

RARAF Staff Photo



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The Radiological Research Accelerator Facility

AN NIH-SUPPORTED RESOURCE CENTER – WWW.RARAF.ORG

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Research Using RARAF

Interest in the “bystander” effect, in which only some cells are irradiated and there is a response greater than would be expected for the fraction of cells irradiated, continued at a high level again this year. Several experiments with a variety of endpoints have been undertaken to determine the size of the effect and the mechanism by which it is transmitted. There is evidence for both direct cell-cell communication through cell membranes and indirect, longer-range communication through some release into the cell medium. In some experiments, the unirradiated cells can be identified due to a different staining and scored directly. Both the microbeam and the track segment facilities continue to be utilized in various investigations of this phenomenon. The single-particle microbeam facility provides precise control of the number and location of particles, but is somewhat limited in the number of cells that can be irradiated. The track segment facility provides broad beam irradiation that has a random pattern of charged particles but allows large numbers of cells to be irradiated.

The experiments performed at RARAF during the period May 1, 2000 through April 30, 2001 and the number of days each was run in this period are listed in Table I. Fourteen different experiments were run during this 12-month period, about the same as the average for 1995-2000. Ten experiments were undertaken by members of the CRR, supported by grants from the National Institutes of Health (NIH), the Department of Energy (DOE) and the Avon Products Foundation. Four experiments were performed by outside users, supported by grants and awards from the NIH and the Ministry of Education, Science, Sports and Culture of Japan. Brief descriptions of these experiments follow.

Investigations involving the on-

cogenic neoplastic transformation of mouse C3H 10T½ cells (Exp. 73) were continued by Satin Sawant of the CRR. Using the microbeam facility, a fraction of the cell nuclei was irradiated through the nucleus to observe the bystander effect. Irradiation of 10% of the cells with 4 or more alpha particles yielded less than 90% clonogenic survival and a transformation rate significantly higher than 10% of the rate when all the cells were irradiated. This indicates the presence of a bystander effect. Cells first given a low dose of 250 kVp X rays before microbeam irradiation showed higher clonogenic survival compared to the corresponding populations treated with same number of alpha particles through

Table I.

Experiments Run at RARAF May 1, 2000 - April 30, 2001

Exp. No.	Experimenter	Institution	Exp. Type	Title of Experiment	Days Run
73	S. Sawant E. Hall	CRR	Biology	Neoplastic transformation of C3H 10T½ cells by specific numbers of α particles	21.5
84	W. Morgan (C.R. Geard)	University of Maryland	Biology	Genomic instability using specific numbers of α particles	2.0
92	S. Amundson	NIH	Biology	Functional genomics of cellular response to high-LET radiation	1.0
94	B. Ponnaiya C.R. Geard	CRR	Biology	Single cell responses in hit and bystander cells: single-cell RT-PCR and protein immunofluorescence	14.5
100	T. Kumaravel (B. Ponnaiya)	NIH	Biology	Comet assay of normal and <i>Ataxia Telangiectasia</i> cells irradiated with specific numbers of alpha particles	4.0
101	K. Komatsu (H. Zhou)	Hiroshima Univ.	Biology	Bystander effect of <i>Ataxia Telangiectasia</i> cells	2.0
102	H. Zhou T.K. Hei	CRR	Biology	Mutagenesis of alpha particle traversal in normal human bronchial cells	7.5
103	G. Jenkins C.R. Geard	CRR	Biology	Damage induction and characterization in known hit versus non-hit human cells	14.5
104	A. Xu T.K. Hei	CRR	Biology	Role of reactive oxygen species in cytoplasmic irradiation by alpha particles using CM-H2DCFDA	15.0
106	B. Ponnaiya C.R. Geard	CRR	Biology	Track segment alpha particles, cell co-cultures and the bystander effect	3.5
107	H. Zhou T.K. Hei	CRR	Biology	Effects of irradiated medium with or without cells on bystander cell response	2.0
108	H. Zhou M. Suzuki T.K. Hei	CRR	Biology	Modulation of adaptive response in alpha-particle-induced bystander effects	7.0
109	A. Balajee C.R. Geard	CRR	Biology	DNA damage induction in microbeam-irradiated cells assessed by the comet assay	3.5
110	H. Zhou D. Roy T.K. Hei	CRR	Biology	Identification of molecular signals of alpha particle-induced bystander mutagenesis	6.0

Note: Names in parentheses are CRR members who collaborated with outside experimenters.

their nuclei.

William Morgan of the University of Maryland, in collaboration with Charles Geard of the CRR, continued use of the microbeam facility to investigate genomic instability (Exp. 84), this time using hamster cells containing human chromosome 4. A fraction of the cells, stained so they could be located by the microbeam imaging system, was irradiated in their nuclei with alpha particles and the neighboring unirradiated (unstained) cells cultured to observe genomic instability in the human chromosome.

An experiment employing cDNA microarray technology (Exp. 92) by Sally Amundson of the National Institutes of Health (NIH) was continued this year to study bystander effects at the level of gene expression. Aluminum half-discs placed under the Mylar-bottomed cell culture dishes for the track segment facility allowed irradiation of the "inducer" portion of the dish with alpha particles having an LET of 125 keV/um while shielding the bystander cells. RNA harvested from the separate halves of the dishes after allowing 4 or 24 hours incubation was used for cDNA microarray hybridization to monitor differences in gene expression. In this preliminary experiment, a pattern of changes in gene expression for unirradiated bystander cells was observed that was distinct from but overlapping with that induced in the directly irradiated cells. Comparison of the genes regulated by direct irradiation with those responding to bystander signaling should identify candidates for genes transmitting and regulating the bystander signal.

Two studies involving the bystander effect were continued by Brian Ponnaiya and Charles Geard of the CRR. A protocol has been developed in which a single cell can be observed for gene expression using reverse transcription polymerase chain reaction (RT-PCR) (Exp. 94). Copies of DNA segments are created by reverse transcription from RNA produced by the cell. The DNA is then amplified by PCR until enough material is available for gel electrophoresis. This method permits observation of individual responses to radiation instead of just the average response of a large number of cells. A fraction of the cells is stained with a nuclear dye that fluoresces blue, used for the microbeam facility to observe nuclei during irradiation. The others are stained with a cytoplasmic dye that fluoresces orange and are not irradiated because they are not visualized in the microbeam system. Irradiated cells and unirradiated (bystander) cells each can be identified after irradiation by using two excitation wavelengths and observing the different color stains. Individual cells are selected using a micromanipulator on the off-line microscope system of the microbeam facility. Immunofluorescence is used to quantify p21/WAF1 induction in irradiated and unirradiated cells as a function of the number of particle traversals through the cell nuclei and time after irradiation. The majority of bystander cells show a response, indicating that the signal from hit cells is widespread. These are the first studies wherein the bystander effect has been directly visualized rather than inferred. The other investigation (Exp. 106) involves use of the track segment facility for broad-beam charged particle irradiations of human fibroblasts and epithelial cells immortalized with telomerase. Stainless steel rings have Mylar epoxied to both

sides, cells are plated on both inner surfaces and the volume is filled with medium. Cells on one surface are irradiated with ^4He ions; cells on the opposite surface are unirradiated because the particle range is much too short. This eliminates all possibility of cell-cell contact. Some experiments involved no cells on the irradiated surface, looking for any effects of irradiating only the cell medium. Cells are observed in situ at 4 time periods up to 72 hours after irradiation with doses from 0.1 to 100 Gy of 125 keV/ μm ^4He ions. Plateau phase cells are scored for cell cycle delay and micronucleus production while log phase cells are scored for chromosomal aberrations. Yields of micronuclei in bystander cells ranged from 1.2- to 1.8-fold higher than controls, with no clear increase with alpha particle number.

A study of the effects of ^4He ions on normal and *Ataxia Telangiectasia* human fibroblasts using the comet assay (Exp. 100) by T. Kumaravel of the National Institutes of Health continued with the collaboration of Brian Ponnaiya of the CRR. This procedure, like the single-cell PCR assay (Exp. 94), is a way to observe effects in individual cells. Because the cells are irradiated using the microbeam facility, the number of ^4He ion traversals is known, so variability in response is solely due to individual variability in the cells and the stochastic nature of the radiation.

Kenshi Komatsu of Hiroshima University in Japan, in collaboration with H. Zhou of the CRR, has also studied the bystander effect on *Ataxia Telangiectasia* (AT) cells (Exp. 101) using the microbeam facility. Much evidence indicates that p53 may play a crucial role in the bystander effect. Atm is a kinase for the phosphorylation of several proteins including p53 and seems to be the sensor of DNA damage or center of signal transduction. AT cells lack Atm and therefore could provide useful information on the role of p53 in the bystander effect. In current experiments, cell survival was measured for radiation sensitive AT cells that received 5 alpha particles in each nucleus and normal Hx cells that received 10 alpha particles. In addition, the bystander effect for HPRT mutation in Hx cells was determined by irradiating 10% of the cell nuclei with 20 alpha particles. Future experiments will be performed to observe bystander effects in the AT cells.

Hongni Zhou and Tom Hei of the CRR are using the single-particle microbeam facility for several experiments, most investigating the bystander effect. One study involves the mutagenesis of normal human bronchial cells with alpha particles (Exp. 102). The cells have been irradiated with a single alpha particle in each nucleus using the microbeam facility and examined for HGRT mutations. Because yield is very low, not enough cells have been irradiated yet to form a determination of the effect. However, a mutation rate above background has been observed using the track segment facility, where orders of magnitude more cells can be irradiated. Doses of alpha particles were used that produced an average of 1-8 particle tracks per cell nucleus. A second study is examining adaptive response in bystander effects (Exp. 108) in human-hamster hybrid (A_1) cells. After low-dose X-ray irradiation, 10% of the cells are traversed by 1 or 20 alpha particles. There is a decrease in the bystander effect for mutation when neighbor cells are traversed by one parti-

cle and a somewhat smaller decrease for traversal by 20 particles. With Debasish Roy of the CRR, they are trying to identify the molecular signals of cell-cell communication in bystander mutagenesis (Exp. 110). AHI-9 cells (A_L cells plus a hygromycin resistance vector in the human chromosome 11) were transfected with either a dominant negative connexin 43 vector, which shut down gap junction communication, or with connexin 43-expressing vector. Using the microbeam facility, 20% of the cells were irradiated with a single alpha particle. The data indicate that A_L cells containing the connexin 43 vector expressed a higher bystander mutagenic yield than that of vector control. In contrast, there was no significant mutagenic effect observed among A_L cells containing the dominant negative connexin 43 vector. These studies provide clear evidence that irradiated cells with alpha particles can induce bystander mutagenic response in non-irradiated neighboring cells, and gap junction cell-cell communication plays a critical role in mediating such a bystander mutagenesis.

Damage induction in irradiated and bystander cells is being studied by Gloria Jenkins and Charles Geard of the CRR (Exp. 103). Using the microbeam facility, specific numbers of alpha particles are delivered to normal human fibroblasts. Bystander cells are stained as described above in Experiment 106 to distinguish them from those irradiated. *In situ* immunofluorescence is used to assay the levels of p53, p21, mdm2 and a number of other damage response proteins in both the irradiated and unirradiated cells. The total amount of protein can be determined as a function of the number of particles delivered to the irradiated cells and the fraction of cells irradiated.

An Xu and Tom Hei of the CRR have continued investigating the role of nitric oxide (NO), an important bioregulatory molecule, in mediating the mutagenicity of cytoplasmic irradiation (Exp. 104) using the microbeam facility. A_L cells were irradiated with $8\ ^4\text{He}$ ions through the cytoplasm in the presence or absence of L-NMMA, which has been shown to competitively inhibit nitric oxide synthases (NOS). Pretreatment with L-NMMA suppressed mutation induction by ~3-fold to near background level. In contrast, the treatment had no effect on the mutagenic yield in cells irradiated by 2 alpha particles through the nucleus.

Using the track segment facility, Hongning Zhou and Tom Hei of the CRR have investigated the effects on bystander cells that are not in contact with irradiated cells (Exp. 107). As in Experiment 106, cells were plated on opposite sides of a special dish with two Mylar surfaces. A_L cells were irradiated with doses from 0.1 to 100 Gy of ^4He particles with an LET of 125 keV/ μm . For some dishes, only the cell medium (no cells on irradiated side of dish) was irradiated. Bystander cells were examined 1 and 48 hours after irradiation for mutation at the S1 locus. Cell survival in the bystander cells after 48 hours was reduced by 20% for the highest dose to the irradiated cells but no statistically significant change in mutation was observed at any dose. Neither survival nor mutation was significantly different for 1 hour of co-culture after irradiation. This implies that cytotoxic factors were released into the cell medium but these had minimal effect on mutation in A_L cells.

Adayabalam Balajee and Charles Geard have begun investigations of damage induction in human fibroblasts and *Ataxia Telangiectasia* cells using the comet assay (Exp. 109). Cells irradiated through the nucleus using the microbeam facility show increasing initial damage with increasing numbers of particles. After 3 hours, much of the damage is repaired. Future experiments will focus on damage in bystander cells using some of the identification methods described above in Experiment 106.

Accelerator Utilization and Operation

Accelerator usage is summarized in Table II. Use of the accelerator for radiobiology and associated dosimetry increased by about 15% over last year and was ~40% higher than the average for 1995-2000. Over 90% of the accelerator use for all experiments was for microbeam irradiations. Because of the relatively low number of cells that can be irradiated in a day, these experiments often require considerable beam time to obtain sufficient biological material, especially for low probability events such as transformation and mutation. In addition, there continues to be interest in "bystander" experiments that produce low yields even for normally frequent responses.

No utilization of the accelerator by radiological physics and chemistry occurred this past year. Two ongoing projects, one for physics and the other for chemistry, are scheduled to run again in November of this year and there are at least two more physics experiments under discussion.

Use of the accelerator for online development doubled over last year and was 50% higher than 1998-99 due to the installation of the new 90° bending magnet and beam components and to increased testing and modification of the microbeam focusing system.

We have continued to minimize the time spent for inspections of the radiation safety system by not inspecting those systems and target stations that are rarely used. Of course, any facility is inspected before it is put back into use.

Accelerator reliability was somewhat better than normal this year. Maintenance and repair time was about 20% lower than last year and slightly less than our long-term average. No major repairs or modifications to the accelerator were performed. A vacuum leak in one of the sections of the acceleration tube is a problem that has troubled us for a couple of years and will require a permanent repair, possibly next year. Whenever we open the accelerator, we have to reseal a

Table II.

Accelerator Use, May 2000 - April 2001 Percent Usage of Available Days

Radiobiology and associated dosimetry	42%
Radiological physics and chemistry	0%
On-line facility development and testing	38%
Off-line facility development	3%
Safety system	2%
Accelerator-related repairs/maintenance	9%
Other repairs and maintenance	6%

portion of one of the 17 glue joints in one of the seven acceleration tube sections. Once sealed it poses no problem until the next opening, when the changes in tank pressure disturb the seal. Since there are no spares, we are dismantling damaged tube sections that were removed previously in order to recover the electrodes. After these electrodes have been reconditioned, new tube sections will be assembled using spare insulators. The assembly process requires heat and pressure to create a strong bond between the insulators to the electrodes using vinyl sealing material. The special oven at Brookhaven National Laboratory that was used for this process no longer exists. We will have to either build a new one and assemble the tube sections ourselves, or send the parts out for assembly to one of the few companies that build acceleration tubes.

Development of Facilities

The considerable development of the single particle microbeam facility is described here briefly:

- Testing of the single electrostatic quadrupole quadruplet continued, using the existing microbeam facility. The beam now has been reduced down to 11 μm diameter for an object aperture with a diameter of 40 μm or smaller and the position is quite steady. Further alignment, modifications and testing will be done to obtain the calculated demagnification factor of 4 so that an object aperture diameter of 8-10 μm will produce a final beam spot diameter of 2-2.5 μm .
- In order to focus the ions better, a "phase space sweeper" was constructed. The beam at the object aperture of the microbeam lens system has a correlation between angular divergence and position in the beam, which interfered with focusing. An electrostatic wobbler was made from sections of the ceramic rods from the first lens prototype and positioned below the object aperture. The beam spot, which is considerably larger than the object aperture, is moved around by voltages applied to pairs of electrodes that are at right angles to each other. The voltage on each pair of electrodes is varied with a different frequency, selected so that there is no repetitive pattern to the motion of the beam.
- A fixture was constructed to rotate the ceramic rods used for the quadrupole electrodes so that their surfaces could undergo ion implantation. Six ceramic rods were implanted with tantalum ions by Dr. Ian Brown of the Lawrence Berkeley National Laboratory. These rods were substituted for the ones in the lens that still were exhibiting some sparking at high voltage. This modification has essentially eliminated sparking and has allowed the voltage on the lens to be increased quickly to the working value of ~15 kV.
- The test laser system obtained from the University of Arkansas has been used successfully to extract ions produced by the laser pulses on aluminum, iron and lead targets. Tests using the analyzing and focusing systems provided information on the yields of different charge states as a function of emission angle, laser power density and target surface condition.
- The new 90° bending magnet was received in January of this year and positioned between the exit of the Van de Graaff and the switching magnet to direct the charged parti-

cle beam to the floor above (Fig. 1). It is capable of bending the heavy ion beams that will be eventually produced by the laser ion source under development. Because there is now less room in this region, several new, more compact beam line components were purchased and installed to replace existing ones. Whereas the older components used o-ring seals, the new components all use metal vacuum seals that have resulted in better vacuum. The magnet was aligned and its bending and focusing characteristics were checked. A cable tray - filled with cables for the magnets and radiation safety system - which was originally directly above the beamline had to be relocated, with the cables still in it. The entire operation lasted five weeks.

- Construction of a new microbeam laboratory on the floor over the exit of the Van de Graaff was completed in June of this year. The room was designed to be essentially the same as the existing microbeam lab and has already been equipped with a cell handling bench, an incubator and the table for the microbeam apparatus. This will house the next-generation microbeam facility with an ultimate beam diameter of <0.5 μm .

Microbeam Workshop

We organized a workshop "Probing Individual Cells: Applications to Signaling, Structure and Function" held in Bethesda, Maryland on March 12-14, 2001. This was motivated by the recent rapid development of new technologies to study biological responses in individual cells, as well as new technologies (in particular single-particle/single-cell microbeams) for inserting perturbations into individual cells or parts of cells.

The Workshop provided a forum to assess how such tools may enrich "conventional" structural biology studies of

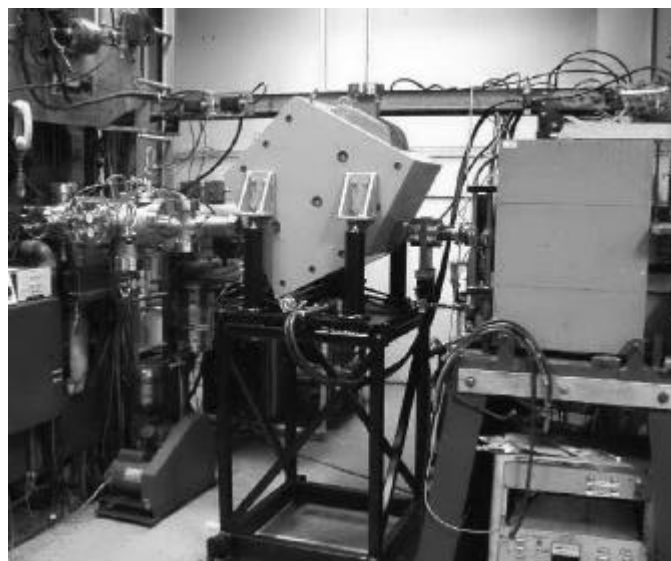


Fig. 1. The new 90° bending magnet on its stand is in the center of the picture. The switching magnet is on the right and the Van de Graaff on the left. The vertical beam-port flange extends just above the cable tray near the top of the picture. Almost all the beam line components between the Van de Graaff and the switching magnet were replaced.

multi-component signaling and DNA repair complexes or chromatin, and to discuss future directions, both technological and biological, in these fields. The meeting brought together individuals from a number of very different fields, and much cross-fertilization took place of ideas and technologies in this emerging area of basic cancer research.

Approximately 55 scientists attended the workshop. The meeting proceedings have been published in the journal *Radiation Research* (vol. **156**: 434-445, 2001).

Personnel

- The Director of RARAF is Dr. David Brenner. The Van de Graaff accelerator is operated by Mr. Stephen Marino and Dr. Gerhard Randers-Pehrson.
- Dr. Alan Bigelow, a postdoctoral fellow, is continuing the development of the laser ion source as part of his duties.
- Dr. Alexander Dymnikov, an expert on ion beam transport who left RARAF in April 2000, returned as a part-time Visiting Research Scientist in June of this year. He is assisting in the design of the electrostatic lenses for the microbeam facility.
- Mr. Mutian Zhang continues as a full-time accelerator technician.
- Dr. Charles Geard, the Associate Director of the CRR and biologist on the RARAF grant, spends much of each working day at RARAF. In addition to his own research, he is collaborating with several outside users on experiments using the single-particle microbeam facility.
- Dr. Satin Sawant, an Associate Research Scientist who spent all his time at RARAF primarily doing experiments utilizing the microbeam facility, left in March of this year.
- Dr. Brian Ponnaiya, formerly a postdoctoral fellow and now an Associate Research Scientist, works at RARAF full-time performing experiments using the track segment and microbeam irradiation facilities.
- There is one full-time biology technician, Ms. Gloria Jenkins.
- Two new postdoctoral fellows are expected to arrive in the year: Dr. Oleg Belyakov and Dr. Stephen Mitchell. They will both work full time at RARAF.
- Biologists from the Center for Radiological Research not supported by the RARAF grant spend various amounts of time at the facility in order to perform experiments:

Recent Publications of Work Performed at RARAF (2000-2001)

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3. Brenner DJ and Sachs RK, Are bystander effects relevant for domestic radon exposure risk estimation?, *Int. J. Radiat. Biol.* (accepted for publication 2001).
4. Geard CR, Jenkins-Baker G, Marino SA and Ponnaiya B, Novel approaches with track segment alpha particles and cell co-cultures in studies of bystander effects, 13th Symposium on Microdosimetry, Stresa, Italy, May 26-June 1, 2001, to be published in *Radiat. Prot. Dosim.*
5. Geard CR, Jenkins-Baker G, Ponnaiya B and Randers-Pehrson G, Microbeam irradiation of cytoplasmic regions of normal human fibroblasts, *Int. J. Radiat. Biol.* (accepted for publication following revision).
6. Geard CR, Jenkins-Baker G, Ponnaiya B and Randers-Pehrson G, Hit cell fraction and bystander cell responses in normal human fibroblasts, *Radiat. Res.* (in press).
7. Geard CR, Jenkins-Baker G, Ponnaiya B and Randers-Pehrson G, Irradiation of inter-cellular medium with microbeam directed alpha particles, *Radiat. Res.* (submitted).
8. Hong J, Craig WW and Hailey CJ, Laboratory tests on neutron shields for gamma-ray detectors in space, *Nucl. Inst. Meth. Phys. Res., Section A* **452**:192-204, 2000.
9. Marino SA and Johnson GW, A microdosimetry chamber for low-energy x-rays, Proceedings of the 13th Symposium on Microdosimetry, Stresa, Italy, May 26-June 1, 2001, to be published in *Radiat. Prot. Dosim.*
10. Milligan JR, Aguilera JA, Paglinawan RA, Ward JF and Limoli CL, DNA strand break yields after post-high LET irradiation incubation with endonuclease-III and evidence for hydroxyl radical clustering, *Int. J. Radiat. Biol.* **77**:155-164, 2001.
11. Ponnaiya B, Jenkins-Baker G, Brenner DJ, Hall EJ, Randers-Pehrson G and Geard CR, Biological responses in individual known microbeam irradiated and non-irradiated bystander cells, *Cancer Res.* (submitted 2001).
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13. Sawant SG, Randers-Pehrson G, Geard CR, Brenner DJ and Hall EJ, The bystander effect in radiation oncogenesis, *Mutation Res.* (submitted, 2001).
14. Sawant SG, Randers-Pehrson G, Metting NF and Hall EJ, Adaptive response and the bystander effect induced by radiation in C3H 10T1/2 cells in culture, *Radiat. Res.* **156**:177-180, 2001.
15. Sawant SG, Randers-Pehrson G, Metting N and Hall EJ, Can adaptive response alter the bystander effect in C3H 10T1/2 cells?, 13th Microdosimetry Symposium, Stresa, Italy, May 26-June 1, 2001, to be published in *Radiat. Prot. Dosim.*
16. Zhou H, Randers-Pehrson G and Hei TK, Studies of bystander mutagenic response using charged particle microbeam, *Radiation Research* **153**:236-237, 2000.
17. Zhou HN, Suzuki M, Geard CR and Hei TK, Effects of irradiated medium with or without cells on bystander cell responses, *Mutat. Res.* (accepted for publication). ■