Scanning Microscopy

Volume 4 | Number 3 Article 13

7-2-1990

Radiation Cataractogenesis Induced by Neutron or Gamma Irradiation in the Rat Lens is Reduced by Vitamin E

W. M. Ross
Defence Research Establishment Ottawa

M. O. Creighton
University of Western Ontario

J. R. Trevithick
University of Western Ontario

Scanning Microscopy, Vol. 4, No. 3, 1990 (Pages 641-650) 0891-7035/90$3.00+.00
Scanning Microscopy International, Chicago (AMF O'Hare), IL 60666 USA

RADIATION CATARACTOGENESIS INDUCED BY NEUTRON OR GAMMA IRRADIATION IN THE RAT LENS IS REDUCED BY VITAMIN E

W.M. Ross[¶], M.O. Creighton[+], and J.R. Trevithick[++,*]

[¶]Nuclear Effects (Radiobiology), Defence Research Establishment Ottawa, Ottawa, Ontario, Canada, K1A 0Z4

[+]Department of Ophthalmology, and [++]Department of Biochemistry, University of Western Ontario, London, Ontario, Canada, N6A 5C1

(Received for publication January 15, 1990, and in revised form July 2, 1990)

Abstract

Although cataract of the eye lens is a known late effect of ionizing radiation exposure, most of the experimental work to date has concentrated on single, acute high doses or multiple, fractionated, chronic exposures. Many papers have dealt with biochemical alterations in metabolism and cellular components, with microscopic and electron microscopic lesions to the epithelial and cortical layers, and with clinical cataract formation. However, the minimum cataractogenic dose for rats has for many years been considered to be about 2 Gy for a single, acute dose of low LET radiation.

Our purpose in designing this pilot study was three fold: firstly, to determine whether any physical damage could be detected after low, acute exposure to neutron radiation (10 and 100 cGy); secondly, to compare the relative effectiveness of fast (14 MeV) neutrons with gamma-rays; and thirdly, to investigate the possibility that vitamin E could protect the lenses from radiation damage.

The results revealed that morphological damage was already discernible within minutes after exposure to neutrons or gamma-rays, that it became greater after 24 hours, that neutrons were more damaging than gamma-rays, and that vitamin E could effectively reduce the cataractogenic damage induced by ionizing radiation. Control, non-irradiated lenses with or without vitamin E, either in vivo or in vitro, showed no damage. Also, it appeared that in vitro irradiation was more damaging to lenses than in vivo irradiation, so this culture technique may prove to be a sensitive tool for assessing early damage caused by ionizing radiation. However it must be noted that at this level of radiation exposure (10-100 cGy), the early damage we have described will probably be repaired so no clinical cataracts will develop, unless other factors contribute to their development.

Key Words: Cataract, vitamin E, radiation, scanning electron microscopy, relative biological effectiveness (RBE), neutrons, in vitro model, in vivo model, rat, lens.

*Address for correspondence:
John R. Trevithick,
Department of Biochemistry, Faculty of Medicine,
University of Western Ontario,
London, Ontario, N6A 5C1,
Canada

Telephone Number: 519 661 3063

Introduction

The main purpose of this pilot study was to determine whether low doses of radiation could induce damage to the ocular lens and whether such damage could be detected very soon after irradiation. Eventually, this damage would be repaired in most cases, rarely leading to cataract formation and then only after several months (rat) or years (human). Alternatively, if not repaired, it might lead to the early development of cataractous damage. The in vitro assay described by Ross et al. (1983a) for detecting early radiation damage to the lens, particularly to the fibre cells, is a sensitive, reproducible technique that causes no detectable damage to non-irradiated control lenses; it has also been used to compare the effect of excess glucose and other sugars on cataractogenesis (diabetic cataract) in isolated rat lenses (Creighton et al., 1980). This in vitro system may serve well in future studies of cataractous and precataractous changes in the lens, not only in terms of morphological alterations, but also for molecular, enzymatic and membrane variations that may precede opacity. Such studies would usually be devised to test for occupational hazard or radiotherapeutic exposure, such as total-body irradiation for bone marrow transplantation (Deeg et al., 1984).

Radiation damage to mammalian cells is primarily caused in the first instance by free radicals formed as the radiation passes through the cell, ionizing water molecules to form hydrogen peroxide, hydroxyl and superoxide radicals (Prasad, 1984; Giblin et al., 1979; Petkau, 1980). The damage by these radicals seems to involve the cell membrane, but may also affect DNA and other critical macromolecules. Consistent with this, damage can be reduced by increasing the concentration of antioxidants and free-radical scavengers, such as glutathione (Ross et al., 1983b), vitamin C (Kuck, 1970; Varma et al., 1982) and vitamin E (Creighton et al., 1980; McKay and King, 1980, Ross et al., 1982).

Cataracts are generally regarded as a late effect of irradiation (Von Sallmann, 1951; Miller et al., 1967; Barron et al., 1970), usually developing as a posterior subcapsular cataract; therefore, several studies have dealt with the long-term development of clinically-detectable cataracts (Cogan et al., 1952; Merriam and Focht, 1957; Di Paola et al., 1978; Deeg et al., 1984). Many studies have been concerned with damage expressed in animal models several weeks to months after irradiation (Lipman et al., 1988); the damage parameters included ultrastructural

changes (Palva and Palkama, 1977), alterations in metabolism (Lambert and Kinoshita, 1967), Na-K ATPase activity (Palva, 1978), cation transport across membranes (Hockwin, 1962), and effects on the epithelial cells (Hanna and O'Brien, 1963; Richards, 1966; Worgul et al., 1982a; Broglio and Worgul, 1985; Worgul et al., 1989) and DNA (Scullica et al., 1962). Many investigators believe that damage to the sensitive epithelial cells is responsible for subsequent cataract formation (Evans et al., 1960; Tokunaga, 1969; Worgul et al., 1982b). Others believe it is due to circulatory impairment (Fukami et al., 1980). Most of the investigations have used relatively high doses of radiation in order to achieve definite effects on the lens and its components, although lower doses in the range of a few hundred cGy have sometimes been used. The doses have been single or fractionated acute exposures or chronic exposures, ranging from 10-20 Gy or even higher (which would be well beyond the lethal dosage if given whole-body instead of to the eye alone) (Cogan and Donaldson, 1951; Broglio and Worgul, 1985; Giblin et al., 1979; Scullica et al., 1962; Hayes and Fisher, 1979) to 0.1 cGy/day (Evans, 1948). Some workers have not seen any cataract formation at doses below 10 Gy, but it is generally conceded that the cataractogenic dose in rats is about 2 Gy (Merriam and Focht, 1957; Prasad, 1984). Cogan and Donaldson (1951) induced opacities in rabbits with 250 cGy; Worgul et al. (1989) used accelerated heavy particles (570 MeV ^{40}Ar) to induce cataractous changes at doses as low as 1 cGy, compared to 250 kVp x-rays from 2-10 Gy. Heavy particles seem to have a much greater cataractogenic effect than sparsely ionizing x- and gamma rays; neutrons appear to fall in this category also (Di Paola et al., 1980).

However, for the purposes of this paper, we have focused on single, acute whole-body doses of gamma rays and 14 MeV neutrons rather than the head or eye alone. As whole-body exposure is used extensively as preparation prior to bone marrow transplantation, we would like to know whether the potential for cataract formation is greater due to early damage to lens cells. Although reports exist in the literature on cataractous changes in the lens at doses in the range of 10-100 cGy, the changes reported have usually been clinical in nature, and weeks to months after exposure, or after heavy particle irradiation. For example, Kodama et al. (1983) used SEM (as well as slit-lamp microscopy) 2-4 months after irradiation (10 Gy to the head only) and found vacuolation and swelling of fiber cells. What we would like to determine is if damage is detectable after only a few cGy, and if it can be detected early (within the first 24 hr) by SEM. Certainly, as Versura and Maltarello (1988) pointed out, SEM can play a very important role in assessing damage to the lens experimentally. It would also be particularly important to show that our in vitro intact lens culture technique can serve as a very sensitive biological dosimeter for early, potentially cataractogenic damage.

Methods

Animals

Female Wistar rats weighing about 100 g (5-6 weeks old) were obtained from the colony at the NRC (Montreal Road, Ottawa) and transported to CRNL the week preceding the experiments to allow recovery from the trip and acclimation to the new environment. They were maintained on normal tap water and Purina laboratory chow ad libitum, housed 2 per cage, at a temperature of 22°C and 55% relative humidity.

Treatment

A total of 40 rats was used for several treatment protocols, in vivo and in vitro, involving vitamin E and irradiation with neutron or gamma radiation.

Vitamin E (VE) treatment: A total of 40 rats was used in two protocols:

1. In Vivo: Rats were injected subcutaneously (ventral surface) daily with 0.1 ml (100 units) D-alpha-tocopherol (vitamin E) in soya bean oil (Covitol; Henkel) for 4 days prior to irradiation. On the fourth day, 2 hr after the final injection, the rats were irradiated as described below. Rats were decapitated with a guillotine, then the eyes removed and the lenses extracted aseptically, as described previously (Ross et al., 1983b). The undamaged, intact lenses were placed immediately in complete M199 medium supplemented with penicillin, streptomycin and 10% horse serum, then incubated at 35.5°C for 24 hr before fixation.

2. In Vitro: Rats were decapitated and lenses removed as described above. The lenses were placed in complete M199 medium with or without vitamin E (2.4 μM D-alpha-tocopherol acetate), and incubated at 35.5°C for 24 hr pre- and post-irradiation. Medium was changed twice pre-irradiation (at 8 hr and immediately before irradiation) and twice afterwards (immediately after irradiation and after 20 hr) prior to fixation at 24 hr.

Fixation

The rat lenses were removed from the incubator 24 hr after irradiation and placed in Karnovsky's fixative (Graham and Karnovsky, 1966) at 4°C. This fixation protocol combines low temperature to slow biochemical processes, paraformaldehyde and cacodylate to rapidly fix the sample, and glutaraldehyde to cross-link appropriately for electron microscopy. These fixation conditions were chosen after experiments in 1976 when we began to examine lenses by scanning electron microscopy. Examination of lenses revealed significantly better fixation using these conditions, when compared to fixation at room temperature or fixation with glutaraldehyde alone.

The 'Quick-Fix' lenses (QF) were fixed immediately after irradiation (within 30 min). After 24 hr at 4°C, the fixative was changed to 0.1 M cacodylate buffer. The lenses were then dehydrated through an alcohol series to acetone, critical-point dried from CO_2, halved as previously reported (Mousa et al., 1979) and the halves or quarters sputter-coated with palladium-gold for subsequent examination with the Hitachi S-650 Scanning Electron Microscope (SEM).

Damage Evaluation

Criteria for grading the degree and extent of damage to the lens were established (see below), and several areas of each code-identified lens were examined visually in the scanning electron microscope to measure the extent of damage using a ruler on the screen surface; based on the distribution and extent of the damage we determined the damage category, which was applied to the lens when identifying codes were decoded at the end of the experiment. This approach has been used in our previous studies (Ross

et al., 1983b; Stewart-DeHaan et al., 1981) and resulted in reproducible and consistent analysis, which we also found to be quite comparable to our previous work on irradiated lens damage (Ross et al., 1983a). The grading system used was as follows:

'0' no damage, normal lens;
'1' slightly granular fibre cells, normal capsule;
'2' 10-15 micrometers depth of granular fibre cells in equatorial region (zonular), normal capsule;
'3' 10-15 micrometers depth of granular fibre cells in equatorial region (zonular), abnormal capsule;
'4' 10-15 micrometers depth of granular fibre cells with holes in underlying cells, mainly at equatorial (zonular) region;
'5' extensive presence of granular fibre cells immediately under capsule all around the lens;
'6' foam under capsule in equatorial region extending 10 micrometers or more in depth, capsule apparently normal;

Irradiation

Doses delivered for both neutron (14 MeV) and gamma (^{60}Co) irradiation were 10 and 100 cGy, at a dose rate of 10 cGy/min(n) and 8 cGy/min(g), respectively.

1. Neutron irradiation was performed using the Fast Intense Neutron Source (FINS) at the AECL Chalk River Nuclear Laboratories (CRNL). The FINS is a CW accelerator providing a 300 kV beam of deuterons (beam current, 12 mA) aimed at a rotating, water-cooled tritium drum target. At an angle of 90° to the deuteron beam, neutrons were produced by $T(d,n)He^4$ at an energy of 14.2 MeV.

Neutron and gamma-ray spectroscopy was done at a distance of 3.72 meters from the target using NE-213 and BF_3 (boron trifluoride) detectors at 90° to the beam line (with nominal beam current of 12 microamperes). Then, folding the neutron and gamma-ray spectra with the respective energy-dependent fluence-to-kerma conversion factors resulted in the kerma components shown in Table 1 for neutron energies.

Thus, the major component (about 85%) of the neutron beam (and of the dose delivered) was 14.2 MeV (average) fast neutrons, and the gamma component "contamination" was about 15%.

1a. In Vivo: Rats were irradiated in a specially-designed rotating 'Ferris Wheel', diameter 80 cm, at 90° to the beam and a rodent-to-target (midpoint of individual cylindrical animal chambers) distance of 80 cm. The wheel rotated at about 1 rpm (Figure 1). This was similar to the setup used by Di Paola et al. (1980), where mice were irradiated head-on to the beam, in individual cages, 20 mice at a time.

For dosimetry, a TE (tissue-equivalent) detector for n/gamma and a GM (Geiger-Müller) detector for gamma-only were placed in individual chambers for one revolution of the wheel to confirm the previous calculations and define the total dose for a single revolution. A TE dosimeter was placed in an empty chamber during each rat irradiation to ensure the correct dose was being delivered.

Each rat was facing the neutron source at a slight angle, and the snugness of fit prevented it from turning around or moving forward or back to any appreciable extent; thus, the distance from source to head (and to either eye) did not effectively vary during irradiation. The average dose rate received by each rat (eye) was determined to be 10 cGy/min (neutron and gamma), which was consistent to within 3% of the static average measured previously. Any movement of the head was considered irrelevant with respect to angular distribution, and to have a negligible impact on the dose delivered to each eye; depth-dose calculations revealed that 1 cm of tissue would decrease the 14 MeV neutron flux by only 7%.

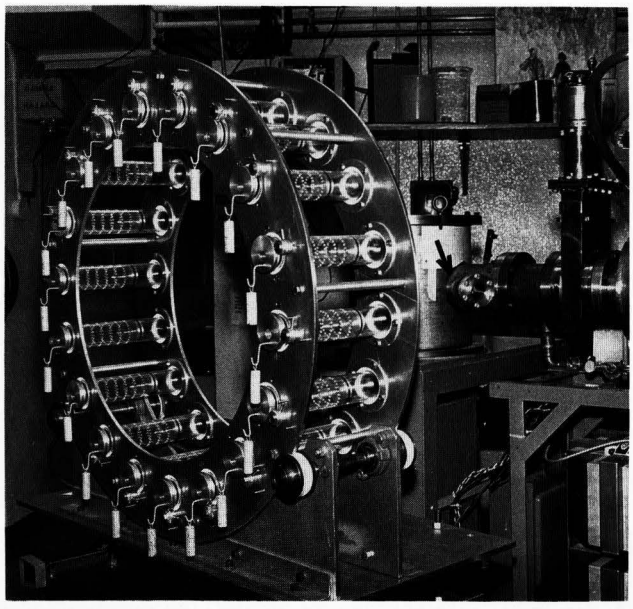

Figure 1: Configuration for neutron irradiation of rats. This "Rodent Ferris Wheel" housed one rat per chamber, revolved at about 1 rpm, and had an external weight to maintain chamber and rat horizontal. Source of 14 MEV neutrons is seen beyond wheel (arrow).

Table 1: Kerma and proportion of total dose for various neutron energies from FINS.

Group (n)	Energy	Kerma*	Fraction (%)
Thermal n	0.2 eV	1.52	0.12
Epithermal n	0.2 eV - 0.6 MeV	8.25	6.73
Fast n	0.6 MeV - 12 MeV	2.85	23.26
Source n	12 MeV - 16 MeV	6.72	54.86
TOTAL n	0 - 16 MeV	1.04	84.9
Gamma	0.3 MeV - 9 MeV	1.84	15.02
Total (n + gamma)		1.23	100

*in cGy/sec.microampere

1b. In Vitro: Rat lenses were placed in 5 ml of complete M199 tissue culture medium, each group (2 lenses) in a sterile 10 ml snap-cap polyethylene tube (Falcon). The tube was supported vertically in a plexiglass box 80 cm from the source and in line with the neutron beam at 90 degrees. This location corresponded to the middle of the ferris wheel used for in vivo neutron experiments at 10 cGy/min. Before and after the brief irradiation (about 1-10 min), during transfer to and from the incubator, the

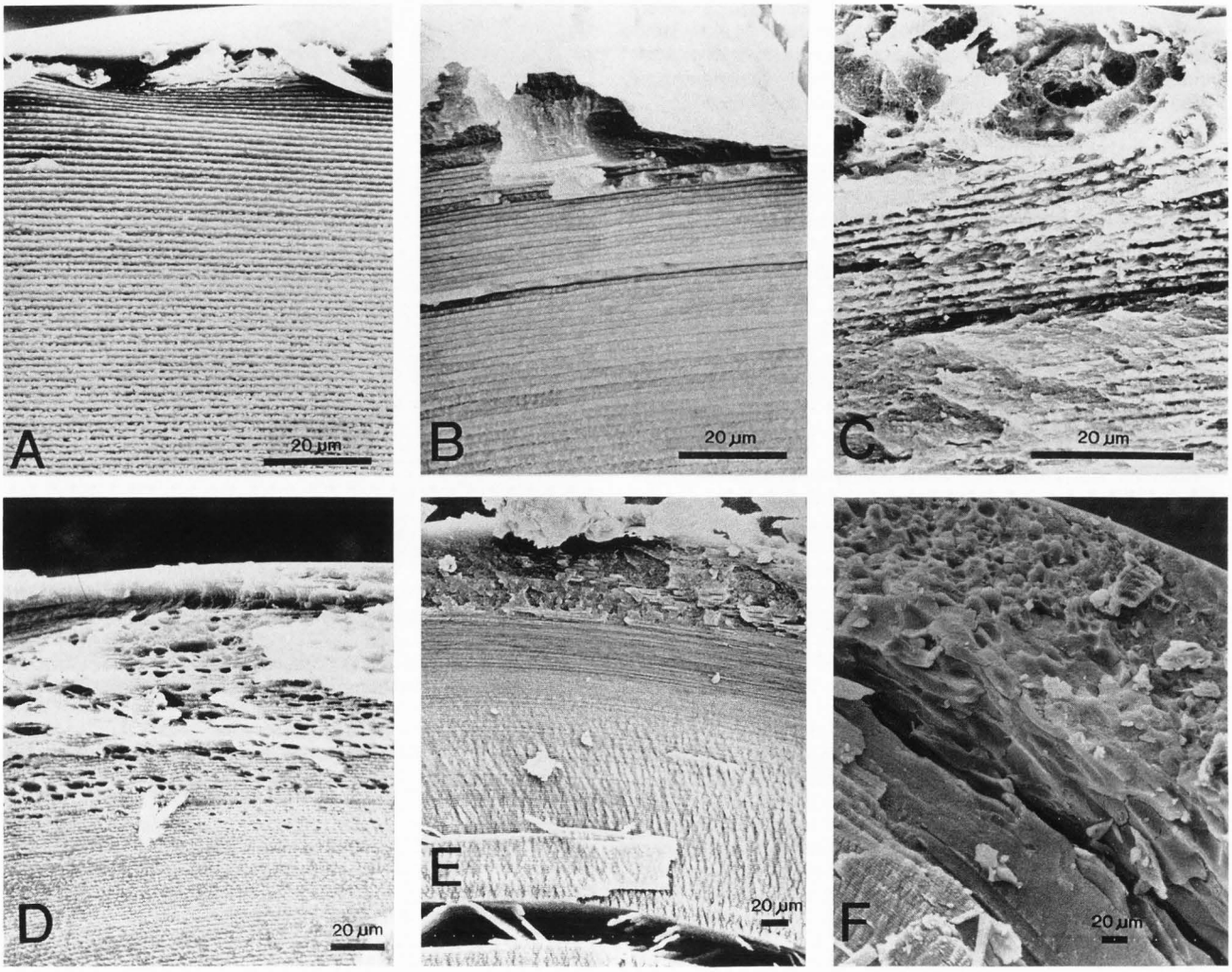

Figure 2. Scanning electron micrographs of the lenses showing various levels of damage in lenses examined by SEM after sham irradiation or exposure in vivo or in vitro to 14 MeV neutron irradiation or gamma irradiation; lenses were incubated 24 hr pre- and post-irradiation, or fixed immediately after irradiation (QF), then prepared for SEM as described in the text. The criteria for the levels of damage are described in the Methods (Damage evaluation section). **(A)** Control rat, no irradiation: level of damage 0: no damage, normal lens; **(B)** level of damage 1: slightly granular fibre cells, normal capsule; **(C)** level of damage 2: 10-15 micrometers depth of granular fibre cells in equatorial region (zonular), normal capsule; **(D)** level of damage 4: 10-15 micrometers depth of granular fibre cells with holes in underlying cells, mainly at equatorial (zonular) region; **(E)** level of damage 5: extensive presence of granular fibre cells immediately under capsule all around the lens; **(F)** level of damage 6: foam under capsule in equatorial region extending 10 micrometers or more in depth, capsule apparently normal;

tubes containing the lenses were kept in a water bath at 35.5°C.

2. Gamma irradiation was performed using a ^{60}Co Gamma-Cell 150 Irradiator (AECL) in a shielded room adjacent to the animal facility (Health Sciences Div., CRNL) near the neutron facility. Measurements of dose rate were taken using the TE ion chamber at 100, 150, 200 and 250 cm from the source along the beam center-line; the dose rates were 14.06, 6.10, 3.62 and 2.32 cGy/min, respectively. The off-axis fall-off of dose rate was only about 3% to a distance of 15 cm, so several rats could be irradiated at once in a stacked box design (up to 8 in four tiers). The dimensions of the individual rectangular plexiglass rat boxes were comparable to those for the ferris wheel chambers, moderately but effectively limiting movement of the rats.

2a. In Vivo: A similar dose rate for the gamma irradiation as for the neutron irradiation was used. The optimal target-to-source distance was 126 cm (8 cGy/min). The boxes were set up in four tiers along an arc 126 cm from the source on a wooden table. The TE dosimeter was placed in an empty box during the irradiation to ensure the correct dose was being delivered.

2b. In Vitro: Isolated rat lenses in complete medium were gamma-irradiated in the same manner as for in vitro neutron irradiations. To obtain a

TABLE 2: Lens Damage Grades Following Neutron or Gamma Irradiation of Rat Lens

Dose (cGy)	Post-Irradiation Treatment*		Damage Grade** Neutron	Gamma
In Vitro Irradiation				
0	C	--	0	0
0	C	VE	0	1
10		--	1-2	4
10		VE	0	3
100	QF	--	0-1	1
100		--	6	3
100		VE	5	2
In Vivo Irradiation				
0	C	--	0-1	0-1
0	C	VE	0	0
10		--	1	1
10		VE	1	0
100	QF	--	2	1
100		--	6	2
100		VE	4	1

*Treatments: VE, vitamin E; C, control; QF, fixed immediately ("quick-fix")
**Damage Grades: For explanation, See legend to Figure 2 on facing page

similar dose rate, tubes were placed in the plexiglass boxes (as for the in vitro neutron irradiation), at 126 cm from the ^{60}Co source. A temperature of 35.5°C was maintained before and after irradiation using a water bath (during transfer to and from the incubator).

Results

The results of the pilot study are illustrated in Figure 2 and summarized in Table 2 indicating the range of measurements observed blind when the samples were examined on the SEM screen; the measurements were later decoded.

There was definite damage evident when 10 cGy lenses were compared to control lenses (in which there was no visible damage). Greater damage was clearly evident in lenses exposed to 100 cGy.

The damage observed in neutron-irradiated lenses was greater than in ^{60}Co-irradiated lenses for all groups, with the exception of one group, 10 cGy ^{60}Co-irradiated in vitro lenses. The damage to this group was rather high in comparison to the other groups, even to those irradiated with 100 cGy gamma, and may be artefactual.

The gamma-irradiated lenses did seem to display slightly more damage than their in vivo counterparts after receiving 100 cGy in vitro, but only marginally for all other groups, if one ignores the slightly anomalous result for 10 cGy gamma.

The results from immediate fixation indicate that damage is visible in lenses fixed immediately after the irradiation instead of after 24 hr of incubation.

More work will be necessary to confirm these results which suggest that the relative biological effectiveness (RBE) for neutron irradiation is greater than for gamma irradiation.

Treatment with vitamin E appeared to alleviate the observed damage for any particular radiation dose in almost every case. This is consistent with its postulated role as a free radical scavenger. Much more work is required to verify this preliminary observation.

Discussion

In Vivo Irradiation

The results of this pilot study on the early damage expressed in the rat lens induced by ionizing radiation reveal that there is indeed morphological damage detectable by SEM immediately after irradiation ('Quick-Fix' lenses). By 24 hr, this damage is somewhat greater, having had more time to develop, but previous reports in the literature have shown that a dose of 200 cGy is likely the minimum cataractogenic dose (Merriam and Focht, 1962; Worgul et al., 1989). Therefore, the assumption is that this damage will be repaired or at least controlled so that visual acuity can be maintained. But, clearly, irradiation does cause immediately noticeable damage.

From the evidence in the literature it is apparent that this early damage to fibre cells that we have observed must be transient since cataracts do not usually develop after such low doses. However, cataracts are multi-factorial in their etiology (Worgul and Rothstein, 1977, Hockwin and Koch, 1975), being influenced by such factors as growth hormone and exogenous noxious substances; if other potentially cataractogenic conditions or substances are also present, the situation could then lead to synergistic action. This could also be the situation for occupational exposures or radiation accidents. This damage may result in precataractous damage which, in the longer times available for cataract development in the human, could result in cataract development after a 20 year delay, as was observed by Hayes and Fisher (1979). Previous papers on either in vivo or in vitro irradiation have dealt mostly with the exposure of one lens alone, the opposite lens and the rest of the body being shielded (Richards et al., 1956; Rini et al., 1983; Worgul et al., 1989). In this study, we are dealing with whole-body irradiation, more practical in the sense of damage to the lens during accidental (e.g., Chernobyl nuclear accident) or radiotherapeutic exposure (preparation for bone marrow transplantation; Deeg et al., 1984).

When the rats were exposed to 10 or 100 cGy, the lens suffered damage particularly in the equatorial region, subcapsularly. After 10 cGy, the lenses exposed to neutrons appeared to have about the same degree of damage as those exposed to gamma radiation (fairly minor). At the higher dose of 100 cGy, damage was markedly greater after neutrons, indicating the expected dose-response relationship. Although the RBE for neutrons is thus clearly greater than 1, it is premature from the results reported here to assign a definitive value for early lens damage. Riley et al. (1988) showed that low energy, fission-spectrum neutrons have an RBE of 7.5 for lens epithelial cell proliferation, while Di Paola et al. (1980) found that for lens opacification the RBE was

24 for 1 MeV and 6 for 600 MeV neutrons. Di Paola et al. (1978) counted individual opacities on the posterior part of the lens using slit-lamp microscopy, and found that technique to be sufficiently sensitive to evaluate opacification in mice at low doses (1-38 cGy) of 14 MeV neutrons (RBE of 9). In their 1980 paper, Di Paola et al., showed that the RBE for all neutron energies increased with increasing age and with decreasing dose; at higher doses, the RBE decreased as the neutron energy increased. Doses ranged from about 1 to 40 cGy for 1, 5, 15, and 600 MeV neutrons, and the corresponding RBE values were 19, 9, 6, and 6, respectively. Mice were irradiated head-on to the beam. These authors suggested that the opacities resulted from radiation-induced non-lethal damage due to defective fiber cell differentiation. Kodama et al. (1983) showed that galactose diet probably reduced X-irradiation-induced cataracts in mice due to inactivation of aldose reductase and hence the polyol pathway. This led to the accumulation of galactose along with decreased formation of dulcitol (galactitol) which are able to scavenge hydroxyl radicals. These authors speculate that decreased osmotic stress also contributed to the cataract prevention. However, these findings were all based on evaluation in vivo several months post-irradiation, whereas our study deals with acute-phase damage.

In Vitro Irradiation

The in vitro system is certainly a sensitive system for detection of early damage, and also has a major advantage that it does not itself induce any damage to the lens (Ross et al., 1983a). Damage by in vitro radiation was slightly greater than seen in the corresponding in vivo irradiations, especially at 100 cGy, as would be expected for a system without many of the variables present in vivo which could influence repair. After 10 cGy, the radiation damage to lenses was about the same for neutrons or gamma (namely very little damage), but after 100 cGy, damage was definitely greater for neutron than for gamma irradiation; this was also observed with the in vivo experiments. Previous workers using in vitro irradiation have dealt with generally higher doses to isolated lenses (calf, ox) in saline solution, monitoring for metabolic changes (Hockwin, 1962), or in M199 medium for 24 hr monitoring the cation permeability changes and hydration (Lambert and Kinoshita, 1967); in vitro results usually paralleled in vivo findings. We do not have any explanation for the apparently greater damage that seemed to be caused in our study by gamma than by neutron irradiation after 10 cGy, but we intend to repeat this work with a larger number of animals and more doses in the near future. This should also lead to a more precise estimation of RBE and a more definitive picture of the damage induced within the first 24 hr.

Relative Biological Effectiveness of Neutrons

The greater RBE of neutrons has been documented in lens as well as in other tissues by several authors (Di Paola et al., 1980; Hei et al., 1988; Riley et al., 1988; Richards, 1966; Keng et al., 1982). We have shown that at a fairly low dose (100 cGy) of either gamma or neutron irradiation, the RBE is detectable with SEM analysis. The damage induced by ionizing radiation, whether neutron or gamma, can also be reduced by antioxidants such as vitamin E, indicating that the type of damage by these two different types of radiation probably shares a similar target site in the cell, the cell membrane. The in vitro technique allows us to make comparisons between different types of radiation (photon or particulate) to determine an RBE for a particular tissue. This technique should prove useful for a variety of analytical studies, not only with SEM, but also metabolic, molecular and membrane-related alterations to cellular function. The probability of cataract formation in an animal may depend on the ability of the individual organism to repair the damage we have reported here before it becomes set and irreversible.

Protection with Vitamin E

The addition of vitamin E (D-alpha-tocopherol) to the system prior to irradiation, either by injection subcutaneously (s.c.) for 5 days or by mixing with the medium added to the lenses in vitro for 24 hr, led to a marked decrease in observed damage in all lenses after 100 cGy. However, after 10 cGy, the protective effect of vitamin E was less obvious because relatively little damage was caused by the 10 cGy irradiation alone, so any protective influence by vitamin E would be difficult to detect.

Oxidative damage has been previously implicated in cataract formation in the eye lens (Petkau, 1980). The Haber-Weiss reaction may be involved in cell membrane damage via oxidation of particular macromolecules (lipids and vital proteins, particularly those containing essential sulfhydryl groups), since free-radical scavengers can attenuate damage (Varma and Mooney, 1986, Ross et al., 1983a). Antioxidants such as vitamin E or vitamin C prevent light-scattering globule degeneration in lenses incubated in vitro with cataractogenic agents such as solumedrol (Creighton et al., 1983), aminoglycoside antibiotics such as hygromycin B (Creighton et al., 1982), elevated sugars (Trevithick et al., 1981; Creighton et al., 1980, 1985; Linklater et al., 1986), hydrogen peroxide (vitamin C but not vitamin E, Stewart-DeHaan et al., 1989), and gamma radiation (Ross et al., 1983a). The similarity of changes in morphology induced by such diverse stresses, coupled with the prevention of cataracts by antioxidants (Trevithick et al., 1981, Ross et al., 1982, 1983b; Stewart-DeHaan et al., 1981) and the induction of lens damage by radiation (known to result from free-radical damage) strongly implies the existence of a common oxidative step in the process of cataract formation. It is generally accepted that radiation, either gamma or neutron, causes cellular damage by ionization in aqueous solutions resulting in the formation of a number of species of damaging oxygen free-radicals and derived oxidizing species. These may include, but are not limited to superoxide anion, molecular oxygen, hydrogen peroxide, hydroxyl radical, hypochlorite, and lipid hydroperoxides. These species may interact with biological molecules to cause damage to DNA (Trevithick et al., 1987), proteins (Wolff et al., 1986), and lipid peroxidation due to chain reactions.

Antioxidants may intervene at many points directly by scavenging these species or by breaking the chain reactions. Oxidative damage may also be autocatalytic (Simpson et al., 1988), so the radiation-induced cellular damage observed in these studies may be a trigger for a cascading series of damaging events which eventually lead to cataract formation as a late effect (Hayes and Fisher, 1979).

The appearance of damage in lenses fixed immediately after irradiation has important implications for the understanding of the process of cataract

formation. The changes observed in these studies occurred during irradiation or were developed by the process of fixation from "latent damage", since lenses fixed immediately afterwards showed altered morphology. The changes observed 24 hours after irradiation were noticeably greater and appeared to increase with the dose delivered, although neutron irradiation definitely caused more damage than an equal dose of gamma radiation. Taken together, these observations indicate that radiation damage to cellular components other than DNA may occur early, resulting in damage to cell membranes or cellular proteins (Patmore and Duncan, 1981). The observations of Tokunaga (1969) of transient vacuoles in the rabbit lens for two weeks after X-irradiation supports the idea that the early changes may involve damage to the cell membrane.

Therefore, the damage to the nucleated epithelial cells can lead to cell lethality in the form of reproductive death or mutations, leading to faulty differentiation into fibre cells (but this takes time to develop and to elicit any noticeable effects). Fibre cells are less sensitive to irradiation, but their ability to form or maintain the necessary tight gap junctions required for proper light transmission (Kuszak et al., 1988) may be impaired.

Worgul and Rothstein (1975) noted that radiocataracts always seem to be preceded by misalignment of the precise order of the meridional rows (MR), critical to the maintenance of transparency. If such misalignment is corrected, opacification is avoided. These authors put forward an explanation for radiocataractogenesis in 1977 that was based on the failure of polymorphic cells of the germinative zone to line up properly. Heterogeneity of cell size and shape prevented proper alignment into coherent rows; once MR disorganization occurred, new cells would be prevented from aligning properly, thus perpetuating disorganization and leading to progressive cataracts. Our results appear to be an early manifestation of the disorganization by direct damage to the cell membrane.

If the severity of the eventual cataract, and the period of latency before its appearance, are dependent on the extent of the initial pre-cataractous morphological damage observed here, then two conclusions could be drawn.

(1) Agents or treatments which cause greater damage to lenses (neutron vs gamma), as seen 24 hr post-irradiation, would be more likely to result in formation of a cataract later, or might require a lower threshold (total dose) to exert their effect.

(2) Antioxidants such as vitamin E are able to reduce at least the initial extent of radiation damage. This would also tend to lower the incidence of cataract, delay the onset, and set a higher threshold for the effective dose of radiation required to induce a cataract.

In summary, we report here the results of a pilot study on an effective in vitro method for treating and examining lenses by SEM for radiation damage. There is noticeable damage after doses as low as 10 cGy, primarily to the equatorial region, and 14 MeV neutrons are more damaging than gamma rays. However, as is evident from extensive work previously reported in this field, doses in this range (10-100 cGy) are unlikely to result in clinical cataract later on. The transient damage reported here will in most cases be repaired, but may remain latent; if other factors come into the situation later, then a cataract may well develop synergistically with these other factors. Also, vitamin E is an effective lens protective agent for in vivo or in vitro irradiation, apparently by scavenging free radicals before they can damage the cell membrane or other critical macromolecules.

Acknowledgements

We acknowledge with thanks the financial support of the Defence Research Establishment Ottawa, and the Medical Research Council of Canada. Thanks are extended to Dr. W. Cross, Physics Div., AECL Chalk River for his help in the use of the FINS, to the Animal Care workers and the Biology Division at Chalk River for assisting in the maintenance and handling of the animals, and to Dr. A. Robitaille, Mr. P. Rushton and Mr. B. Hoffarth (DREO) for the efficient, precise measurements and calculations of the dosimetry involved.

References

Barron A, McDonald JE, Hughes WF (1970). Long-term complications of betaradiation therapy in ophthalmology. Trans. Am. Ophthalmol. Soc. 68: 113-125.

Broglio TM, Worgul BV (1985). Lens epithelium and radiation cataract. V. Observations on acid phosphatase and meridional row nuclear fragmentation. Exp. Eye Res. 40: 263-271.

Cogan DG, Donaldson DD (1951). Experimental radiation cataracts. I. Cataracts in the rabbit following single X-ray exposure. Arch. Ophthalmol. 45: 508-522.

Cogan DG, Donaldson DD, Reese AB (1952). Clinical and pathological characteristics of radiation cataracts. Arch. Ophthalmol. 47: 55-70.

Creighton MO, Stewart-DeHaan PJ, Ross WM, Sanwal M, Trevithick, JR (1980). Modelling cortical cataractogenesis I. In vitro effects of glucose, sorbitol and fructose on intact rat lenses in medium 199. Can. J. Ophthalmol. 15: 183-188.

Creighton MO, Trevithick JR, Sanford SE, Dukes TW (1982). Modelling cortical cataractogenesis IV. Induction by hygromycin B in vivo (swine) and in vitro (rat lens). Exp. Eye Res. 34: 467-476.

Creighton MO, Sanwal M, Stewart-DeHaan PJ, Trevithick JR (1983). Modelling Cortical Cataractogenesis V. Steroid cataracts induced by solumedrol partially prevented by Vitamin E in vitro. Exp. Eye Res. 37: 65-75.

Creighton MO, Ross WM, Stewart-DeHaan PJ, Sanwal M, Trevithick JR (1985). Modelling cortical cataractogenesis VII. Effects of Vitamin E treatment on galactose-induced cataracts. Exp. Eye Res. 40: 213-222.

Deeg HJ, Flournoy N, Sullivan KM, Sheehan K, Buckner CD, Sanders JE, Storb R, Witherspoon RP, Thomas ED (1984). Cataracts after total body irradiation and bone marrow transplantation: a sparing effect of dose fractionation. Int J. Radiat. Oncol. Biol. Phys. 10: 957-964.

Di Paola M, Bianchi M, Baarli J (1978). Lens opacification in mice exposed to 14 MeV neutrons. Radiat. Res. 73: 340-350.

Di Paola M, Coppola M, Baarli J, Bianchi M, Sullivan AH (1980). Biological responses to various

neutron energies from 1 to 600 MeV. II. Lens opacification in mice. Radiat. Res. 84: 453-461.

Evans TC (1948). Effects of small daily doses of fast neutrons on mice. Radiology 50: 811-824.

Evans TC, Richards RD, Riley EF (1960). Histological studies of neutron- and x-irradiated mouse lenses. Radiat. Res. 13: 737-750.

Fukami Y, Shimizu S, Matsushima H, Kaneko T, Yamada T (1980). Studies on the cause of radiation cataract. Acta Soc. Ophthalmol. Jpn. 84: 594-601.

Giblin FJ, Chakrapani B, Reddy VN (1979). The effects of x-irradiation on lens reducing systems. Invest. Ophthalmol. Vis. Sci. 18: 468-475.

Graham RC, Karnovsky MJ (1966). The early stages of absorption of injected horseradish peroxidase in the proximal tubules of mouse kidney: ultrastructural cytochemistry by a new technique. J. Histochem. 14: 291-302.

Hanna C, O'Brien JE (1963). Lens epithelial cell proliferation and migration in radiation cataracts. Radiat. Res. 19: 1-11.

Hayes RP, Fisher RF (1979). Influence of a prolonged period of low-dosage x-rays on the optic and ultrastructural appearances of cataract of the human lens. Brit. J. Ophthalmol. 63: 457-464.

Hei TK, Hall EJ, Waldren CA (1988). Mutation induction and relative biological effectiveness of neutrons in mammalian cells - Experimental observations. Radiat. Res. 115: 281-291.

Hockwin O (1962). Early changes of lens metabolism after X-irradiation. Exp. Eye Res. 1: 422-426.

Hockwin O, Koch H-R (1975). Combined noxious influences. In: Cataract and Abnormalities of the Lens, Bellows JG (Ed.), Grune and Stratton, NY. pp. 243-254.

Keng PC, Lee AC, Cox AB, Bergtold DS, Lett JT (1982). Effects of heavy ions on rabbit tissues: cataractogenesis. Int. J. Radiat. Biol. 41: 127-137.

Kodama T, Reddy VN, Giblin F, Kinoshita JH, Harding C (1983). Scanning electron microscopy of X-ray induced cataract in mice on normal and galactose diet. Ophthalmic Res. 15: 324-333.

Kuck, JFR (1970). Chemical constituents of the lens. In: Biochemistry of the Eye, Graymore CN (Ed.), Thomas, Springfield, OH, p. 207.

Kuszak JR, Khan AR, Cenedella RJ (1988). An ultrastructural analysis of plasma membrane in the U1866A cataract. Invest.Ophthalmol.Vis.Sci. 29: 261-267.

Lambert BW, Kinoshita JH (1967). The effects of ionizing radiation on lens cation permeability, transport, and hydration. Invest. Ophthalmol. Vis. Sci. 6: 624-634.

Linklater HA, Dzialoszynski T, McLeod HL, Sanford SE, Trevithick JR (1986). Modelling cortical cataractogenesis VIII. Effects of butylated hydroxytoluene (BHT) in reducing protein leakage from lenses in diabetic rats. Exp. Eye Res. 43: 305-314.

Lipman RM, Tripathi BJ, Tripathi RC (1988). Cataracts induced by microwave and ionizing radiation. Survey of Ophthalmology 33: 200-210.

McKay PB, King MM (1980). Vitamin E: Its role as a biological free radical scavenger and its relationship to the microsomal mixed-function oxidase system. In: Vitamin E: A Comprehensive Treatise, Machlin LE (Ed.), Marcel Dekker, NY. pp. 289-317.

Merriam GR Jr., Focht EF (1957). A clinical study of radiation cataracts and the relationship to dose. Am J. Roentgenol. 77: 759-785.

Merriam GR Jr., Focht EF (1962). A clinical and experimental study of the effect of single and divided doses of radiation on cataract production Trans. Am. Ophthalmol. Soc. 60: 35-51.

Merriam GR Jr., Worgul BV (1983). Experimental radiation cataract - its clinical relevance. Bull. N.Y. Acad. Med. 59: 372-392.

Miller RJ, Fujino T, Nefzger MD (1967). Lens findings in atomic bomb survivors. Arch. Ophthalmol. 78: 697-704.

Mousa GY, Creighton MO, Trevithick JR (1979). Eye lens opacity in cortical cataracts associated with actin related globular degeneration. Exp. Eye Res. 29: 379-391.

Palva M (1978). The effect of X-irradiation on the sodium-potassium activated adenosine triphosphatase (Na-K-ATPase) activity in the epithelium of the rat lens. Acta Ophthalmol. 56: 431-438.

Palva M, Palkama A (1977). Ultrastructural lens changes in X-ray induced cataract of the rat. Acta Ophthalmol. 56: 587-598.

Patmore L, Duncan G (1981). The physiology of lens membranes. In: Mechanism of Cataract Formation in the Human Lens, Duncan G (Ed.), Academic Press, London. pp. 193-217.

Petkau A (1980). Radiation carcinogenesis from a membrane perspective. Acta Physiol. Scand. 492 (Suppl.): 81-90.

Prasad KN (1984). CRC Handbook of Radiobiology, CRC Press, Boca Raton, FL. pp. 39-40, 234-236.

Richards RD (1966). Changes in lens epithelium after x-ray or neutron irradiation (mouse and rabbit). Trans. Am. Ophthalmol. Soc. 64: 700-734.

Richards RD, Riley EF, Leinfelder PJ (1956). Lens changes following X-irradiation of single and multiple quadrants. Am J. Ophthalmol 42 (4, part 2): 44-50.

Riley EF, Miller RC, Lindgren AL (1988). Recovery of murine lens epithelial cells from single and fractionated doses of X-rays and neutrons. Radiat. Res. 114: 567-578.

Rini FJ, Worgul BV, Merriam GR Jr. (1983). Scanning electron microscopic analysis of radiation cataracts of the eye lens I. X-radiation cataractogenesis as a function of dose. Ophthalmic Res. 15: 146-159.

Ross WM, Creighton MO, Stewart-DeHaan PJ, Sanwal M, Hirst M, Trevithick, JR (1982). Modelling cortical cataractogenesis: III. In vivo effects of vitamin E on cataractogenesis in diabetic rats. Can. J. Ophthalmol. 17: 61-66.

Ross WM, Creighton MO, Inch WR, Trevithick JR (1983a). Radiation cataract formation diminished by vitamin E in rat lenses in vitro. Exp. Eye Res. 36: 645-653.

Ross WM, Creighton MO, Trevithick JR, Stewart-DeHaan PJ, Sanwal M (1983b). Modelling cortical cataractogenesis VI. Induction by glucose in vitro or in diabetic rats: Prevention and reversal by glutathione. Exp. Eye Res. 37: 559-573.

Scullica L, Grimes P, McElvain N (1962). DNA synthesis in the rat lens epithelium after roentgen irradiation. Arch Ophthalmol. 68: 792-800.

Simpson PJ, Fantone JC, Lucchesi BR (1988). Myocardial ischemia and reperfusion injury: Oxygen radicals and the role of the neutrophil. In: Oxygen radicals and tissue injury, Halliwell B (Ed.), Federation of American Societies for Experimental Biology, Bethesda, MD. pp. 63-77.

Stewart-DeHaan PJ, Creighton MO, Sanwal M, Ross WM, Trevithick JR (1981). Effects of vitamin E on cortical cataractogenesis produced by elevated temperature in intact rat lenses in medium 199. Exp. Eye Res. 32: 51-60.

Stewart-DeHaan PJ, Sanwal M, Creighton MO, Inch WR, Trevithick JR (1989). ^{51}Cr Release and oxidative stress in the lens. Lens and Eye Toxicity Res. 6: 183-202.

Tokunaga T (1969). Transient lenticular vacuoles in the rabbit eye for two weeks after x-ray irradiation. Folia Ophthalmol. Jpn. 20: 7-10.

Trevithick JR, Creighton MO, Ross WM, Stewart-DeHaan PJ, Sanwal M (1981). Modelling cortical cataractogenesis 2. In vitro effects on the lens of agents preventing glucose- and sorbitol-induced cataracts. Can. J. Ophthalmol. 16: 32-38.

Trevithick JR, Chaudun E, Muel AS, Courtois Y, Counis MF (1987). Effect of x-irradiation and vitamin C on DNA degradation and endogenous DNase in embryonic chick lens cells. Curr.Eye Res. 6: 1275-1280.

Varma SD, Mooney JM (1986). Photodamage to the lens in vitro: implications of the Haber-Weiss reaction. J. Free Radicals in Biol. Med. 2, 57-62.

Varma SD, Srivastava VK, Richards RD (1982). Photoperoxidation in lens and cataract formation. Preventive role of superoxide dismutase, catalase, and vitamin C. Ophthalmic Res. 14, 167-175.

Versura P, Maltarello MC (1988). The role of scanning electron microscopy in ophthalmic science. Scanning Microscopy 2: 1695-1723.

Von Sallmann L (1951). Experimental studies on early lens changes after roentgen irradiation. I. Morphological and cytological changes. Arch. Ophthalmol. 45: 149-164.

Wolff SP, Garner A, Dean RT (1986). Free radicals, lipids and protein degradation. Trends Biochem. Sci. 11: 27-31.

Worgul BV, Rothstein H (1975). Radiation cataract and mitosis. Ophthalmic Res. 7: 21-32.

Worgul BV, Rothstein H (1977). On the mechanism of radiocataractogenesis. Medikon 6: 5-14.

Worgul BV, Low S, Merriam GR Jr. (1982a). The lens epithelium and radiation cataract III. The influence of age on the nuclear fragmentation of the meridional row cells following x-irradiation. Radiat. Res. 91: 181-185.

Worgul BV, Rothstein H, Medvedovsky C, Merriam GR Jr., Wilson MA (1982b). Radiation cataracts in the amphibian lens. Ophthalmic Res. 14: 73-82.

Worgul BV, Merriam GR Jr., Medvedovsky C (1989). Accelerated heavy particles and the lens III. Cataract enhancement by dose fractionation. Radiat. Res. 118: 93-100.

Discussion with Reviewers

B.V. Worgul: There is a substantial body of information which militates against a number of points raised by this research. I believe that for other than a few exceptions, e.g., dosimetry (which by the way is not sufficiently enlightening in terms of eye position and depth considerations) the authors have not addressed the overriding issues. I will limit myself to two, what I believe to be, major points. The suggestion that the radiation has an immediate effect on the lens, at a dose which, at least for low LET radiation, has not been reported to cause detectable damage, even in late stages, requires a more convincing set of data for support. In addition, the paper is suffused with unsupported facts that culture has no effect on lens architecture. The fallout from this is illustrated in Figure 2, where the pictures all show different regions of the lens yet are purported to show radiation dependent damage. To truly compare whether something is damaged versus control, one must compare the "damaged area" to the same region on an untreated or control lens. What is called damage is clearly not so. I submit, for example, that Figure 2E shows an otherwise normal bow region. One can clearly see the "cigar shaped" nuclei of bow cells. It is generally recognized that the lens fibers tend to "break" intracellularly in this region resulting in the appearance of disorganization.

Authors: Reports of damage to the lens after x-ray or neutron irradiation have been made, a recent example being Riley et al. (1988). Mouse lens epithelial cells were irradiated, and by 1 hr there was significant damage to wound response (recovery noticeable within 1 week); neutrons were 7.5 times more effective than x-rays (single dose). Di Paola et al. (1980) also found a similar RBE (anterior opacification, 20 months, doses up to 40 cGy).

We agree that culture is an artificial state, and in many cases workers have seen damage in so-called control lenses in vitro. However, we have consistently found an absence of damage in all control lenses with our culture technique for several years. In contrast, lenses incubated in the same medium with excess glucose, or after gamma or microwave irradiation display definite damage to cortical fibre cells.

It is not practical to show all data (photos, etc.) of all lenses studied. We have reported a summary of observations in the table and micrographs.

We agree that it would be desirable to show exactly the same region of the lens for each damage category, however the process of cutting the critical-point dried lenses at the equator resulted in some random differences even though every effort was made to standardize the technique. Damage was mostly in the equatorial area, but extended laterally subcapsularly and more deeply with increasing severity. We are confident that the damage category assigned to the lens examined was accurate. Several regions on each lens were scanned for damage, and the resultant diagnosis recorded. Not all sections were cut and mounted for SEM at the identical angle, which made it difficult to obtain fully comparable photos; the scanning of several regions and sections of each lens did compensate for this, and provided an overall assessment of the state of each lens.

B. Worgul: Another problematic area is the failure to recognize the existing body of literature. If one demands that radiation cataracts reflect a direct effect on the membranes of epithelial cells, how is it possible then that the central zone of the lens, (and the fiber substrate), can receive up to 30,000 rads of protons (as in the Constable study, Rad. Res., 65, 304, 1976), or thousands of rads (as in the Putenney and Schoch studies, Trans. Am. Ophthalmol. Soc., 51, 285, 1953, and Am. J. Ophthalmol. 38, 673, 1954), and yet not result in cataracts? How is it, as in the Rothstein et al. studies (see e.g., Ophthalmol. Res.,

14, 215, 1982) that thousands of rads can be applied to the entire lens and yet, when mitosis is prevented, cataracts do not follow? For surely if the direct damage, as purported in these studies, is the basic causative influence those earlier observations could not have been made!

Authors: The effect on cell membranes is suspected to be indirect by means of free-radical damage, whether to epithelial cells or fibre cells, but several studies have indeed reported cataracts within 2-3 months from head or eye irradiation (e.g. Kodama et al., 1983). Also, we have stated several times that the damage we observed will usually be repaired, but additional stresses may result in its being expressed synergistically.

B. Worgul: That the wholebody exposure is "more practical in the sense of damage to the lens during accidental (e.g., Chernobyl nuclear accident) or radiotherapeutic exposure (preparation for bone marrow transplantation, Deeg et al., 1984)" is silly on the face of it. Whole-body radiotherapy is used sparingly and in those cases cataract is the least of the problems to the individual. Localized radiotherapy to the head region is a more common reality. Whole body doses which can produce cataracts in the life time of the individual are generally compromising to survival although there are subsets of large populations, such as the Hiroshima and Nagasaki data which did have partial body shielding or received doses exceeding the minimum cataractogenic level of 2 Gy (these are not discussed).

Authors: Whole-body irradiation (usually up to 10 Gy) is used regularly for preparation of patients prior to bone marrow transplantation in the treatment of leukemia. Working under the assumption of recovery and long term survival, if there is a potential late effect likely to develop, considering all the other treatments the patient is receiving (including combined chemotherapy), it would be advantageous to consider preventive treatment after the major disease has been conquered. We limited our investigations to whole-body exposure, to model this situation.

Reviewer III: The authors have used Karnovsky's fixative for lens fixation. The rationale behind this choice escapes this reviewer. Kuszak and Rae (Exp. Eye Res., 35, 499-519, 1982), and Rafferty (Curr. Eye Res. 3, 463-471, 1984) have both shown that the proper fixation for lens morphology, as ascertained by SEM, should produce radial cell column faces with undamaged capsule, and the lens epithelium and fiber cells without shape distortion or membrane damage. I suggest that the authors try 2.5% glutaraldehyde in 0.07M sodium cacodylate buffer, pH 7.2. Karnovsky's fixative is particularly harsh on most superficial of lens cells. High quality stereopair SEM micrographs have been published of such lens preparations in this journal (Kuszak et al., Scanning Electron Microsc., 1984;III: 1369-1378). If the authors had produced micrographs of this quality, then the parameters they set up to grade damage evaluation could have easily been assessed in both the experimental and control lenses. ... This is unfortunate because in theory I believe they are absolutely correct, radiation damage within 24 hours is likely to be significant and should be assessed.

Authors: Lens cells have a high protein content, and are notoriously difficult to fix. Karnovsky's fixative exerts a double action, achieving swift penetration and fixation without damaging the cells. It has been used by several leading eye researchers (see e.g., Hollenberg MJ et al. (1976) Cell Tissue Res. 167: 425-438). We have, of course, also tried other fixation techniques, varying the components, concentrations, time and temperature (including the one suggested above). However, none gave markedly better results; most were less effective, and usually required longer to fix.