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Cytogenetic dose–response and adaptive response in cells of ungulate species exposed to ionizing radiation

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Abstract

In the studies reported here, the micronucleus assay, a common cytogenetic technique, was used to examine the dose–responses in fibroblasts from three ungulate species (white-tailed deer, woodland caribou, and Indian muntjac) exposed to high doses of ionizing radiation (1–4 Gy of ^{60}Co gamma radiation). This assay was also used to examine the effects of exposure to low doses (1–100 mGy) typical of what these species experience in a year from natural and anthropogenic environmental sources. An adaptive response, defined as the induction of resistance to a stressor by a prior exposure to a small ''adapting'' stress, was observed after exposure to low doses. This work indicates that very small doses are protective for the endpoint examined. The same level of protection was seen at all adapting doses, including 1 radiation track per cell, the lowest possible cellular dose. These results are consistent with other studies in a wide variety of organisms that demonstrate a protective effect of low doses at both cellular and whole-organism levels. This implies that environmental regulations predicated on the idea that even the smallest dose of radiation carries a quantifiable risk of direct adverse consequences to the exposed organism require further examination. Cytogenetic assays provide affordable and feasible biological effects-based alternatives that are more biologically relevant than traditional contaminant concentration-based radioecological risk assessment.

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1. Introduction

Environmental releases of low levels of radionuclides associated with general mining, nuclear power and weapons production, and the medical use of radionuclides is a topic of continuing public concern. Human exposures from environmental radionuclide releases can be minimized by limiting access to contaminated areas; however, this is not feasible for non-human organisms, and resulting exposures can be significantly higher than those from natural background sources. Nevertheless, non-human exposures are typically characterized by doses and dose-rates far below those expected to produce significant mortality.

It is well known from numerous studies using diverse biological endpoints in a variety of organisms that for sparsely ionizing radiation, there is a reduction in the deleterious effect of a given dose when the dose-rate is lowered or the dose is fractionated (National Council on Radiation Protection and Measurements, 1980). This reduction in harmful effects per unit dose generally applies over a range of dose-rates between a few Gy h^{-1} and a few mGy h^{-1}. There often appears to be a minimum dose-rate, below which further reductions in dose-rate result in no further diminution of response per unit dose, i.e. a plateau is reached (Ulsh et al., 2001). Yet much of the data on the effects of radiation on non-human organisms are based on studies employing doses and dose-rates far higher than are typical for environmental exposures, and use acute (within 30 or 60 days) individual mortality as an endpoint.

Evidence of a radioadaptive response in a wide assortment of organisms suggests that biological responses and environmental risks from very low dose and dose-rate exposures ($<$100 mGy h^{-1}) are more complex than is commonly assumed. An adaptive response is generally defined as the induction of resistance to a stressor by a prior exposure to a relatively small "adapting" stress. Radioadaptive responses have been observed in organisms ranging from yeast and other single-celled organisms (Boreham and Mitchel, 1993), insects (Schappi-Buchi, 1994), and fish (Kurihara et al., 1992), to humans (Broome et al., 2002), rabbits (Flores et al., 1996), cows (Flores et al., 1996), and laboratory rodents (Azzam et al., 1994; Wolff, 1996) at the chromosomal and cellular levels. Early research with a variety of organisms (Luckey, 1980), and more recent studies with mice (Mitchel et al., 1999) demonstrate that this is also true at the organismal level, though how this might translate to the population, community, or higher levels of biological organization is less clear. It is also clear that this adaption phenomenon is not radiation-specific, but rather is a specific example of a more general stress response (Boreham et al., 1991). Cross-adaption has been observed between radiation and metals (Cai and Cherian, 1996), chemicals (Fiores et al., 1996; Mitchel et al., 1990), and hyperthermia (Boreham et al., 1997).

In the studies reported here, a cytogenetic biomarker of radiation exposure was used to examine chromosomal effects in three ungulate species, white-tailed deer (*Odocoileus virginianus*), Indian muntjac (*Muntiacus muntjac*) and woodland caribou (*Rangifer tarandus caribou*). Previous studies in caribou (Roed et al., 1991) and plants (Grinikh and Shevchenko, 1992) exposed to Chernobyl fallout and in turtles (Ulsh et al., 2000, 2001) have demonstrated the utility of cytogenetic techniques for environmental biodosimetry. The biomarker employed in this study, the frequency of micronuclei in exposed fibroblasts, is widely used in cells from humans and laboratory rodents, and this assay has the advantages of sensitivity and modest cost compared to whole-organism studies. Furthermore, this endpoint is more sensitive than acute mortality, allowing an investigation of effects at environmentally relevant doses and dose-rates. The frequency of micronuclei is used as a biomarker of residual radiation damage and reflects the capacity of the cells to repair DNA double strand breaks.

The goals of the experiments reported here were: (1) to investigate the utility of the micronucleus assay for the detection of the effects of radiation exposure in non-human species; (2) to determine the radiosensitivity of these species relative to that of humans, and (3) to test whether an adaptive response was evident at environmentally relevant doses and dose-rates.

2. Materials and Methods

2.1. High dose–response experiments

White-tailed deer skin biopsies were obtained from deer supplied by local hunters in the Chalk River, Ontario, Canada, area. Woodland caribou skin biopsies were obtained from caribou near Detour Lake, north of Cochrane, Ontario. Samples were transported to Chalk River Laboratories, where cell cultures were established (Miller et al., 1999; Miller, 2002). Indian muntjac fibroblasts were purchased from the American Type Culture Collection (CCL 157, passage number unknown). Fibroblasts were grown to confluence in T25 tissue culture flasks, then subcultured into 10 cm^2 chambered slides at non-confluent cell density. Skin fibroblasts were exposed to 0, 1, 2, or 4 Gy of ^{60}Co gamma radiation (0.278 Gy s^{-1}, GammaCell 220, Atomic Energy of Canada Ltd) at 0 °C in phosphate buffered saline solution (PBS). Following irradiation, the PBS was replaced with 37 °C growth medium and cells were incubated for 48 h in the presence of 2 μg ml^{-1} cytochalasin B. Cells were allowed approximately 48 h to attach prior to irradiation treatments. Immediately before irradiation, the chambered slides were completely filled with ice-cold PBS. Skin fibroblasts were exposed to 0, 1, 2, or 4 Gy of ^{60}Co gamma radiation (0.278 Gy s^{-1})(GammaCell 220, Atomic Energy of Canada, Ltd). Following the irradiation, the PBS was replaced with 3 ml 37 °C growth medium and cells were incubated for approximately 48 hours in the presence of cytochalasin B (2 μg ml^{-1}), then fixed and stained using standard methods. Micronuclei were scored using criteria modified from Fenech (1993). Each experiment had three replicate slides per treatment and 500–1000 binucleate cells were scored per slide. The caribou

experiment was repeated twice, the Indian muntjac experiment three times and the white-tailed deer experiment once.

2.2. Low dose experiment

White-tailed deer skin fibroblasts were cultured in chambered slides, as described previously (Miller et al., 1999; Miller, 2002). Prior to adapting irradiation, the chambered slides were completely filled with 37 °C medium. Some samples received 0, 1, 10, or 100 mGy adapting doses delivered at low dose rate (5 mGy min^{-1}, GammaBeam 150 ^{60}Co irradiator, Atomic Energy of Canada, Ltd) at 37 °C. Cultures were then incubated for 3 or 6 hours at 37 °C. At the end of this incubation, 4 Gy challenge doses at high dose rate (0.278 Gy s^{-1}) were delivered as above and the cultures were incubated and fixed also as described above. Some samples received only the 4 Gy challenge dose. Three replicate cultures were prepared for each treatment.

While the same protocols were used for the two experiments (with the exception of adapting doses in some treatments), it should be noted that different cell lines were used, and different cytochalasin B lots and concentrations were used between the experiments. It is our experience that using different lots and/or concentrations of cytochalasin B can affect the micronucleus frequency. The optimal concentration of cytochalasin B was found to be 5 μg ml^{-1} for the low dose experiment.

2.3. Statistics

Data was tested for significant differences between treatments with and without adapting doses using a single-factor analysis of variance (ANOVA). The two 100 mGy adapted treatments (3- 6-h incubations before challenge) were compared to each other and to the unadapted treatment using one-tailed T-tests. Both the ANOVAs and t-tests were performed in Microsoft Excel 2000.

3. Results and Conclusions

3.1. High dose–response experiments

The micronucleus assay proved to be a sensitive and useful indicator of radiation damage in the three species investigated. Indian muntjac cells had fewer binucleate cells (BNCs) with micronuclei than woodland caribou cells and white-tailed deer cells ($p < 0.02$) at all doses ≥ 1 Gy (Fig. 1). Woodland caribou and white-tailed deer cells showed no significant differences in their dose responses for micronucleus formation induced by high radiation doses ($p > 0.05$) (Fig. 1), indicating that caribou and white-tailed deer cells had similar resistance to gamma-radiation as measured by this assay.

Woodland caribou are exposed to high, whole body doses of radiation because of a bioaccumulation of natural radionuclides in their diet. Lichens (*Cladonia* spp.) are the main source of food for caribou during winter and, as lichens have no roots, they absorb radionuclides from the atmosphere along with other nutrients

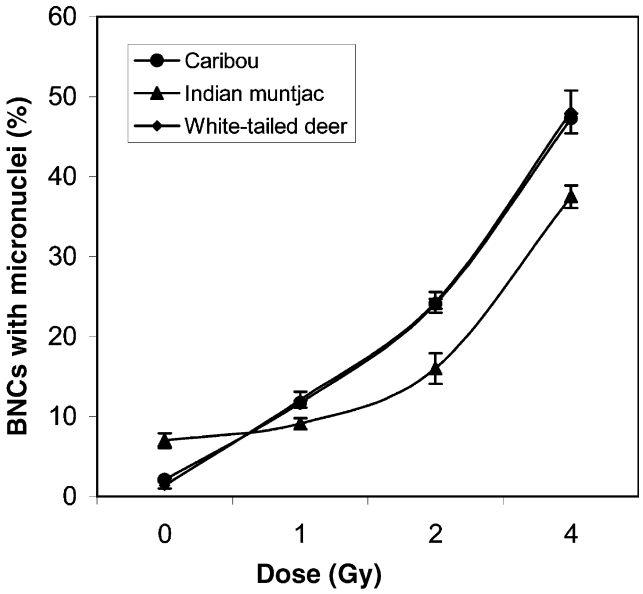

Fig. 1. Effect of gamma radiation on the frequency of micronucleus formation in woodland caribou, Indian muntjac, and white-tailed deer skin fibroblasts. Data points indicate the frequency of binucleate cells (BNCs) with micronuclei ± standard error. There was no significant difference in the data for woodland caribou and white-tailed deer cells ($p > 0.05$) but muntjac cells were significantly different ($p < 0.02$).

(Holleman et al., 1979; Holm and Persson, 1979; Macdonald et al., 1996; Thomas, 1994). The radionuclides with the greatest impact on radiation doses are ^{210}Pb ($t_{1/2} = 22.6$ y) and ^{210}Po ($t_{1/2} = 138.8$ d). ^{210}Po accumulates in the liver and kidney of caribou and is also found in bone as a decay product of ^{210}Pb absorbed by bone (Holm and Persson, 1979; Macdonald et al., 1996; Thomas, 1994; Thomas et al., 1994). When ^{210}Po decays to ^{206}Po, it emits high energy (5.4 MeV) alpha particles, which can result in doses of radiation exceeding 500 mGy per year (weighting factor = 10 for alpha radiation)(Macdonald et al., 1996; Thomas, 1994). Lead and polonium do not accumulate evenly throughout the body, thus exposure to specific tissues within target organs (e.g. liver, bone and kidney) may exceed 500 mGy y^{-1} (Macdonald et al., 1996). Since the level of radiation resistance seen in white-tailed deer is not different from caribou, the results suggest that the high background experienced by caribou has not led to elevated resistance to acute radiation damage.

These results were compared to published human fibroblast data (Dolling et al., 1998). The human cell response to high doses was similar to that of both caribou and white-tailed deer, while Indian muntjac had lower induced micronucleus frequencies. Earlier work with plants (Sparrow, 1964) had suggested that for cells with similar total DNA contents, radiosensitivity increased with decreasing chromosome number. All three ungulate species have total cellular DNA contents similar to that of humans, but have very different chromosome numbers; Indian

muntjac cells have only a few large chromosomes ($2N = 7$), while caribou and deer cells have many smaller chromosomes ($2N = 70$). However, our work showed that muntjac cells were more resistant, which does not support the postulated inverse relationship between chromosome number and radiosensitivity. Our results indicate that DNA organization in muntjac cells may be contributing to more proficient DNA repair.

3.2. Low dose experiments

The results of the low dose experiments demonstrated that doses as low as 1 mGy induce radioresistance in the white-tailed deer fibroblast cell line studied. The cells that received adapting doses (1, 10, or 100 mGy at a low dose rate) 3 or 6 h prior to a challenge dose of 4 Gy had significantly lower frequencies of cells containing micronuclei than similarly treated unadapted cells (single factor ANOVA, $p = 2.09 \times 10^{-5}$)(Fig. 2). There were no significant differences among any of the adapted or unadapted controls (cells that received no challenge dose) (ANOVA, $p > 0.05$). The pattern observed in Fig. 2 is remarkably similar to that observed

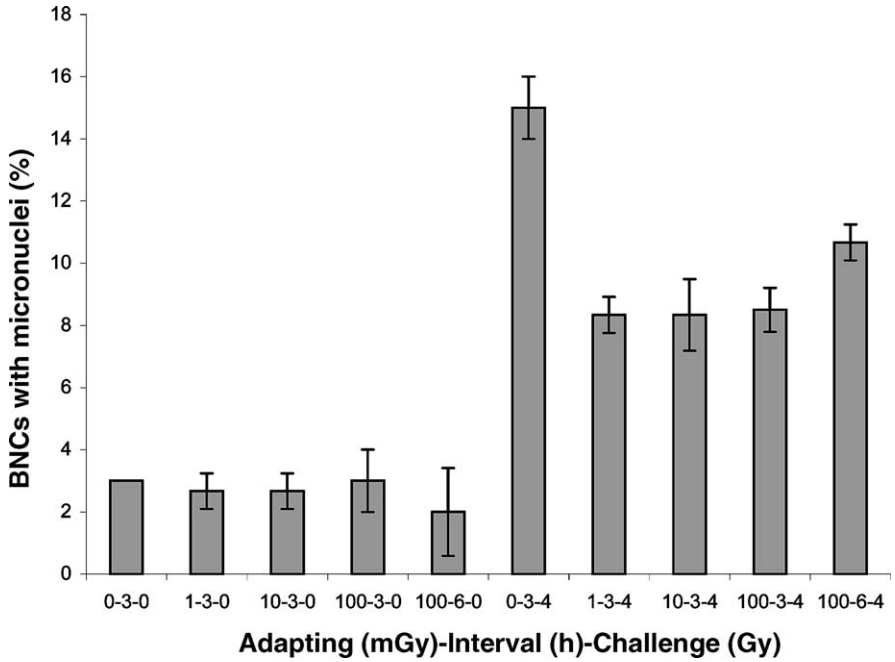

Fig. 2. Frequency of binucleate cells (BNCs) containing micronuclei in white-tailed deer skin fibroblasts exposed to low, adapting doses of gamma radiation. On the *X*-axis, the adapting dose (mGy) is listed first, followed by the interval between adapting and challenge doses (h), followed by the challenge dose (Gy). Bars are mean frequencies of BNCs containing micronuclei (500 BNCs per replicate, three replicates per treatment except for the 100-6-0 and 100-3-4 treatments, which had two replicates each), and error bars show 1 standard deviation.

in human fibroblasts subjected to the same experimental protocol (Broome et al., 2002).

The frequency of white-tailed deer BNCs with micronuclei in the unadapted cells exposed to the 4 Gy challenge dose in the low dose experiment was approximately one-third that observed in the white-tailed deer cells exposed to 4 Gy in the high dose-response experiment.

The frequency of BNCs containing micronuclei 6 h after a 100 mGy adapting dose was significantly higher than the frequency 3 h after the same adapting dose (one tailed t-test, $p = 0.04$)(Fig. 2), though both were significantly lower than the unadapted treatments (one tailed t-test, $p = 1.89 \times 10^{-3}$ for 3 h vs. unadapted, $p = 7.29 \times 10^{-3}$ for 6 h vs. unadapted). This suggests that the effect of the adapting dose may decrease with time in cells held in culture. The duration of protective effects of radioadaption in ungulates *in vivo* is unknown, but the effects in mice *in vivo* have been shown to last for months (Mitchel et al., 1999; Mitchel et al., 2003).

When the impact of low doses on the environment is considered, it is important to consider the influence of physics on the lowest dose that is possible in cells. Ionizing radiations such as X- or gamma-radiation deposit energy in tracks (via Compton scattering or photoelectrons), as the photons pass through a cell. The lowest possible dose to a cell is therefore the dose deposited by a single photon. For ^{60}Co-gamma radiation, 1 mGy corresponds to an average of one track per cell, and is therefore the lowest possible dose to a cell (Bond et al., 1988). At doses less than 1 mGy, not all cells receive a track of damage, but those that do still receive 1 mGy. The radioadaptive results presented here for deer cells include data at 1 mGy and therefore represent the response of these cells to the lowest dose physically possible. However, because of the random nature of radiation, exposures at low doses are not uniform and at 1 mGy not all cells receive exactly one track of radiation. Some cells will receive two tracks but about half of the cells will receive no track, i.e. no dose. The data shows, however, that at 1 mGy all cells respond in the same way as they do when all cells certainly receive multiple tracks of damage at 10 or 100 mGy. This result indicates that cells that actually receive a track (dose) of radiation, communicate that fact to other cells that do not receive any dose. This phenomenon is termed the "bystander effect" and has been previously observed in human fibroblasts at these doses (Broome et al., 2002; Mothersill and Seymour, 2002). The existence of bystander effects at low doses is particularly significant for environmentally relevant exposure levels. The presence of this response indicates that at very low doses where not all cells in a multicellular organism actually receive a track of radiation (dose), the protective effect generated in the cells actually hit by the radiation will be amplified by transmission to the surrounding cells.

When the radioadaptive response to low doses in the deer cells (Fig. 2) was compared to the radioadaptive response in normal human fibroblasts (Broome et al., 2002) the response pattern was indistinguishable. That result suggests that mammals as diverse as humans and ungulates respond in the same adaptive way to the lowest possible doses of ionizing radiation.

The results of these studies demonstrate that using cytogenetic biomarkers of radiation exposure for environmental biodosimetry represents a useful methodology for conducting exposure assessments for organisms inhabiting radionuclide-contaminated environments, and would provide genetically relevant measurement endpoints for ecological risk assessments. These results also suggest that consequences on organismal health from very low doses of radiation may not be negative, since exposures of this type seem to induce adaptive responses in exposed organisms. While the adaptive response results presented here are from one experiment in one species, they essentially replicate the observations reported for the same experiment in human fibroblasts (Broome et al., 2002). Further research might focus on differential adaptability of different populations or species of organisms. It also seems clear that the assumption that any dose, even one radiation track per cell, presenting ecological risks is not supported by this cellular evidence and needs to receive further examination.

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References

Azzam, E.I., Raaphorst, G.P., Mitchel, R.E., 1994. Radiation-induced adaptive response for protection against micronucleus formation and neoplastic transformation in C3H 10T1/2 mouse embryo cells. Radiat. Res. 138, S28.

Bond, V.P., Feinendegen, L.E., Booz, J., 1988. What is a 'low dose' of radiation? Int. J. Radiat. Biol. 53, 1.

Boreham, D.R., Mitchel, R.E., 1993. DNA repair in Chlamydomonas reinhardtii induced by heat shock and gamma radiation. Radiat. Res. 135, 365.

Boreham, D.R., Trivedi, A., Mitchel, R.E.J., 1991. Radiation and stress response in *Saccharomyces cerevisiae*. In: Prasad, R. (Ed.), Yeast Molecular Biology and Biotechnology. Omega Scientific Publishers, New Delhi, India, pp. 294.

Boreham, D.R., Dolling, J.A., Maves, S.R., Miller, S., Morrison, D.P., Mitchel, R.E., 1997. Heat-induced thermal tolerance and radiation resistance to apoptosis in human lymphocytes. Biochem. Cell Biol. 75, 393.

Broome, E.J., Brown, D.L., Mitchel, R.E., 2002. Dose responses for adaption to low doses of (60)Co gamma rays and (3)H beta particles in normal human fibroblasts. Radiat. Res. 158, 181.

Cai, L., Cherian, M.G., 1996. Adaptive response to ionizing radiation-induced chromosome aberrations in rabbit lymphocytes: effect of pre-exposure to zinc, and copper salts. Mutat. Res. 369, 233.

Dolling, J.A., Boreham, D.R., Brown, D.L., Mitchel, R.E., Raaphorst, G.P., 1998. Modulation of radiation-induced strand break repair by cisplatin in mammalian cells. Int. J. Radiat. Biol. 74, 61.

Fenech, M., 1993. The cytokinesis-block micronucleus technique and its application to genotoxicity studies in human populations. Environ. Health Perspect 101 (Suppl. 3), 101.

Flores, M.J., Pinero, J., Ortiz, T., Pastor, N., Mateos, J.C., Cortes, F., 1996. Both bovine and rabbit lymphocytes conditioned with hydrogen peroxide show an adaptive response to radiation damage. Mutat. Res. 372, 9.

Grinikh, L.I., Shevchenko, V.V., 1992. Cytogenetic effects of ionizing radiation in *Crepis tectorum* growing within 30 km of the Chernobyl atomic power station. Sci. Total Environ. 112, 9.

Holleman, D.F., Luick, J.R., White, R.G., 1979. Lichen intake estimates for reindeer and caribou during winter. J. Wildl. Manage 43, 192.

Holm, E., Persson, B.R.R., 1979. Comparative studies of natural and artificial alpha-emitters (actinides) in the lichen-reindeer-man food chain. In: Wrenn, M.E. (Ed.), Actinides in Man and Animals. RD Press, Salt Lake City, UT, pp. 525.

Kurihara, Y., Rienkjkarn, M., Etoh, H., 1992. Cytogenetic adaptive response of cultured fish cells to low doses of X- rays. J .Radiat. Res.(Tokyo) 33, 267.

Luckey, T.D., 1980. Hormesis with Ionizing Radiation. CRC Press, Boca Raton, FL.

Macdonald, C.R., Ewing, L.L., Elkin, B.T., Wiewel, A.M., 1996. Regional variation in radionuclide concentrations and radiation dose in caribou (*Rangifer tarandus*) in the Canadian Arctic. Sci. Total Environ. 182, 53.

Miller, S. M., 2002. Characterization of the radiation response of woodland caribou (*Rangifer tarandus caribou*), Indian muntjac (*Muntiacus muntjac*) and white-tailed deer (*Odocoileus virginianus*) fibroblast cells; and preliminary development of fluorescence *in situ* hybridization probes for ecobiodosimetry. MSc Thesis, Department of Biology, School of Graduate Studies and Research Laurentian University, Sudbury, Ontario, Canada.

Miller, S.M., Mallory, F.F., Mitchel, R.E.J., Morrison, D.P., Boreham, D.R., 1999. Characterization of the radiation response of caribou cells. Oral and poster presentations at the Eleventh International Congress of Radiation Research, Dublin, Ireland.

Mitchel, R.E., Gragtmans, N.J., Morrison, D.P., 1990. Beta-radiation-induced resistance to MNNG initiation of papilloma but not carcinoma formation in mouse skin. Radiat. Res. 121, 180.

Mitchel, R.E., Jackson, J.S., McCann, R.A., Boreham, D.R., 1999. The adaptive response modifies latency for radiation-induced myeloid leukemia in CBA/H mice. Radiat. Res. 152, 273.

Mitchel, R. E. J., Jackson, J. S., Morrison, D. P., Carlisle, S. M., 2003. Low doses of radiation increase the latency of spontaneous lymphomas and spinal osteosarcomas in cancer prone, radiation sensitive *Trp53* heterozygous mice. Radiat. Res. 159, 320.

Mothersill, C., Seymour, C., 2002. Relevance of radiation-induced bystander effects for environmental risk assessment. Radiats. Biol. Radioecol. 42, 585.

National Council on Radiation Protection and Measurements, 1980. Influence of dose and its distribution in time on dose-response relationships for low-LET radiations. Publication 64, National Council on Radiation Protection and Measurements, New York, NY.

Roed, K.H., Eikelmann, I.M., Jacobsen, M., Pedersen, O., 1991. Chromosome aberrations in Norwegian reindeer calves exposed to fallout from the Chernobyl accident. Hereditas 115, 201.

Schappi-Buchi, C., 1994. On the genetic background of the adaptive response to X-rays in *Drosophila melanogaster*. Int. J. Radiat. Biol. 65, 427.

Sparrow, A.H., 1964. Comparisons of the tolerances of higher plant species to acute and chronic exposures of ionizing radiation. J. Genet. 40, 12.

Thomas, P.A., 1994. Dosimetry of ^{210}Po in humans, caribou, and wolves in northern Canada. Health Phys. 66, 678.

Thomas, P.A., Sheard, J.W., Swanson, S., 1994. Transfer of ^{210}Po and ^{210}Pb through the lichen-caribou-wolf food chain of northern Canada. Health Phys. 66, 666.

Ulsh, B.A., Muhlman-Diaz, M., Whicker, F.W., Hinton, T.G., Congdon, J.D., Bedford, J.S., 2000. Chromosome translocations in turtles: a biomarker in a sentinel animal for ecological dosimetry. Radiat. Res. 153, 752.

Ulsh, B.A., Whicker, F.W., Congdon, J.D., Bedford, J.S., Hinton, T.G., 2001. Chromosome translocations in *T. scripta*: the dose-rate effect and in vivo lymphocyte radiation response. Radiat. Res. 155, 63.

Wolff, S., 1996. Aspects of the adaptive response to very low doses of radiation and other agents. Mutat. Res. 358, 135.