where slab inner surfaces have been polished with an accuracy of 0.1 μm (collimator aperture down to 2×2 μm^2). Previous prototypes employed nickel slits of the same thickness. Another version of this kind of collimator, using 400-μm-thick tantalum slabs, will also be tested. Much effort will be spent to optimize the geometry of the collimator slabs to reduce the maximum ion halo (multiscattering) in air. The ultimate aim is to construct a one-piece gold collimator consisting of a disc with a central hole 2 μm in diameter and 2 mm long; some attempts are now in progress.

Cell Visualization and Positioning System

This element is in the assembling phase. The project for a cell recognition, positioning and revisiting system is based on micropositioning stages and an optical microscope. The phase-contrast Olympus microscope will be placed so that the axes of the objectives will be parallel to the beam pipe axis. The object image will be taken by a CCD camera and will be transferred to a PC with appropriate image acquisition and analysis software (Microimage, Casti Imaging) that is able to calculate and log the X-Y coordinates for every cell. The cell positioning apparatus employs a helicoidal guide moving system for the cell holder platform, to let it pass from a position under the microscope objectives, where the holder will be positioned horizontally, to a vertical position in front of the beam pipe. The fine motion on the cell dish X-Y plane will use a remotely controlled system, based on two translation stages (0.1 μm positioning resolution and unidirectional repeatability, no backlash; M-511.DDB Series, Physik Instrumente) driven by software able to reproduce, in front of the beam, the cell map (semiautomatically) taken before irradiation. By placing a CR-39 foil just before cell sample, it will be possible to log every particle's track position and overlap this image with the cell map acquired before irradiation to check every projectile's impact point relative to the cell positions.

Preliminary Results

Initial tests have concerned the performance of the collimator and of the motorized alignment system (*3*); both of these can be assessed in terms of the quality of the energy spectrum. The energy spectrum of collimated particles in air is measured using a silicon surface barrier detector (500 μm depletion region thickness; 50 mm^2 active surface) and conventional spectroscopy instrumentation. In addition, solid-state nuclear track detectors (CR-39 detectors) are used to check both the energy and spatial distributions of the particles in air. Several energy spectra of 10 MeV (corresponding to 5.9 MeV in air) ^{4}He ions, collimated by an 8×8-μm^2 titanium collimator, have been acquired for different orientations of the collimator. These spectra show a progressive improvement of the collimator alignment prior to achieving the optimal condition: Here the low-energy scattering component is reduced and the high-energy particle fluence shows an increase; the FWHM of the peak shows an energy spread of about 3%.

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Session III

Chair: P. Rossi

INFN, Padua, Italy

The Leipzig High-Energy Ion-Nanoprobe LIPSION: Design of Single-Ion Bombardment of Living Cells

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The Leipzig High-Energy Ion-Nanoprobe became operational in October 1998. It consists of a single-ended electrostatic accelerator by HVEE, a 3.5 MV Singletron, and a nuclear microprobe by Marco, Melbourne, equipped with quadrupole pair lenses in the split Russian quadruplet arrangement. Relevant specifications for 2.25 MeV protons are: less than 30 V ripple, long-term voltage stability better than 100 V/8 h, reduced brightness of larger than 20 pA/mrad^2 m^2 MeV (*1–3*), demagnification up to 130 in x and y, minimum focused beam size at 0.1 fA of 40 nm (*4*). This beam has been tested by scanning over an atomically flat interface of GaAs/InGaP. Thus far this is the smallest proton beam worldwide.

Contrary to existing microprobes with collimated beams approximately 1 μm in diameter, we are now in a position to aim at cells and subcellular structures using the beam scanning and single ion facilities. A difficulty of our system is that the beam is horizontal, in contrast to the existing systems at the Gray Laboratory in the UK and at Columbia University in New York, which have vertical beams. To extract the beam into ambient air as required for the bombardment of living cells, we shall use an irradiation platform that can be inserted into one of the CF160 flanges of the target chamber and that is equipped with a 100-nm thin, 1 mm × 1-mm Si_3N_4 window. The energy loss and lateral straggling for protons and α particles should be quite small according to TRIM simulations. We plan to use the following scheme:

First, cells are sowed onto miniaturized petri dishes with a central bore covered with a 0.5- or 3-μm thin Mylar foil and with two or more fiducial markers. A cell observation system detects the cells and stores the coordinates of the cell nuclei and the fiducial markers. Then the petri dish is transferred to the irradiation platform, where it is positioned in front of the ion exit window with a precision of about 1 μm using an *x,y* translation stage. The fine *z* positioning will be accomplished by a piezoelectric transducer so as not to damage either the ion exit window or the Mylar foil and to minimize the air gap in between. Next we aim at the fiducial markers using secondary electron detection or scanning transmission ion microscopy. Thus we carefully avoid hitting the medium/cells. After we determine the coordinates of the fiducial markers, we hit our target, i.e. a cell nucleus, the cytoplasm, intracellular structures, or the medium, with a predetermined number of particles, starting from a single ion and moving upward, using the coordinates from the cell observations. The ions traversing the exit window, the Mylar foil, and the cell covered with a thin layer of medium will be detected in a particle detector immediately behind the cell. Thus we obtain a signal to close the beam gate, and we also know the amount of energy deposited. Preliminary tests at the Gray Laboratory showed that AG 01522 fibroblasts adhere to the Mylar foil for 5 to 10 min when placed in a vertical position as required for our horizontal beam. This is more than we need for investigations of the bystander effect, where only a single hit or a small number of hits are required. After bombardment, we transfer the petri dish back to the cell observation system and verify that the original positions have been retained. The same experiments will be carried out with human arterial endothelial cells. The end point will be micronucleus formation and apoptosis, accompanied with TUNEL assays, including morphology, and flow cytometry.

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Single-Particle Detection for the MIT Charged-Particle Microbeam

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Introduction

The development of a biological charged-particle microbeam is under way at the Laboratory for Accelerator Beam Applications at MIT. The facility is comprised of a vertical beam from a 1.5 MV single-ended electrostatic proton/α-particle accelerator for the irradiation of cells *in vitro*. The system is being designed such that it is fully automated; the cells are imaged with a coupled epifluorescence microscope, the cellular or subcellular targets are recognized, and their positions are recorded under computer control. The stage, which has a precision of 1 μm, is instructed to position each cell in turn at the beam aperture, and the controlling software opens an electrostatic shutter in the beam until a preset number of particles have irradiated the cell.

To know precisely the energy deposited in each target, particle detection with ~100% efficiency is required. The detector configuration adopted at the MIT microbeam is that of positioning the detection system between the collimator and the cell substrate. One can envision placing the detector to intercept particles after they have traversed the cells, but this would significantly hamper the ability to support and irradiate cells in medium. Furthermore, such a post-cell detection system would not be appropriate for experiments in which the particles deposit all their energy in the cell. Therefore, a pre-cell detection system was implemented at MIT, as is the case at some other microbeam facilities (*1, 2*). Such a configuration imposes significant constraints on the ultimate design. For example, a thin, transmission-type detector is necessary to minimize energy loss of the particulate beam to a few hundred keV. A BC400 thin

film plastic scintillator (Saint-Gobain Crystals & Detectors, Newbury, OH) has been chosen for this purpose, because it has a very fast decay time (*1*) and is available in thicknesses >5 μm. However, such an organic scintillator has poor efficiency (*3*), and careful consideration must be given to the positioning and coupling of the associated photomultiplier tube(s) (PMTs).

Scintillator-PMT Configuration

With pretarget detection systems, the general configuration adopted by other biological microbeam facilities has been to position a scintillator film at the beam exit, with the PMT above the cell dish, such that the tube views the light from the film in a head-on arrangement (*1, 2*). An alternative configuration still positions the film at the beam exit, but it has the PMT optically coupled to the side of the scintillator. The use of a light guide, when properly coupled, can increase the light collected by the PMT by effectively increasing the solid angle that the tube subtends. In addition, using a light guide for a biological microbeam allows for the separation of the detection system from the sample stage and the imaging systems, in contrast to the conventional approach that positions the PMT above the dish in the turret of the associated microscope. Finally, a light guide facilitates the use of coincidence counting as a means of noise reduction, which may prove critical to the success of single-particle counting with the MIT biological microbeam.

Coincidence counting is the preferred means of noise reduction for systems in which the signal amplitude is not significantly larger than the amplitude of the noise events. At MIT, the 1.5 MeV protons are predicted to give rise to a PMT signal with a mean of ~12 primary photoelectrons per particle traversal in 10 μm of scintillator. This calculation is based on the energy deposited in the film, determined by the software SRIM (*4*), a film conversion efficiency of ~2% (*1, 5*), a film-PMT separation of 1 cm, a PMT photodiode diameter of 8 mm, and a quantum efficiency of 18% (Hamamatsu R7400 P). The statistical distribution associated with such a small mean value will certainly have some overlap with the low amplitude distribution of the dark current and electronic noise, and therefore coincidence counting may be necessary.

^{241}Am Testing

Experiments were conducted with a 37-kBq ^{241}Am source, with a primary emission of 5.5 MeV α particles, in direct contact with the 10-μm-thick scintillator. The pulse-height amplitude spectrum was measured with the PMT positioned in the head-on configuration with 1.0 ± 0.2 cm between the photocathode and the film. A similar spectrum was acquired with the PMT optically coupled to the side of the film with a Lucite light guide. The same PMT was used in both experiments, and no changes were made in the spectroscopic electronics used to generate the spectra. The centroid of the peak in the light-guide spectrum was found to be 60% of the centroid in the head-on spectrum, indicating that the new geometry is less efficient in light collection than the conventional head-on approach.

To test the absolute efficiency of the light guide, the coupled PMT was run in coincidence with a silicon surface barrier (SSB) detector in the head-on geometry. The ratio of the coincident event rate to the silicon detector event rate, as a function of the amplitude threshold set on the PMT signal, was investigated. At the lowest threshold setting on the PMT signal, a maximum ratio of 99% was achieved with a 5-μs resolution time in the coincidence circuitry.

Last, a spectrum was acquired to assess the distribution in time between the signals arising from the same α-particle traversal generated in the PMT on the light guide and the SSB detector looking at the source directly. The width of this distribution arises from the statistical fluctuations in the signal pulse size and shape from each detector, and it is an important parameter for the ultimate coincidence system. For the best noise reduction, a small width for the distribution is preferred. From the time spectrum generated with the PMT and SSB detector, a width of 0.045 μs was observed (σ of the Gaussian peak). A good compromise between false positives and negatives is therefore the use of 0.5 μs for the coincidence circuit resolving time, which would have a corresponding random coincidence rate of $\sim 1 \times 10^{-4}$ Hz. Such a rate would lead to a 0.1% probability of a false positive event occurring in a 10-s cell irradiation.

Conclusions

The preliminary source-based investigations have indicated that the signal amplitude in the light guide is, at present, less than that achieved with the PMT in a head-on geometry. However, these studies have also shown that there is a 99% probability of a detectable signal generated in the PMT on the light guide, as seen with coincidence measurements with an SSB detector, for a minimum threshold setting on the PMT output. Last, experiments with the PMT on the light guide and an SSB detector head-on have shown that it is possible to achieve a 0.1% probability of noise/false positive events for a 10-s cell irradiation and a 0.5-μs coincidence resolution time. Further gains in signal from the light guide will be necessary before this system is useful for 1.5 MeV protons, and the investigation of a reflective coating as well as geometry optimization will be pursued. In addition, coincidence system testing with two PMTs on the light guide, with the ^{241}Am source, 1.5 MeV protons, and 3 MeV α particles, is required before final conclusions can be made on the feasibility of this approach to single-particle detection for the MIT biological microbeam.

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Irradiation of Collimated Heavy-Ion Beams on Individual Cells: The Method for Detecting Ion Tracks at Irradiation Time

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Heavy-ion microbeam irradiation is a unique method for controlling both the number and the position of ion hits on a target. Applying this method to irradiation of individual cells, we can transfer a limited amount of energy to specific cells, thus enabling us to study the cellular response to low-dose radiation. When studying low-dose effects with a microbeam, obtaining accurate information about the radiation each cell receives, i.e., the number of ions traversing the cells and the localization of dose within each cell, is quite important for the interpretation of the behavior of cells after irradiation. We determined that the best way to achieve this goal is to detect the place where the ion tracks actually hit the samples at the time of irradiation. We therefore developed a method for detecting the ion-hit tracks on the target at the time of irradiation.

The heavy-ion microbeam apparatus, which was installed under a ver-

tical beamline of the AVF cyclotron at JAERI-Takasaki, was used for cell irradiation. The number of ions traversing the sample was detected and counted with a plastic scintillator-photomultiplier tube assembly, and the irradiation was terminated with a beam shutter actuator, which was governed by a preset counter module. The cells were attached to a CR-39 ion track detector (100 μm thick) and then irradiated with 17.5 MeV/μm ^{20}Ne or 11.0 MeV/μm ^{40}Ar ion beams. Just before irradiation, the medium was removed to allow the ions to penetrate the cells and the CR-39 film. The beam was collimated with a 20-μm-diameter aperture, and the each cell was irradiated with 10 counted ions. Immediately after irradiation, the cells were re-covered with medium; then the CR-39 film was etched from the side opposite to the cell with an alkaline-ethanol solution at 37°C for 15 min. After a rinse with distilled water, the etched samples were observed under a phase-contrast microscope. The cell samples were then incubated continuously at 37°C to observe the effect of this etching treatment on the cell growth.

After 15 min of the etching treatment at 37°C, we could observe the ion-track pits on the CR-39 film. At each irradiation point, 9 to 12 ion-track pits were observed. Almost all ion-track pits were concentrated within the collimated diameter range. No significant effect of the etching treatment on cell growth was observed.

These results indicate that this method will provide us accurate information about the spatial distribution of the radiation from the ions. This means that, with this method, we can observe the position and the number of ion hits within and around the target cells at the beginning of the postirradiation incubation of cell samples. When studying low-dose effects, especially the effects of hits by single ions, the accuracy of information about the radiation is quite important. Therefore, this method will be quite useful in studies of the cellular effects of low-dose irradiation, especially in single-ion hit experiments.

Implications of Single-Particle Experiments for Track Theory, Therapy and Radiation Protection

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''Because the predominant exposure of cells in humans is to single isolated tracks, a critical question is what effects a single track is capable of producing and with what probabilities.'' ''Most of the current biophysical models. . . make the clear prediction that a single track can produce virtually all of the detrimental stochastic effects of interest.'' ''But one model, in particular (the amorphous track model of Katz and coworkers), disagrees fundamentally that a single low LET track has the ability to cause the cellular changes.'' ''This model leads to very dramatic differences in the predicted risk at low doses. . . '' ''Because of the very major implications this would have if true, there may be strong grounds for critical evaluation of the model'' (*1*).

That critical evaluation is now possible in single-particle experiments that are the subject of this workshop. Thus far, calculations from published equations, from published radiosensitivity parameters, and thus from predictions in real time have yielded agreement with measurements of cell survival after proton and α-particle bombardments, as well as with single α-particle-induced oncogenic transformations (*2*). From their experiments, the authors have concluded that ''the measured oncogenecity from exactly one alpha particle was significantly less than for a Poisson-distributed mean of one alpha particle, implying that cells traversed by multiple alpha particles contribute to most of the cancer risk'' (*3*).

Recall that our predictions are based on the application of the theory to high-fluence experiments with a variety of bombarding ions, up to argon, at energies up to 10 MeV/nucleon.

This theory also provides the motivation for ion-kill dosimetry (*4*), now being investigated as the basis for evaluation of the qualitative effects of high-LET radiations. Ion kill is also suggested as the basis for the differences in tissue response, for the loss of the benefits of fractionation, and for late effects in neutron and high-LET radiation therapy (where overdose can be ruled out as the cause) and also as a basis for space dosimetry in regard to the protection of astronauts from the deleterious effects of cosmic rays. For these purposes, experiments based on the Biostack principle offer the greatest versatility in projectile type and energy (*5, 6*). Since the physical parameter that determines single-particle response is z^*/β, we propose that the response of a tissue or cell to different particles should match at the same value of this parameter.

Single proton bombardments in particular are needed to test both Katz's theory and the assumption that a single (electron or proton) track has the ability to cause cell killing, and transformation, as demanded by the linear, no-threshold extrapolation to low dose which forms the basis of radiation protection affirmations. This is an excellent opportunity for experiment to test both theory and the conventional wisdom.

There is frequently a hidden agenda in physical units and measurements. Thus dose, energy per unit mass, implies multiple random transits of charged particles through a designated target. Cross section is a probabilistic concept, not a geometric concept. It relates to the probability of an interaction between randomly placed targets and random trajectories of projectiles within a channel. The theory of RBE requires knowledge of cellular properties and the full particle energy spectrum in a radiation field, and it is not restricted to a sub-class of secondary interactions, like all secondary protons. Heat and temperature imply thermal equilibrium, which is not achieved in a ''thermal spike''.

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Session IV

Chair: E. J. Hall

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Upgrading of the Gray Laboratory Soft X-Ray Microprobe with Aluminum K-Shell X Rays and Measurement of the Effect of a Carbon K-Shell X-Ray Beam of Different Size Focused into V79 Cell Nuclei

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The X-ray microprobe developed at the Gray Laboratory was originally designed to produce carbon K-shell X rays (278 eV) by electron bom-

bardment and to focus them into a spot of a few hundred nanometers by using a circular diffraction grating with increasing line density (zone plate) (*1*). Shallow reflection on a silica mirror, placed between the X-ray source and the zone plate, eliminates most of the bremsstrahlung component from the beam, allowing the individual irradiation of subnuclear targets with a near monochromatic carbon K-shell X-ray beam (97% purity). Cell imaging, computerized image analysis, and micropositioning techniques, as for the microbeam facility, are also employed for a fast and accurate target location, irradiation and revisiting. The very fine focus achieved (<0.25-μm-radius spot) and the highly localized energy deposition of carbon K-shell X rays (photoelectron range <7 nm) represent unique tools to investigate modern radiobiological phenomena. Recent improvements have been directed to increase the dose rate (to irradiate a higher number of cells per experimental day and to broaden the spectrum of experiments performed) and to evaluate the possibility of using higher-energy photons. To increase the production of carbon K-shell X rays, the electron current striking on to the graphite target has been increased from 300 μA up to 1 mA while the electron energy had to be decreased to 9 kV to avoid overheating of the target. The overall improvement was a factor of about 2.5 in the production of carbon K-shell X rays. A more significant improvement resulted from the employment of new zone plates optimized for carbon K-shell X rays and for the microprobe geometry. The new zone plates (made by the PSI, Zurich) are made of 250-nm-thick Si_3N_4 and can be as big as 800 μm in diameter with 50-nm structures. Their efficiency reaches about 15% in the first-order focus, leading to a final dose rate of about 6 Gy/s for a typical mammalian cell. Improvements have also been made regarding the use of higher-energy X rays. Measurements using an aluminum target have confirmed the possibility of producing a nearly monochromatic aluminum K-shell X-ray beam (1.48 keV) without drastic alteration of the microprobe source. The bremsstrahlung component of energy higher than 1.5 keV is again significantly reduced by the use of the silica mirror (although set for a smaller reflection angle than for the carbon K-shell photons) while 3 μm Mylar filters the low-energy photons. As a result, a near monochromatic aluminum K-shell beam with 95% purity is achieved. A preliminary germanium zone plate that focuses the aluminum K-shell beam into a submicrometer size spot has been tested successfully. The final dose rate corresponds to about 1.5 Gy/s for a typical mammalian cell. By considering the small focusing spot (<250 nm), the high absorption of the carbon K-shell and aluminum K-shell photons and the low range of the secondary electrons produced, it is possible to deliver very precise doses to cytoplasmic targets or to a particular region of the cell nucleus. Because of their different absorption characteristics, aluminum K-shell and carbon K-shell X rays will also produce different dose distributions inside the sample for any given dose. Dosimetric calculations show that after carbon K-shell irradiation, there will be a factor of ~50 difference between the energy absorbed at the entrance and at the exit of the cell nucleus but only a factor of ~2.5 for aluminum K-shell X rays. The efficiency of the microprobe system has been tested by assessing the clonogenic potential of V79 cells irradiated with carbon K-shell X-ray beams of different sizes (5 and 0.25 μm radius). Particular interest has been directed to the low-dose region (<0.5 Gy), where discrepancies from the linear-quadratic model were expected based on previous experiments. Although the two sets of data do not show statistically significant discrepancies, a linear-quadratic fit described the data obtained with a 5-μm beam well, while a modified linear-quadratic model that includes hypersensitive and adaptive response at low doses fits the 0.25-μm data better. This is in good agreement with theoretical estimation of the surviving fraction after partial irradiation of the cell nucleus performed by Scholtz. According to such calculations, bigger differences should result by comparing the effect of focused and unfocused aluminum K-shell X-ray beams, for which experiments are already planned. Recently, we have used the microprobe facility to investigate some aspects of the bystander effect. The data show a ~10% decrease in survival after the irradiation of a single V79 cell. This effect is constant over a large dose range (up to 2 Gy) but follows a dose response below 0.2 Gy. By irradiating a single cell and following the fate of all the neighboring cells whose coordinates have been recorded, it is also possible to perform critical analyses of the spatial distribution of the damaged cell. The analysis indicated no correlation between the bystander effect and the distance of the damaged cells from the irradiated one. However, a statistically significant clustering of damaged cells has been observed in the irradiated dishes relatively to the control in which an area with no cell has been irradiated.

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Spatially Resolved Single-Cell Irradiator to Study Bystander Responses to Low-LET Radiation

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Currently, the standards for human exposure to ionizing radiation are determined by extrapolating data obtained at high doses to the low-dose region using a linear, no-threshold model. This extrapolation of the radiation risks presented from low doses implicitly assumes that cells in an irradiated population respond individually, not collectively (*1*). When α particles enter tissue, they traverse only a few cells before stopping. Therefore, unless the fluence is extremely high, only a small portion of the exposed cell population receives a direct dose. For such high-LET radiation, it has been shown that the number of cells responding to the radiation exceeds the number of cells actually hit (*2*). The bystander effect refers to the observation of a biological response in the absence of direct irradiation. Although bystander effects have been demonstrated for high-LET radiation, they have not been shown for low doses of low-LET radiation. In an effort to address this latter case directly, we developed a novel single-cell irradiation device. This device has been designed such that high-energy electrons deposit energy in a preselected subset of cells for which the unirradiated neighbors can be identified easily. By targeting individual cells with a highly spatially resolved dose, the biological responses of a single irradiated cell or a bystander can be studied.

The device consists of a pulsed electron beam capable of operating at energies from 10 to 80 keV. Rather than making use of a tightly focused beam that makes it difficult to achieve sufficiently low electron fluxes, we have instead chosen to use a broad source (~5 mm) combined with a spatial collimator. The electron gun is housed in a standard vacuum chamber pumped by a turbo molecular pump (base pressure 1×10^{-7} Torr). The chamber is equipped with a Faraday cup for monitoring beam current and an optical shutter to ensure no electron dark current between pulses.

The spatial resolution of the device is achieved in two stages by passing the electron beam first through a pre-collimator and then through a very high aspect ratio hole (~15:1) in the final collimator/interface platen. The pre-collimator is fabricated from an aluminum disk with a 0.005″ hole for transmitting electrons. Ten micrometers of gold is plated on the exit side of the pre-collimator. The pre-collimator serves to reduce the number of electrons hitting the final collimator and decreases the chances for production of X rays at the cell interface.

After they exit the pre-collimator, the electrons hit the collimator platen, which establishes the final spatial resolution of the electron beam. The platen has been designed to minimize the production of X rays while optimizing the spatial resolution of the delivered dose. It is fabricated from a 0.15″ thick aluminum disk. A 0.05″ hole is drilled, leaving an aluminum membrane 25 μm thick, sufficient to stop 50 kV electrons. This membrane is then coated with 10 μm of gold. To provide the final beam collimation, a 2-μm hole is laser drilled through the aluminum/gold membrane. Both the collimator and pre-collimator can be constructed with one hole or a series of holes depending on the biological experiment of interest.

The vacuum interface is obtained by coating the platen with a 200-nm polyimide window. The irradiation device is interfaced with a biological irradiation chamber mounted on an X-Y scanning stage of a standard optical microscope. Cells are plated on a 1.5-μm Mylar membrane that is place directly on the electron gun interface. Thin, low-density films of polyimide and Mylar are highly transparent to energetic electrons. The device can deliver doses of a few to hundreds of electrons per cell. At very low fluences, the number of electrons actually passing through the hole will be governed by Poisson statistics. The feasibility of the design has been investigated by a Monte Carlo simulation of the irradiation of a cellular monolayer (*3*). The results indicate that the majority of the calculated beam spreading would be contained within a volume typical of cells from mammalian cell lines.

The dosimetry of the device has been measured using several different methods. The electron beam is characterized by measuring the current on a Faraday cup both before and after collimation. Very low currents are measured in pulse counting mode using a channeltron. Imagining of the beam is done on a ruggedized phosphor screen.

Dosimetric measurements are performed using GAFCHROMIC® HD-810 dosimetry film. These films are suitable for beam profiling and dose mapping over a wide range of absorbed dose. The films are composed of materials with low atomic number, which produce minimal alteration of the radiation field. Film calibrations were made at various doses using a ^{60}Co source. Films were analyzed using a CCD camera/optical microscope arrangement to measure optical density differences and indicated good spatial localization of the dose. Using a Canberra germanium spectrometer, measurements have also been made to check for the production of X rays under various conditions. We find that the contribution from X rays is a minimal component of the total dose delivered to the cell.

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Soft X-Ray Microbeam in the Water Window Energy Region Using a Capillary Plasma Source

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Introduction

In the last few years, many types of sources and X-ray focusing devices have been developed to obtain micrometric spots (*1*). In this area, tabletop soft X-ray sources for application in radiobiology and biological imaging are very interesting. With this aim, we have constructed a capillary plasma source, and we are in the process of developing an optical system, based on the use of zone plates as optical elements, in the water window region (284–532 eV). With this experimental setup, we plan to develop a soft X-ray microbeam and an imaging system for irradiated biological samples.

X-Ray Source

The X-ray emission of the source is based on the production of plasma in a capillary pipe (15.5 cm log and 4 mm in diameter) in which argon gas has been introduced. Plasma is produced by discharging a capacitor with a strong current (30–35 kA) impulse quickly across the gas.

The plasma by this process produced collapses radically (pinch effect) because of the magnetic field generated by the current itself. By the collisional excitation of ions, soft X rays are emitted. The MARX High Tension generator (350 kV) charges the capacitor that is suddenly discharged on the capillary when the spark gap is closed. Our source, which uses argon gas in the capillary, can produce X rays at an electron temperature in the plasma of about 70 eV. In the future, we also plan to use nitrogen and krypton gas. To optimize the efficiency of the X-ray emission and to determine the characteristics of the radiation, we have done measurements of the intensity of the radiation as a function of the argon pressure in the capillary and of the source–detector distance using a PIN diode filtered with 0.5 μm of vanadium. The vanadium filter selects X rays between 300–510 eV. The measured X-ray flux is about 4×10^{13} ph/cm^2 at a distance of 40 cm from the source with a duration of 50 ns and argon pressure optimized at 0.4 Torr. On the capillary axis, the measured source diameter, during the pinch effect, is less than 300 μm.

X-Ray Optical System

Our experimental setup is based on the use of zone plates (*2*). We will use two different types of zone plates, a condenser zone plate and microzone plate. The condenser zone plate focuses and monochromatizes the X-ray beam on the sample with different focal lengths, and the microzone plate produces an image of the biological sample on a CCD detector. We can use two condenser zone plate types. The first one is made with the e-beam technique by Fastec (diameter 920 μm, zone number 920, outer zone dimension 250 nm), and the other is made holographically by a group at Göttingen University (D. Rudolph and G. Smahl, Institut fur Rontgenphysik, George-August Universitat Göttingen, Germany, diameter 9 mm, zone number 41890, outer zone dimension 53.7 nm). The depth of focus of the zone plate, which indicates the size of the permitted displacement from the ideal focal plane for which the intensity on optical axis is reduced by only 20%, is a very important parameter in relation to the acceptable thickness for the biological samples. In fact, with a sample thickness greater than the depth of focus, the material outside that region would not be irradiated uniformly, and the image would be seen with lower resolution. In our case, the condenser zone plates by Fastec and by Göttingen have, respectively, average depths of focus of 80 μm and 4 μm. The first condenser zone plate is convenient for thick samples, while the second is convenient for thin samples. The size of the focal spot depends on an Airy distribution in the focal plane and on the size of the source. In our case, for a distance of 60 cm from the source and for $\lambda = 30$ Å, we obtain a 35-μm focal spot size for the condenser zone plate from Fastec and 75 μm for the condenser zone plate from Göttingen (*3*). In conclusion, from the radiation characteristics of the source and from the optical features of the beamline, it is possible to obtain a soft X-ray microbeam well suited for radiobiological applications.

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Low-LET Microbeam Dosimetry

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We describe here the dosimetry calculations supporting the design of several low-LET irradiation facilities and the interpretation of experiments performed with them. The computations involve event-by-event, detailed-history Monte Carlo simulations of low-energy electrons and soft X rays interacting in a low-*Z* homogeneous medium. The spatial variation of absolute event frequency and the distribution in specific energy are typically obtained, and from these one can estimate the dose for arbitrary cellular monolayers growing on a thin substrate. This approach represents an extension of Berger's point-kernel treatment into the microdosimetry realm.

Cellular Targets

Preliminary calculations were performed with realistic cell-like target boundaries, obtained by confocal microscopy of HeLa cell monolayers (*1*). Two cells (designated A and B) were selected for dosimetry calculations. The average radii of the two nuclei and cytoplasms were similar; however, the thickness of the nucleus of cell A was approximately 25% greater than that of cell B, even though the total cell thicknesses were comparable (6 and 7 μm, respectively, for A and B). Hence the cytoplasm above the nucleus was much thinner for cell A than for cell B. This difference in cell components had a significant effect on the mean energy imparted in the two cases and emphasizes that the actual cell morphology will be important in evaluating the dosimetry in radiobiology experiments.

25 keV Electrons

Extensive dosimetry calculations have been made, aimed at characterizing the spatial variation of the stochastics of the energy deposited by the slowing and stopping of individual energetic electrons (*2*). Electrons of 25 keV were simulated and energy deposition distributions, scored in 1-μm spheres located at varied penetration and radial distances up to 15 μm from the point of incidence. The 1-μm scoring-sphere size was selected as a compromise between large enough to get reasonable statistics within acceptable computational time and still small compared to real mammalian target cells. The 25 keV electrons were selected for a first study because the energy deposited will be contained mostly within a typical mammalian cell. Single tracks were scored because their energy deposition distributions are elementary. Computations for higher doses (multiple tracks) are under way, and results will be compared with single-track mean values combined by binomial statistics.

The PITS code set (*3*) was used to generate the electron tracks by replacing the continuum δ-ray source module with a delta function in energy. Single event distributions were computed for penetration and radial distances until the event probability fell to less than about 0.1%. Sample sizes ranged from 100,000 to 1 million tracks, depending on penetration and radial distance.

Over the range of 10 μm in penetration, *h*, and radial distance, *r*, the event frequency decreases from 1 to less than 10^{-4}. After the first micrometer, the event frequency decreases along the *h* direction approximately exponentially. The event frequency is more varied radically; at small penetration, the event frequency decreases rapidly with radial direction, reflecting the low probability of very large angle scattering by the electron near its initial energy. At deeper penetration, the event frequency dependence takes on a more isotropic shape, as a result of many random small-angle scatterings. The variation of the mean energy deposited (frequency-mean lineal energy, y_F) varies from about 1.5 keV/μm at the lowest to roughly 3 keV/μm near the end of the electron range. y_F initially decreases along the *h* direction, passes through a minimum at about 4 μm, and then increases again. As the electrons penetrate, two things happen: They scatter away from their initial direction, and they slow down; i.e., their stopping power increases. These two effects account for the variation of y_F with penetration. Near the starting point, the electrons will have nearly diametric paths across the 1-μm-diameter site. As they penetrate and before they have lost significant energy, scattering begins to have an effect; some electrons will have shorter paths within the site and hence will deposit less energy on average. This accounts for the initial decrease in y_F with penetration, *h*. Eventually the slowing, with associated greater stopping power, dominates and y_F increases with further penetration and radial distance. Furthermore, near the end of the electron track, scattering produces such a tortuous path that the mean path length within the site will actually increase and therefore will also contribute to the increase in energy imparted.

Soft X Rays

A soft X-ray module has been added to our PITS suite of Monte Carlo radiation simulation tools. It is specifically designed to simulate the interactions of low-energy photons, e.g. the characteristic X rays of carbon (278 eV), aluminum (1487 eV) and titanium (4509 eV). The three most important interactions at low energy are included; they are the photoelectric effect, coherent (Rayleigh) scattering, and incoherent (Compton) scattering. The photoelectric effect, since it is by far the most important, is simulated by ''table lookup''. The other two interactions are simulated by the Monte Carlo rejection method using Klein-Nishina theory and published form factors and scattering functions. Bremsstrahlung production and beam focusing are features yet to be included. Atomic additivity is assumed for the absorbers and appropriate atomic source data have been assembled for all elements through calcium (except the noble elements) plus iron.

Calculations are under way for the cell A and B target morphologies described above with a 0.9-μm Mylar-equivalent entrance foil and ICRU's recommended composition for spleen as absorber medium. Preliminary results for aluminum X rays (1487 eV) indicate that the fraction of photons that deposit energy in the target compartments are as follows:

	Cell A	Cell B
Nucleus	0.41	0.31
Cytoplasm	0.058	0.20
Above	0.40	0.35
Foil	0.14	0.14
Backscatter	0.002	0.002

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Low-LET Microbeam Dosimetry

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Several laboratories are using microbeams to characterize bystander responses induced by low-LET radiation. This extended abstract describes dosimetry calculations supporting the design of low-LET microbeams and the interpretation of data obtained from these facilities. One approach to low-LET microbeam irradiation is to place a mask with micrometer-size holes over an electron gun (see Resat *et al.* in these proceedings). Monte Carlo simulations by Miller *et al.* (*1*) suggested that individual mammalian cells in a confluent monolayer could be targeted for irradiation by 25 to 100 keV electrons with minimal dose leakage to their neighbors. The PITS (*2*) code set was used to generate electron tracks by introducing a monoenergetic δ-ray source module. Tracks simulated in a uniform water medium were scored in a simple model that assumed the target cell was cylindrically symmetrical and had concentric cytoplasm and nucleus. Radial profiles, the lateral extent of cytoplasm and nucleus as a function of depth into a cell, were obtained from confocal microscopy of HeLa cell monolayers.

To obtain a more realistic model of cellular targets, we used unstructured grids to define a coordinate system in a reconstructed 3D image of the HeLa cell monolayer. Cell morphologies quantified in this way can be used to calculate the probability that a specified amount of energy will be deposited in each volume element defined by the grid; however, more meaningful results are usually obtained by grouping the volume elements that define an organelle, such as the nucleus. The amount of cellular structure that can be included by this method is limited only by the experimental capabilities of confocal microscopy.

In general, one cannot expect confocal microscopy to be available for every cell targeted in a microbeam irradiation experiment. Nevertheless, information about the feasibility of single-cell irradiation of a given cell type at various beam energies can be obtained with only a few confocal images that capture the typical morphology of the cell line under investigation. Merging data on cell morphology with spatial patterns of energy deposition in a homogeneous water medium provides a qualitative picture of the microdistribution of dose expected when a particular cell type is used in experiments with microbeam irradiation. Wilson *et al.* (*3*) calculated the spatial variation of energy deposited in a uniform water medium by individual energetic electrons. The stochastics of energy deposition by multiple tracks can be obtained from these results using binomial statistics. The accuracy of this method has been tested by comparison with simulations of pulses containing two, four and eight electrons.

Focused X-ray sources are another approach being used to obtain low-LET microbeam irradiation of cells (see Schettino *et al.* in these proceedings). A soft X-ray module has been added to the PITS suite of Monte Carlo track structure simulation tools. The code has been used to simulate electron tracks generated by the absorption of characteristic X rays of carbon (278 eV), aluminum (1487 eV) and titanium (4509 eV). The photoelectric effect, which is by far the most frequent interaction of low-energy photons with matter, was simulated by a table-lookup method. Less frequent events due to coherent (Rayleigh) scattering and incoherent (Compton) scattering were simulated by a Monte Carlo rejection method that used Klein-Nishina theory as well as published form factors and scattering functions. Atomic additivity was assumed for the absorber and atomic source data have been assembled for all elements (except noble gases) through calcium plus iron.

Calculations have been carried out with the model of HeLa cells developed by Miller *et al.* (*1*) and ICRU's recommended atomic composition of spleen as the absorbing medium. Preliminary results for aluminum X rays showed that about 15% of the incident photons were absorbed in a 0.9-μm Mylar-equivalent entrance foil, approximately half of the photons deposited energy in the target cell, and about 35% were absorbed in the medium above the cellular monolayer. Work in progress will add Bremsstrahlung production and beam focusing to these simulations.

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Session V

Chair: K. M. Prise

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ALBA: Alpha Light-Based Analysis. A New Approach to Single-Cell Irradiation

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Several nuclear techniques employ few-MeV ion microbeams to analyze a sample localized to micrometric resolution. In 1998, B. L. Doyle and collaborators at Sandia National Laboratories proposed to replace beam focusing with the measurement of the impact position of the single ion of a broad beam (*1*; Doyle *et al.*, results presented at ICNMTA 2000). Although this is possible only for a low-rate beam, with less than 10^5 particles/s, nonetheless, a large amount of physics could be done concerning the material analysis and microelectronics with the techniques IBICC (ion beam induced charge collection), IBIL (ion beam induced luminescence) and SEU (single-event upset). There are two ways to identify the ion position, exploiting either the secondary electrons emitted from the sample surface or the light coming from a scintillating layer attached or coated to it. The latter method, called IPEM (ion photon emission microscopy), although less precise (resolution not better than 1 μm), has the advantage of ''in-air'' irradiation and reduced cost, by using a standard optical microscope to collect light and enlarge the impact position for a subsequent measurement in the image plane with a position-sensitive detector (PSD). Thus IPEM ''in-air'' radiobiology experiments look possible in principle.

With the contribution of some of the authors, a simplified version of IPEM, in which the accelerator ion beam is replaced with an α-particle emitter placed somewhere close to the sample, has also been envisioned. This version, which is low in cost and is only as bulky as an optical microscope, is the object of a feasibility study called ALBA (alpha light based analysis), recently funded by the Italian research institution INFN and whose early outcomes are reported here.

Although only one ion species (an α particle) with limited energy choices can be used, ALBA seems poised to become a remarkable experimental resource. In addition to material science and microelectronics applications, biological and medical studies can be carried out. Presently, single-cell radiobiology employs collimated ion beams, obtained with holes of a few micrometers and affected by an unavoidable halo, which renders true micrometric precision difficult. Focused microbeams will be used in a few laboratories, but no one can guarantee that the vacuum window will not deteriorate the beam. Regardless, a system like ALBA looks appealing for its simplicity, and it could support more sophisticated equipment. The kind of experiments to be carried out is somewhat similar to that performed years ago exploiting broad beams and stacks of CR-39 solid-state nuclear track detectors. In these experiments, there are random hits on a cell culture and then a subsequent identification of the positions of the hits. The great advantage of the present system is that this position is defined online and not after long and demanding microscope measurements, allowing detailed statistics to be collected.

One should let cells grow directly on a scintillating blade of 10–20 μm, which will then be placed horizontally on the microscope object plane. Three isotopes look appealing as possible sources: ^{148}Gd (3.18 MeV, 75 years), ^{210}Po (5.407 MeV, 138 days), and ^{241}Am (5.48 MeV, 433 years). The source has to be put close to the sample because of the limited range of α particles in air, but the source cannot be too close to avoid irradiation at undefined angles and a discrepancy between the light spark and the irradiation position. In practice, a sample inspected area with a diameter of 1 mm, an α-particle source surface of similar size, and a distance between them of about 10 mm looks reasonable. The source could be kept in place either by a holder adjacent to the lens or by direct insertion in a small cavity in the lens itself. A 5.5 MeV α particle would ultimately reach a backward surface barrier silicon detector, since its range is about 40 μm in water. That would allow a rough check of whether a cell has actually been hit, by measuring the energy loss, and above all trigger a signal for the position detector readout. A possible scintillating material is a doped plastic like Pilot-B or Bicron, easily workable in thin blades (*2* and Yang *et al.*, results presented at ICNMTA 2000) and suitable as cell culture support. It gives about 70 ph/μm when hit by α particles of a few MeV. The overall detection efficiency depends also on lens numerical aperture, the optical transmission coefficient, and PSD quantum efficiency.

There are three questions a feasibility study should answer: Can the α-particle detection efficiency be close to 100%, which is absolutely necessary in radiobiological experiments? Can the source be active enough to give cell-hit rates larger than dozens per second, as required for a reasonable irradiation experiment time, while keeping a good energy resolution? Can spatial resolution be close to 1 μm? To answer these questions at least partially, tests with microscope, α particles, and a scintillating blade have recently been carried out.

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Probing the Origins of Radiation-Induced Biological Damage in Normal Human Cells with a Charged-Particle Microbeam

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The selective exposure of parts of intact individual cells to high-energy radiation was recognized many years ago by Zirkle and Bloom (*1*) as having the potential to provide information about the mechanisms whereby radiation produces such ''strikingly injurious effects in living systems'', and to ''aid in analyzing the normal functions of the various cell parts by selectively altering them''. Accelerator-based microbeams and collimated or microneedle coated isotopic α-particle sources helped to establish one of the basic paradigms of radiation biology: that it is the hit cell nucleus from which deleterious effects originate. In 1962, a symposium on partial and microbeam irradiation, reported on by Smith (*2*), was held at which ''All participants were persons who are actively interested or working in this rapidly growing field.'' It now appears that the growth of the field stalled for a number of years, with a resurgence in interest developing in part from concern about the effects of single α particles and from low-dose studies with isotopic α-particle sources. The results of these latter studies challenged the basic paradigm of radiation action. Low fluences of α particles were delivered to mammalian cells such that only a small fraction of cells were likely to have had their nuclei hit by an α particle. Significantly more cells showed changes in sister chromatid exchange frequencies, in chromosomal changes, and in gene expression than were traversed by α particles; hence some responding cells were bystanders of hit cells. These end points along with other cellular/molecular end points relevant to the risks associated with exposure to ionizing radiation were not available in the early 1960s. The development of pertinent risk and mechanistically oriented assays may perhaps be considered to be the greatest incentive for the burgeoning current interest in microbeams.

Studies with broad-beam α-particle sources, either isotopic or accelerator-based, cannot of course readily distinguish between known hit cells and known bystander cells, nor can the relative contributions of nuclear hits, of cytoplasmic hits, or even of medium hits to the final biological response be ascertained. The ability to establish the origin of the biological responses of individual cells is, however, attainable with a microbeam, consistent with the original expectations of Zirkle and Bloom (*1*).

We have used the Columbia University RARAF microbeam and specifically devised protocols to target individual cell nuclei, or cell cytoplasm, and to miss cells completely, hitting intercellular medium. Using these protocols, all sites or a known proportion of sites can be irradiated with a precisely known number of α particles. That is, some cells can be hit cells while the remaining proportion will be bystander cells. Alternatively, cells can be labeled with two vital dyes then plated in known proportions and only one cell population irradiated. After irradiation, the hit cells can be discriminated from the bystander cells, with the relative positions of each cell at the time of irradiation being recorded. End points examined include frequencies of micronuclei, cell growth by monitoring cell numbers (all cells on a dish are examined), progression of cells through the cell cycle by monitoring bromodeoxyuridine uptake, and the expression of the stress-related genes TP53 and CDKN1A (p21, WAF1/Cip1). The latter is undertaken on cells *in situ* using immunofluorescence, and also after the removal of individual cells of known radiation history with a micromanipulator and carrying out single-cell RT-PCR.

The conclusions from an extensive series of studies with normal human fibroblasts using RARAF microbeam protocols are as follows:

1. Deliberately missing cells and irradiating the culture medium between them produces no detectable response.
2. Irradiating cell cytoplasm (including the medium surrounding the cell) produces no detectable increase in the frequency of micronuclei, but does induce some cell cycle delay in synchronized G_0/G_1-phase cells. No delay is seen in cycling cells.
3. Irradiating cell nuclei (including surrounding cell cytoplasm and medium) results in fluence dependent increases in micronuclei, cell cycle delay, and increases in gene expression. This response specifically includes exactly one α particle.
4. Irradiating 50% of cells through their nuclei produces a response in known non-hit bystander cells, which is not dependent on the number of particles through the hit cells.
5. Reducing the proportion of hit cell nuclei results in a proportional lessening of the response in bystander cells.

6. Irradiating hit cells through the cytoplasm does not produce a response in bystander cells. The expression of a bystander effect in non-hit cells originates from insult to the nuclei of hit cells.

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Large DNA Fragment Distributions in Directly Hit Cells from Low-Dose, High-LET Radiation

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We modeled DNA double-strand breaks (DSBs) produced by low-dose high-LET radiation. Our approach considers stochastic DSB distributions along chromosomes on large DNA scales, from 100 Mbp down to ≈2 kbp, using analytical techniques and Monte Carlo computer simulations in a mechanistic model of chromatin geometry acted on by ion tracks. In the model, called DNAbreak, a high-dose case corresponds to the juxtaposition of many ion tracks interacting with a chromosome. Low-dose extrapolation by DNAbreak was produced by one-track action. The efficiency of a track in the creation of DSBs along a chromosome is characterized by a single parameter, *Q*: the radiation quality parameter. This parameter has been determined for a number of ion velocities and charges. In the one-track action, we have introduced an impact parameter: the distance between the track core and a given chromosome in the cell nucleus. Both random-impact and ''precise'' parameters were considered. A deterministic model of the radial energy distribution of the track allowed for the interaction between an ion and a chromosome through the ion's penumbra. The radiation energy profile was computed from both ionizations and excitations.

DSB distributions are non-random in the genome as a result of the propensity of DSBs to cluster in space for high-LET radiation. Our chromosome random-walk model neglected molecular details but systematically incorporated the increase in average spatial separation between two DNA loci as the number of base pairs between the loci increased. All chromosomes in a human cell nucleus were simulated. The results lead to biophysically based estimates of DNA damage by one-track action, which dominated at low doses. Clustering of DNA damage was observed for high-LET HZE and α particles in comparison to low-LET radiation. It was shown that the action of ions with wide penumbras is similar to low-LET radiation for cells hit by penumbras only.

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Direct Evidence for CDKN1A (p21) Focus Formation at Sites of Particle Traversal and the Association with ATM Protein

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The cellular response to radiation-induced DNA damage is complex and includes cell cycle checkpoint activation, DNA repair, and changes in gene transcription (*1*). Late radiation effects like genomic instability and transformation depend on the outcome of this network of processes. Therefore, to assess these biological end points, it is crucial to understand the signal transduction mechanisms that connect initial DNA lesions with the overall DNA damage response. In view of the application of heavy-ion beams in radiotherapy, this task has become increasingly important for high-LET radiation.

The examination of immunofluorescence-stained proteins localized to individual tracks of heavy ions in human cell nuclei using confocal laser scanning microscopy provides a means to monitor the cellular response to radiation within defined subnuclear regions. We have recently reported the very rapid formation of localized foci of CDKN1A correlated to sites of ion traversal (*2*). CDKN1A is known to have a central function in radiation-induced growth arrest (*1*). Direct evidence for the spatial correlation of CDKN1A foci to particle tracks has now been obtained using broad-field particle irradiation in conjunction with the retrospective determination of actual ion traversals through individual cells as an alternative to microbeam irradiation (*3*).

Aiming to understand the functional significance of this very rapid relocalization of CDKN1A to sites of heavy ion damage, we used immunostaining of individual cells to further investigate the association of CDKN1A with other proteins known to be involved in the early steps of the DNA damage response pathway. Here we present results showing colocalization of the ATM protein to radiation-induced CDKN1A foci. However, AT cells are still capable of CDKN1A focus formation.

Spatial Correlation between CDKN1A Foci and Particle Tracks

For the determination of actual sites of heavy-ion traversal, an experimental method described by Soyland *et al.* (*4, 5*) for absolute dosimetry of α-particle hits per cell was applied with slight modifications for heavy-particle irradiation. Cells (AG1522B human diploid fibroblasts, Coriell) were grown on CR-39 plates, irradiated with 10 MeV nucleon^{-1} calcium ions at the UNILAC (Darmstadt), and subjected to immunofluorescence analysis using antibodies against human CDKN1A as described (*2*). After acquiring images of CDKN1A foci, CR-39 plates were etched for track detection. Reference tracks produced by preirradiation of the CR-39 plates allowed a precise matching of images. A clear spatial correlation between the CDKN1A fluorescence response pattern and the calcium-ion tracks was determined (*3*). This technique is an alternative to the microbeam irradiation, and it provides direct evidence for the radiation-induced localization of CDKN1A to tracks of heavy ion damage.

Co-localization of ATM and CDKN1A in Subnuclear Foci

ATM is a protein kinase that is activated after exposure to ionizing radiation to phosphorylate many checkpoint proteins including TP53 (*1*). ATM plays a key role in DNA damage-activated signaling pathways and is likely to be involved in the detection of DNA damage (*6*).

Considering the very early response observed for CDKN1A and the upstream position of ATM in the radiation-induced signaling cascade, we examined the subnuclear localization of both of these proteins in confluent primary human fibroblasts (AG1522B) after exposure to heavy particles. The technical approach based on immunofluorescence analysis using confocal laser microscopy was essentially as described for CDKN1A (*2*). However, an additional incubation in hypotonic Hepes extraction buffer (containing 0.1% Triton X-100) was included prior to the fixation of cells to remove soluble proteins and to reduce the level of diffuse nuclear background staining of ATM. Mouse anti-Atm (Ab-2) and rabbit anti-CDKN1A (Ab-2), used for double-staining, were obtained from Oncogene; mouse anti-Cdkn1a was from BD. The secondary antibodies were carrying either Alexa 488 (goat anti-mouse, green) or Alexa 568 (goat anti-rabbit, red) as fluorescent labels.

After exposure of cells to 3.2 MeV nucleon^{-1} uranium ions (LET ≅ 14500 keV/μm), immunostaining for ATM revealed easily recognizable subnuclear foci in the wild-type human fibroblasts. The bright clear ATM foci were detected directly after irradiation, i.e. with cells kept on ice no longer than 15 min postirradiation. At 3×10^6 particles cm^{-2}, an average of about eight particles are expected to traverse the nuclear area of about 230 μm^2 (mean). This number correlates well with the number of foci per nucleus counted in a preliminary evaluation. Irradiation at the lower fluence of 1.5×10^6 particles cm^{-2} confirmed the correlation of foci formation with nuclear particle hits.

As described previously for CDKN1A focus formation induced by lead-ion irradiation, the ATM foci obtained at sites of uranium-ion traversal persisted for at least 18 h. In contrast, cells irradiated with variable doses of sparsely ionizing radiation lacked detectable ATM foci at any time. Dual immunostaining using anti-ATM and anti-CDKN1A antibodies (from different hosts) revealed the formation of nuclear foci of both proteins in the same individual cell after exposure to uranium ions. The patterns of CDKN1A immunoreactive sites were identical and were superimposable on those obtained for ATM, demonstrating the co-localization of these proteins. However, one must bear in mind that the co-localization of these two proteins may not necessarily involve their physical or functional interaction. Instead it could arise from their association with different types of DNA lesions persisting localized within the heavy-ion tracks.

To determine whether a physical interaction of CDKN1A and ATM was necessary for the association of CDKN1A to damaged DNA, and to confirm that Ab-2 anti-ATM foci formation was specific for ATM, fibroblasts (GM2052C, Coriell) from an AT patient defective in expression of ATM were used. The AT cells were irradiated with 2×10^6 particles cm^{-2} of 3.5 MeV nucleon^{-1} nickel particles (LET ≅ 4000 keV/μm) and tested for *in situ* immunoreactivity to anti-ATM and anti-CDKN1A. As expected, these cells failed to localize ATM to nuclear foci after irradiation. However, radiation-responsive CDKN1A focus formation was still observed in these ATM-deficient fibroblasts (scored immediately or 30 min after irradiation), indicating that the association of CDKN1A to tracks of heavy-ion-induced DNA damage is not affected by the absence of functional ATM. Furthermore, since ionizing radiation-induced early phosphorylation and activation of TP53 is abrogated in AT cells, the preliminary results provide evidence for CDKN1A focus formation being independent of TP53 and the known function of CDKN1A in growth arrest.

From the immediate co-localization observed for CDKN1A and ATM, we infer that both proteins may have an early function in the response to DNA damage. Our results from the experiments with AT cells are indeed supportive for a new role of CDKN1A in the DNA damage pathway, possibly as a sensor of DNA lesions. However, although CDKN1A and ATM appear to coexist within a common nuclear substructure at sites of particle traversal, association of CDKN1A to DNA is not directly mediated by ATM. Thus the mechanisms underlying CDKN1A focus formation remain to be elucidated.

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Session VI

Chair: N. F. Metting

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Bystander Mutagenesis Induced by a Single Alpha Particle is Mediated by Cell–Cell Communication

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Radon, a well-known lung carcinogen in underground miners, is a colorless, odorless gas present in indoor environments including homes and schools. At high exposure levels, the bronchial epithelial cells at risk may be traversed by several α particles, whereas for individuals exposed in homes at a normal level of domestic radon, it is unlikely that any cells

at risk will be traversed by more than one α particle in a lifetime. Because of a lack of direct epidemiological evidence linking indoor radon and lung cancer, risk assessment models for the general population have been based on extrapolation from higher exposures in studies of underground miners. Over the past several years, there have been many reports on the induction of bystander effects in mammalian cells by α particles (*1–7*). When only a fraction of a cell population is irradiated by α particles, the biological effects observed have been to a significantly higher proportion of cells than those estimated to have been traversed by an α particle. Although the mechanisms of the bystander effects are unclear, these investigations surely raise one important issue: how to assess the cancer risk of low-dose exposure to radon. How does the bystander effect modulate the perceived risk?

It has been difficult to measure the induction of genetic changes in cell populations in which only a small fraction of the cells were traversed by exactly one α particle, particularly in the case of mutations, where the frequencies observed are low and are more correlative with cancer incidence. A precision charged-particle microbeam can solve this problem easily since under the control of an image analysis system, a small, randomly selected fraction of cells or parts of cells can be traversed by an exact number of α particles, including a single α particle. Using the microbeam of Columbia University and the highly sensitive A_L cell mutagenic assay, we show here that irradiation of 5–20% of randomly selected cells each with a single α particle through the nucleus results in mutant fractions that are significantly higher than expected assuming no bystander modulation effect. It is of interest to note that the mutation fraction induced among A_L cells in which 10 to 100% of the cell populations were irradiated with a single α particle each was not much different. The decrease in bystander mutant yield could indicate that the production of the mediators of mutation was saturated because the number of nonirradiated cells in direct contact with an irradiated cell between the 10 and 20% population was not much different. The observation that mutant yields are unchanged irrespective of whether all or only a fraction of the cells are subject to the same level of damage is clear evidence for a strong bystander mutagenic effect, which indicates that unirradiated cells are responding to damage induced in irradiated cells.

To explore the mechanisms involved in the bystander mutagenic effects, experiments were performed to investigate the contribution of gap junction intercellular communication between traversed and nontraversed cells. Two complementary research approaches were conducted. In our first set of studies, we treated A_L cells with a 1 m*M* concentration of octanol, which inhibits gap junction-mediated intercellular communication (*8*), 2 h before and 3 days after irradiation. We found that pretreatment with octanol significantly decreased the mutant yield ($P < 0.01$). These results indicate that gap junction cell–cell communication plays a critical role in mediation of such a bystander mutagenesis. Octanol, however, is not specific to gap junctions, and other cell structures are affected by it as well. Since connexins are the principal protein component of gap junctions and there is much evidence that connexins alone (assembled in a lipid bilayer) are responsible for the generation of gap junction channels (*9–11*), we used genetically engineered A_L cells with either functional or defective gap junction communication to examine the bystander mutagenic effect.

In our second approach, we transfected AH1-9 cells (a variant of A_L cells with a high background as well as induced mutant yield) with either a dominant negative connexin 43 vector that shut down gap junction communication or a vector control. Using the scrape-loading technique (*12*), we found that Lucifer yellow migrated a longer distance in cells transfected with connexin 43 than with a vector alone, and the transfer of dye was completely blocked in cells carrying the dominate negative vector. Using these transfected cells, we irradiated 20% of the various cell populations with a single α particle to see if there were any changes in the bystander mutagenic effects. We found that AH1-9 cells containing the connexin 43 vector expressed a higher bystander mutagenic yield than that of the vector control. In contrast, there was no significant bystander mutagenic effect observed among AH1-9 cells carrying the dominant negative connexin 43 vector. These data clearly showed that gap junction intercellular communication was critical in mediating the bystander mutagenesis, although the nature of the signaling molecules involved in the communication between α-particle-traversed and nontraversed cells remains to be established.

Our studies provide clear evidence that a cell irradiated with a single α particle can induce a bystander mutagenic response in neighboring cells not directly traversed by an α particle, and they strongly suggest that gap junction intercellular communication plays a critical role in mediating the bystander mutagenesis. These results, if applicable *in vivo*, would have significant consequences in terms of extrapolation radiation risk to low doses, implying that the relevant target for radiation genotoxicity is larger than an individual cell. Thus a simple linear extrapolation of radiation risk from high doses to lower doses might not adequately reflect the risk of late radiation effects.

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Low-Dose Studies of Cell Survival with Microbeams: Bystander and Direct Cell Killing

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Understanding of low-dose responses has been advanced significantly by the availability of microbeam approaches. A major emphasis of studies in recent years has been the role of radiation-induced bystander responses. These are responses in which cells that are not directly traversed by radiation respond when their neighbors are irradiated. We have been determining the effectiveness of targeted particles and X rays at inducing bystander responses using two microirradiation facilities we have developed within our group. Our charged-particle microbeam (*1, 2*) allows us to collimate protons or helium ions with ± 2 μm resolution and deliver single particles with >99% efficiency. Our soft X-ray microprobe (*3*) focuses carbon characteristic soft X rays down to <250 nm. Studies in V79 cells have been comparing the effectiveness of high-energy protons (11 keV/μm) or focused carbon K-shell X rays (278 eV) in cell killing under conditions where every cell is targeted or where only a single cell has been selected. In this cell line, the phenomenon of low-dose hypersensitivity is observed at doses below 0.5 Gy, so we have also determined the interactions between the bystander response and low-dose hypersensitivity.

Cells are seeded 3 h prior to irradiation on specially constructed Mylar-based dishes. Cells are located automatically after staining with Hoechst 33258 using a computerized cell recognition and revisiting system. Typically around 100–150 single cells are present on each dish (5×5-mm area) at the time of irradiation. For measurements of direct cell killing, each of these cells is selected and the required number of particles or dose of soft X rays is delivered through the center of the nucleus. For bystander-induced cell killing, a single cell is selected at random near the center of the dish and irradiated. For control bystander experiments, the same number of particles or dose of soft X rays is delivered to a location in the center of the dish where no cells are present. For all dishes, incubation is continued for an additional 3 days. After this time cells are restained with Hoechst, and each of the original cell locations is revisited to determine whether colony formation has occurred. In some experiments, after a single cell was targeted, dishes were uniformly exposed to low doses of conventional X rays (240 kV).

For studies with protons, a significant bystander effect in the form of cell killing was detected. The degree of bystander-mediated killing relative to controls was around 5% when cells were targeted with either 20 or 50 protons, equivalent to a nuclear dose of 0.44 or 1.11 Gy, respectively. In experiments where only a single proton (0.02 Gy) was delivered to a single cell, no significant bystander response was observed. Further studies are confirming the dose–response relationship in this low-dose region. The history of each cell during these experiments is recorded so that the fate of the targeted cell relative to the bystander cells can be followed. After irradiation of a single cell, bystander-induced cell killing could be observed with equal probability anywhere on the dish ($\sim 5 \times 5$ mm). Given that these cells are exposed at very low density, this points to a cell medium-borne factor being involved.

Parallel experiments were also performed with focused soft X rays. Here a significant bystander response is also observed when a single cell is targeted. In contrast to the proton data, a larger bystander response was induced (10–12% cell killing) that also saturated with increasing dose to the cell (0.2–2 Gy).

In summary, for two different radiation types, we have direct evidence for a bystander effect measured as increased cell killing in populations in which only a single cell has been targeted. The response saturates with dose for both radiation types. This dose–response relationship appears to be a consistent finding with bystander effects, because it has been observed by many workers, including our own studies in different cell lines with different end points (*4*). Taken together, the data we have obtained here predict important radiation quality dependences for the degree of bystander effects in mammalian cells exposed at low doses.

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3D Targeting of a Porcine Ureter Tissue Fragments with a Charged-Particle Microbeam

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We have recently found evidence for a bystander effect after irradiation of porcine ureter tissue samples with a microbeam (*1, 2*). The main aim of the present work was to investigate the mechanisms involved in the bystander effect after low-dose microbeam irradiation in porcine ureter sections.

The ureter connects the kidney and bladder. It is covered inside with four layers of uroepithelial cells. Only the basal cells, located next to the *lamina propria*, proliferate. The next two cell layers are semi-differentiated, and the last layer, next to the lumen, consists of only fully differentiated cells. Ureter samples were trimmed from connective tissue, opened longitudinally, in buffered culture medium. All irradiations were performed with the Gray Cancer Research Trust charged-particle microbeam.

Tissue samples were explanted after microbeam irradiation and incubated for 7 days to form an outgrowth. The ureter primary explant technique (*4*) enables us to study bystander effects under *in vivo*-like conditions where stem and differentiated cells are present. The uroepithelial outgrowth reconstructs the *in vivo*-like microstructure with normal epithelial stratification. This approach is an intermediate stage between an *in vivo* system and cell culture. The total cell damage (fractions of micronucleated and apoptotic cells) was scored in the whole explant outgrowth (*5*). Apoptosis was measured according to morphological criteria. Another end point measured was differentiation, which was estimated with antibodies to Uroplakin III, a specific marker of terminal uroepithelial differentiation.

Results of pilot experiments demonstrated a similar magnitude of the bystander-induced cell damage and premature differentiation in explant outgrowth after microbeam preirradiation of ureter tissue samples within the basal, intermediate and superficial layers. It suggests that there is signaling of the bystander response from the damaged superficial layer, which consists of only terminally differentiated cells, to the dividing basal

level, which is responsible for formation of the explant outgrowth. This contrasts with our earlier experiments where we measured a bystander effect in the explant outgrowth after irradiation with 10 $^{3}He^{2+}$ particles of 10 cells on the periphery, where actively proliferating cells are located. Under these conditions no significant increase in cell damage was found after irradiation of 10 cells within the center of the explant outgrowth, where only terminally cells are present (*1, 2*). It indicates that the normal 3D ureter tissue microstructure is essential for manifestation of the bystander effect.

The discovery of a bystander effect is important for understanding the dose–response mechanisms relevant to low-dose irradiation *in vivo.* One crucial question is whether the bystander effect is a protective mechanism or whether, conversely, it amplifies the number of cells damaged by the isolated radiation tracks of low-dose exposures. One theory, supported by the experimental data obtained during this project, is that a main function of the bystander effect is to decrease the risk of transformation in a multicellular organism exposed to radiation. We speculate that individual cells within a tissue may not have the ability to detect irradiation such that an individual cell response is not expressed. An integrated multicellular system may be able to detect damage from irradiation and respond to it by removing a functional group of cells, which could be potentially damaged. This mechanism would work only for low doses of radiation, because only in this case is the damage localized within a small fraction of the cell population. In some systems, the most convenient way to remove potentially damaged cells is by cell death. In particular, apoptosis allows the removal of affected cells without a negative impact on other cells. Another way to isolate damage is to prompt potentially affected cells into irreversible differentiation. A normal 3D tissue microarchitecture is essential for manifestation of the bystander effect.

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Are Bystander Effects Important?

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At low doses of high-LET radiation and at very low doses of low-LET radiation, some cells are directly hit by the radiation, but a significant number of cells are not—at least in an appropriate time frame for development of oncogenic damage. This inhomogeneous energy deposition is of potential relevance to public health because there is convincing evidence, at least *in vitro*, that irradiated cells can send out signals that can lead to damage to nearby bystander cells. The evidence for bystander effects is particularly strong for high-LET radiation for a broad variety of *in vitro* end points.

Relevant low-dose high-LET exposure scenarios include home-dwellers exposed to domestic levels of radon, radiation workers or air flight personnel exposed to neutrons, and astronauts in deep space exposed to galactic cosmic rays; of these, the dominant public health concern is domestic exposure to radon. Direct epidemiological assessment of the risks from domestic radon exposure is difficult, resulting in risk estimates with wide confidence intervals. Consequently domestic radon risk estimates are currently based on extrapolation of data from the studies of miners, largely at considerably higher exposures (*1*). There are very different proportions of non-hit bystander cells in individuals exposed domestically to radon compared to miners exposed to higher levels of radon (*1*). Given that risk estimates from the miner data are used as the basis of a linear extrapolation of risk down to domestic radon exposure levels, it is important to understand whether the different proportions of bystander cells in these two situations might result in misleading conclusions from such a linear extrapolation.

The single-cell microbeam is a useful tool for studying bystander effects, allowing the experimenter to identify and irradiate some cells while other neighbor cells—also identifiable—are not irradiated. Specifically, microbeam irradiation facilities (*2*) have made it possible to define precisely what proportion of cell nuclei are traversed by an exactly defined number of particles (including exactly one), rather than relying on estimates of probabilities.

We have used the Columbia University single-particle/single-cell microbeam (*3*) in a series of studies (*4, 5*) to probe bystander effects induced by α particles. Broad conclusions (*6*) were that (1) an irradiated cell can indeed send out a signal which can lead to an oncogenic response in a ''bystander'' cell, i.e., a cell whose nucleus is not hit; (2) the cell population appears to contain a small subpopulation which is hypersensitive to transformation by the bystander signal; and (3) the response of bystander cells is likely to be a binary ''all or nothing'' effect; i.e., once a bystander cell has received sufficient signal, further signal does not further increase its probability of response.

While such results suggest that bystander effects for end points relevant to cancer can be important, it is also clear that there must also be a component of radiobiological damage that is ''direct'', in the sense that it involves damage in a cell by a radiation track that directly deposits energy in that cell nucleus. We have discussed (*6, 7*) possible high-LET radiation dose–risk relationships using an approach (denoted *BaD*) that incorporates radiobiological damage both from a bystander response to signals emitted by irradiated cells and also from direct traversal of high-LET radiations through cell nuclei. The approach produces predictions consistent with the series of studies (*4, 5*) of the bystander phenomenon using the Columbia University microbeam, with the end point of *in vitro* oncogenic transformation.

Within the *BaD* framework, the addition of a saturating bystander response to a linear direct response results in an overall risk varying nonlinearly with dose. Different assumptions about the prerequisites for emission of a bystander signal by hit cells, and about whether directly hit cells can also show a bystander response, can produce predictions consistent with *in vitro* data, but result in significantly different extrapolations of radon risks from high to low exposures (*7*).

Comparisons of such models with epidemiological data (miner data at high doses, domestic case–control studies at low doses) are, however, hindered by our limited knowledge of the appropriate cellular targets in the bronchial epithelium (e.g. basal cells or secretory cells, nucleus or whole cell), as well as uncertainties in the appropriate time scales in which to consider numbers of α particles traversing those targets. However, when the most likely scenarios are considered, the most likely outcomes appear to be either (a) the same (linear) dose–risk relationship

would apply both for domestic radon exposure and for high radon exposures in mines, implying the validity of a linear risk extrapolation from high to low exposures, or (b) linear extrapolation of radon risks to low doses based on high-exposure miner data could overestimate domestic radon risks by (misleadingly) including a bystander component which could be present at high but not low radon exposures (*7*).

Both these scenarios are consistent with current epidemiological data for radon. Further laboratory-based studies on the patterns of the bystander effect at low doses, and particularly about its temporal aspects, should yield more insights concerning the appropriate extrapolation of radiation risks from intermediate to low doses, both for high- and low-LET radiations.

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