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RESPONSE OF AVIAN EMBRYONIC BRAIN TO SPATIALLY SEGMENTED X-RAY MICROBEAMS

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Absbwt - Duck embryo was studied as a model for assessing the effects of microbeam radiation therapy (MRT) on the human infant brain. Because of the high risk of radiation-induced disruption of the developmental process in the immature brain, conventional wide-beam radiotherapy of brain tumors is seldom carried out in infants under the age of three. Other types of treatment for pediatric brain tumors are frequently ineffective. Recent findings from studies in Grenoble on the brain of suckling rats indicate that MRT could be of benefit for the treatment of early childhood tumors. In our studies, duck embryos were irradiated at 3-4 days prior to hatching. Irradiation was carried out using a single exposure of synchrotron-generated X-rays, either in the form of parllel microplanarbeams (microbeams). or as non-segmented broad beam. The individual microplanar beams had a width of 27 pm and height of 111 mm, and a center-to-center spacing of 100 μm. Doses to the exposed areas of embryo brain were 40,80, 160 and 450 Gy (in-slice dose) for the microbeam, and 6, 12 and 18 Ciy for the broad beam. The biological end point employed in the study was ataxia. This neurological symptom of radiation damage to the brain developed within 75 days of hatching. Histopathological analysis of brain tissue did not reveal any radiation induced lesions for microbeam doses of 40-160 Gy (in-slice), although some incidences of ataxia were observed in that dose group. However, severe brain lesions did occur in animals in the 450 Gv microbeam dose groups, and mild lesions in the 18 Gy broad beam dose group. These results; indicate that embryonic duck brain has an appreciably higher tolerance to the microbeam modality, as compared to the broad beam modality. When the microbeam dose was normalized to the full volume of the irradiated tissue, i.e., the dose averaged over microbeams and the space between the microbeams, brain tolerance was estimated to be about three times higher to microbeam irradiation as compared with broad beam irradiation.

Key words: Microbeam radiation therapy, microplanar beams, developing brain, tissue sparing, pediatric brain tumors

INTRODUCTTON

Although radiation therapy is the principal method of treatment for most primary and metastatic brain tumors, the efficacy of the conventional methods is limited by the radiosensitivity of normal tissues surrounding the tumor. The limitation is particularly severe in infants and children, and when the type of tumor is radioresistant, Despite considerable recent progress in the fields of stereotactic and conformal radiation therapy, radiation treatment of central nervous system (CNS) tumors is still generally avoided in infants (below the age of three), and is used judiciously in older children because of the risk of adverse normal tissue morbidity. The study described here employs a novel experimental radiotherapy modality, microbeam radiation therapy (MRT). Developed first at the National Synchrotron Light Source (NSLS), Brookhaven National Laboratory (BNL) in the late 1980s and early 1990s, MRT research is now being pursued both at the NSLS (5,6,14,15), and at the European Synchrotron Radiation Facility, Grenoble, France (7). MRT uses arrays of parallel "microplanar" beams (microbeams),

microscopically thin planar slices of synchrotron-generated X-rays, The microplanar beams used at the NSLS have been 27 to 100 μm wide, spaced 100-400 μm center-to-center, with median beam energies ranging from 50 to 140 keV. The first finding with microbeams was that they are tolerated by the adult rat brain at very high doses (14). Furthermore, both unidirectional and cross-fired microbeams have proved highly effective in the treatment of certain experimental rat brain tumors, with minimal impact on adjacent normal CNS tissue (5,6,15).

Synchrotron radiation is produced when ultra-relativistic electrons in an electron storage ring pass through magnetic fields. The characteristics of synchrotron radiation include small source size, high intensity, natural forward collimation of the beam, and a broad and continuous spectrum ranging from infrared to X-rays. These properties facilitate: a/ tailoring of X-ray beams of different energy spectra, either by using beam filtration or by introducing a monochromator in the beam and b/ production of very narrow beams at very high dose rates. With beam filtration techniques, the median energy of the X-ray spectrum can be adjusted to facilitate dose delivery at different depths in tissue and/or to optimize the sharpness of the beam's geometrical edge at the given tissue depth. Medical research programs using synchrotron radiation are underway in the USA, Europe and Japan. Although at the present time MRT can be implemented only at certain large synchrotron facilities that have been designed to deliver high flux rates of high energy X-rays in narrow beams, compact synchrotron machines for medical applications are also being developed (16).

The present study has used duck embryo as a model for assessing the effects of MRT on the human infant brain. A second group of duck embryos was irradiated with broad beams from the same synchrotron beamline for comparison. Ducks are utilized frequently in a wide range of biomedical fields, including neurology. By targeting eggs, the studies avoided the need for anesthesia. This study complements the recent studies with suckling rat brain at ESRF (7) as important components in the development of the MRT modality as a potential therapy for the treatment of pediatric CNS tumors. The findings of this preliminary study are reported here.

MATERIALS AND METHODS

Pekin duck eggs supplied by the Cornell University Duck Research Laboratory (CUDRL), Eastport, New York, and by the Crescent Duck Farm, Aquebogue, New York were used The eggs were incubated at the companies' sites for the first 20 days of incubation, prior to delivery to BNL. Incubation was continued at BNL until the irradiations, which were administered on days 24 ot 25 of incubation. The incubation continued at BNL until hatching, which happened mostly about the day 28 of incubation. Incubation at BNL used a simple incubator in which the eggs were turned manually. After hatching, the ducklings were kept in two 60 cm x 90 cm brooders, equipped with a 25 Watt heat lamp, for 10 days where they were observed several times a day. The microbeam-irradiated ducks were then moved to larger brooders at BNL with no lamp-heating for another 2 weeks, and then to large rooms with outdoor pans. The broad-beam-irradiated ducks were kept in the brooder at BNL up to 10 days of age, and then were transferred to CUDRL. In both groups, feed and water were provided *ad libitum*.

Dosirnrtry

The dose evaluations repotted in this paper were reached by combining the results of several dosimetrical measuremets, together with dose calculations, as described below.

Dosimetric measurements included: a/ thermoluminescence dosimeters (TLDs). b/ radiochromic films of two types, HD810 (for the dose range of 80-200 Gy) and MD-55 (for the dose range of 12-60 Gy), both from Nuclear Associates (Carte Place. NY, USA), and c/ ion chamber measurements.

Analytical calculations using the code "Source" based on the code "Photon". Chapman et al. (2)], provided the X-my energy spectra. These calculations provided the X-ray energy spectrum and intensity for a given ring energy, ring currens wiggler design and its magnetic field, and beam filtration as a ti.mction of the vertical angle of the emitted beam. The beam was then integrated vertically to provide the average energy spectrun.

The spatial distribution of dose from microbeam arrays was evaluated by Monte Carlo simulations. using the code EGS4. The simulations used the code's recent upgrades that include: a/ a subroutine to introduce the effect of linear polarization of the synchrotron X-rays (9). and the subroutine LSCAT for Iow-energy scattering (10. 11). These simulations followed the basic concepts of the work by Slatkin et al. (13) which used an older version of the EGS4 code, and the recent work by Orion et al (12). The simulations incorporated: a) the linear polarization of the synchrotron X-rays at the 90% level: b/ the beam's penumbra, caused by the 0.9 mm wide hotizontal size of the beam spot at a distance of 28 m from the collimator (with the collimator positioned about 9 cm for the front surface of the subject); c/ an ellipsoidal shape for the egg, and d/ uniform values for composition of the egg shell and for the embryonic tissue.

Irradiations

Irradiations were carried out at the X-my storage ring of the NSLS which was operated at 2.584 GeV during the experiments reported in this paper. The X 17B 1 beamline used for this research is fed by a superconducting wiggler (a periodic array of fixed magnets of alternating polarities) operated at 4.7 tesla. The source-to-subject distance was 28 m, which made the beam quasiparallel. The irradiation system consisted of at a beam filter to adjust beam energy/intensity; b/ a sliding beam-shutter, controlling exposure time; c/ a single-slit collimator producing a single 3.8 mm high microbeam: d/ chromographic film for dosimetry; e/ subject-positioning apparatus: f/ an ionization chamber for dosimetty, and .g/ a phosphor screen for beam viewing.

The embryos were irradiated 34 days before hatching. The eggs were irradiated in the upright position, with the wider side up. To show the skull for targeting the embryo brain, X-ray fluorescent radiography was used. However, the positioning information obtained in this way did not allow us to achieve a consistent angle between the beam and the brain's axis. The doses delivered to the exposed areas of embryo brains were 40, 80, 160 and 450 Gy (in-slice) in the microbeam irradiation

Protocols, and 6, 12 and 18 Gy in the broad beam protocols. The corresponding egg-shell-entrance doses were 70,139,278 and 800 Gy (in-slice) for microbeams, and 9.5, 19 and 28.5 Gy for broad beam (the doses referred to in this report are to the brain unless stated otherwise). The beam fihrations used were 3.7 mm Si and 0.5 mm Cu for the ifiadiations of% 80 and 160 Gy; 3.7 mm Si and 0.25 mm Cu for the microbeam irradiation of 450 Gy and 3.7 mm Si and 9.77 mm Gu for the broad beam irradiations. A much thicker Cu filter was used for the broad beams to ensure uniform irradiation because our broad-beam irradiation method entailed scanning of the subject vertically in the beam using a fixed horizontal slit (see irradiation techniques below). For this reason dose rates had to be considerably lower than those in microbeams to avoid "hot spots" at the beginning and the end of the scan range.

Exposure to the microbeam entailed the use of a single-slit, vertical collimator and stepping the egg transversely across the beam by means of a computer-controlled translational stage of 1 urn step size. The microplanar beam produced by this collimator was vertical, 27 μm wide and 3.8 mm high. Beam heights of 11.4 mm were achieved by irradiating the eggs with 3 tiers of such 3.8 mm beams. The spacing used was 100 μm from center-to-center of each beam; the number of microplanar beams in the arrays was 110. The broad beam irradiations scanned the egg vertically at a uniform speed with a stationary, horizontal, line beam, 11 mm wide and 0.27 mm high. The irradiation fields for both the microbeams and the broad beams were 11 mm high x 11 mm wide,

Due to logistical considerations, surviving ducks that had been irradiated with broad beam, and their concomitant controls, were humanely euthanized at 98 days after hatching. The euthanasia used injection in the foot vein of 65 mg/kg of Na pentobarbital. Ataxia-free surviving ducks that had been irradiated with the microbeams, and their concomitant controls, were euthanized at 250 days after hatching. Brains were removed and fixed in 10% neutral buffered formalin. Fixed brain tissues were processed, embedded in paraffin, sectioned at 5 or 6 μm and stained with hematoxylin and eosin according to standard procedures. Brains of the microbeam-irradiated birds were sectioned first in horizontal (i.e., coronal) and then in sagittal (near the midbrain) slices to reduce the chance of missing microplanar lesions with uncertain orientations in the brain.

RESULTS

Dosimetry

The doses cited in this paper were determined on the basis of averaging the results of 5 different dosimetric measurements: two with TLDs, 3 with radiochromic films. The deviation among the results of these methods was less than 10%. Our analytical calculations showed that the median beam energy was about 66 keV for the microbeams, and 130 keV for the broad beams. The corresponding dose rates for the 200 mA ring current were about 650 Gy/s and 9 Gy/s, respectively.

Our Monte Carlo simulations using the code EGS4 provided the spatial distribution with a precision of about 2 μm (Fig. 1). The simulation treated the egg as an ellipsoid, with a 0.6 mm thick calcified shell. The height of the 'valley' (i.e. the leakage of radiation to the regions between individual microbeams) in the center of the array and in the center of the egg is about 9% of the peak dose. The width of the valley, full width at half maximum (FWHM), was about 72 μm.

Radiation response

A total of 37 irradiated eggs (-5 per dose group) and 28 unirradiated control eggs were used. Of the irradiated embryos 76%, and of the unirradiated embryos 79% hatched. Microbeam irradiation at doses of 40- 160 Gy had no appreciable effect on the number of eggs hatched. At the high dose of 450 Gy the level of hatching was reduced to 33%. Irradiation with broad beam at doses of 6-18 Gy did not affect the incidence of hatching. The following are the numbers of the hatched embryos in each dose group: 5,4,3 and 2 in the microbeam groups of 40,80,120 and 450 Gy, respectively, and 3, 5 and 6 in the broad beam groups of 8,12 and 18 Gy, respectively.

Radiation damage was manifest in juvenile ducks as general weakness and lameness (ataxia). Ataxia was chosen as the biological endpoint for the following analysis. It was deftnedas the stage in which the animal is weak or lamed to the extend that cannot move around to reach food and water. The animal was then euthanized. Fig. 2a and 2b shows post-hatching, ataxia-free survival of the embryos irradiated with microbeams and broad-beams, respectively. The results indicate that the time of occurring of ataxia was dose-related in the cases of microbeam irradiated embryos. In the broad-beam irradiated embryos, ataxia occurred only in the highest dose group, i.e. of the 3 dose groups used, ducks in the 6 and 12 Gy groups were symptom t?ee, but ducks in the 18 Gy dose group all developed ataxia (Fig. 2b). It is evident

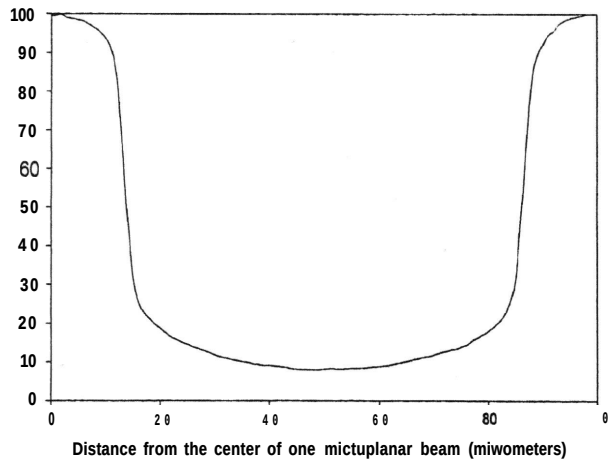


Fig. 1 Dose distribution calculated by the EGS4 Monte Carlo code for two adjacent microplanar beams in the

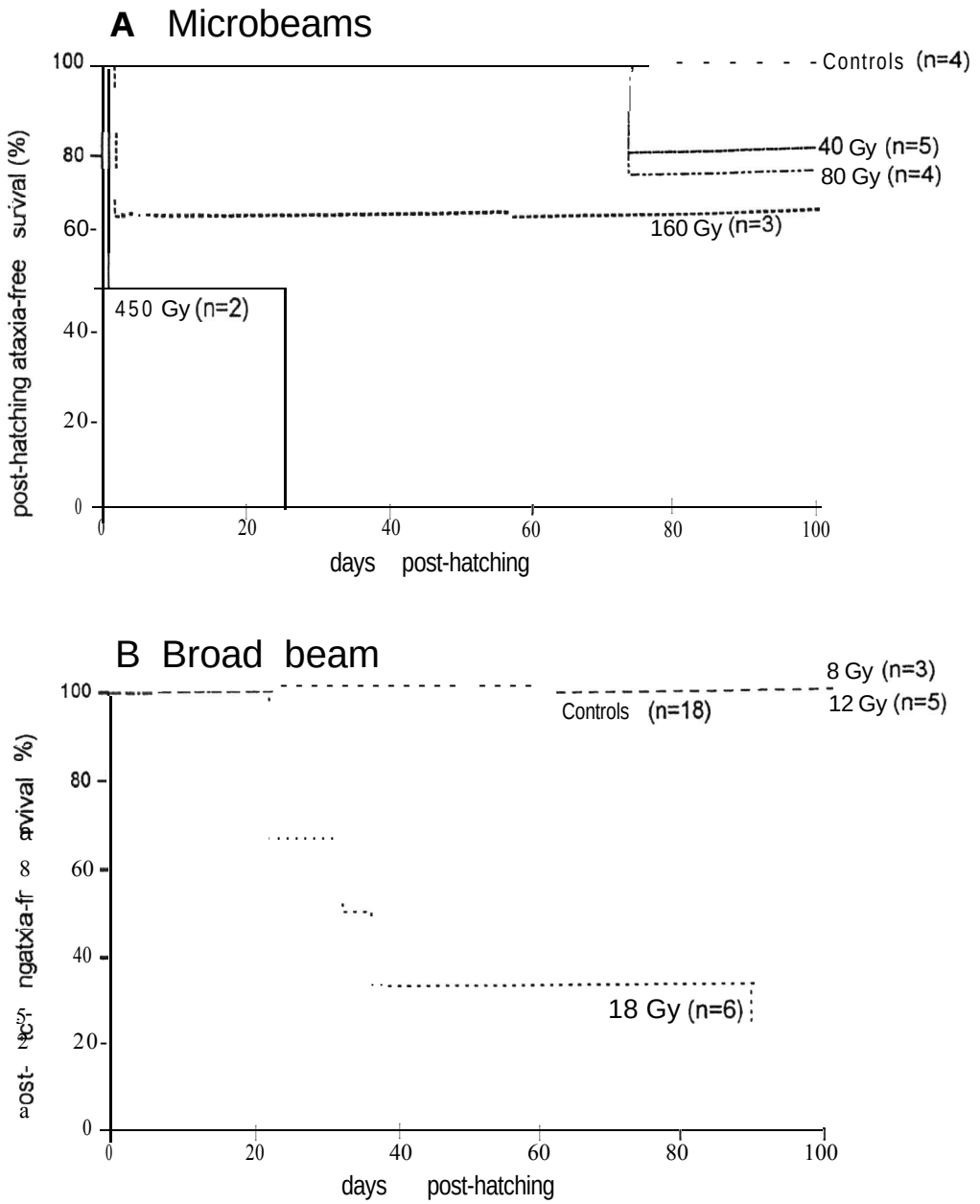
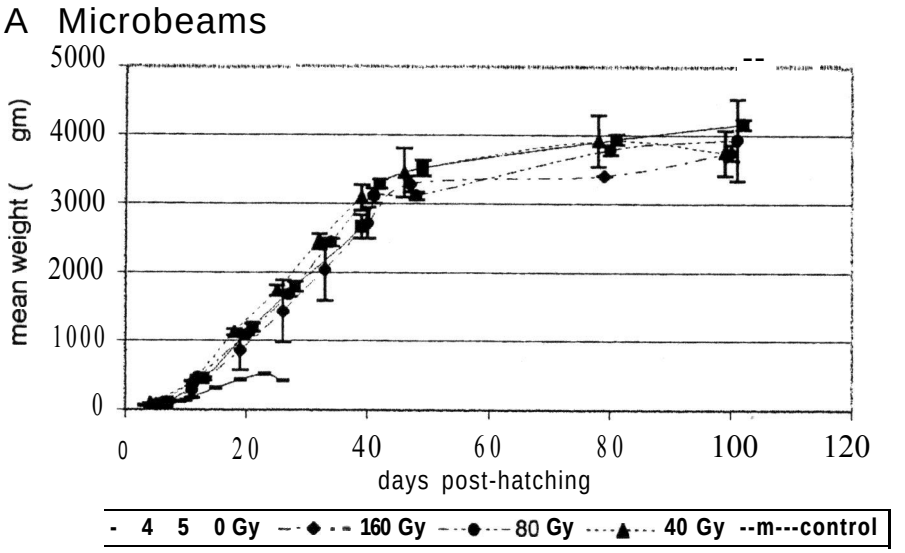


Fig. 2 Post-hatching time-related changes in the proportion of ducks surviving without ataxia in the microbeam and broad beam dose groups. A) microbeam; B) broad beam

from these preliminary results that considerably higher doses of radiation were tolerated by embryos irradiated using the microbeam modality, as compared with embryos irradiated with the broad beam.

As indicated above the ataxia-free surviving ducks in the broad-beam dose groups were euthanized at 98 days after hatching, while ducks in the microbeam and control groups were maintained until ~250 days after hatching. All cases of ataxia in the microbeam-irradiated birds developed before the age of 75 days (Fig. 2). All those ducks who survived the first 75 days without ataxia stayed ataxia free to the age of 250 days with the exception of two that were found dead, both from unknown causes; a duck irradiated with 80 Gy microbeams died on day 137 after



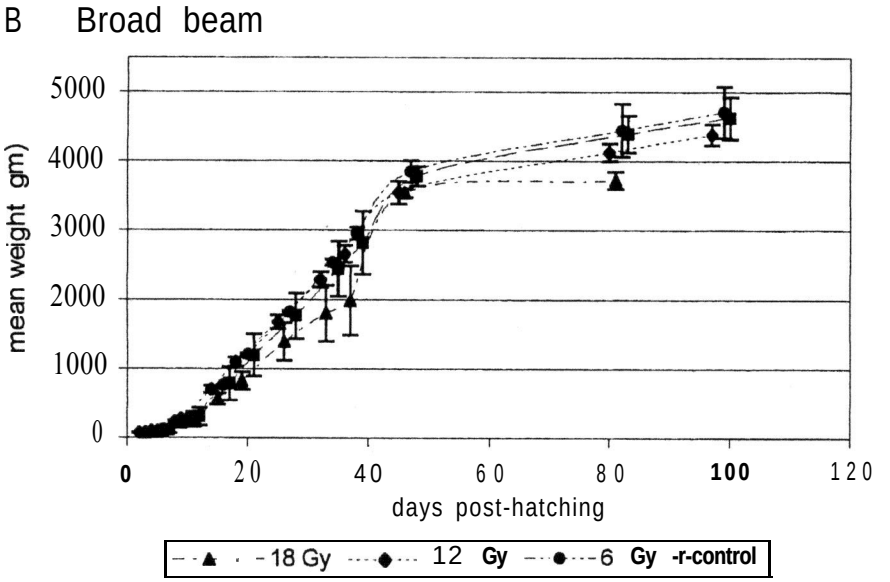


Fig. 3 Post-hatching time-related changes in the body weight of surviving ducks in the microbeam and broad beam dose groups. A) microbeam; B) broad beam

hatching, and a duck in the control group died on day 183 after hatching. The only body deformation in the 40-160 Gy microbeam groups was one duck in the 80 Gy group with deformed beak.

The irradiated ducks and the unirradiated controls were weighed periodically (Fig. 3). With the exception of the 450 Gy microbeam study, the weight gain profiles of irradiated and control animals were comparable. In terms of mean values, the rate of weight gain was dose related in both microbeam and broad-beam irradiated dose groups. However, with the exception of the highest dose groups (160- and 450-Gy microbeam and 18-Gy broad-beam) values did not differ significantly $p>0.05$ student t-test) from the controls (Fig. 3A, 3B). Standard behavioral tests, such as the level of spontaneous activity or avoidance response were not undertaken. However, periodic qualitative observations by Drs. William Dean and Tirath Sandhu of CUDRL did not reveal any behavioral abnormalities.

Comparing the microbeam and the broad-beam ataxia-free survival plots of Fig. 2A and 2B shows that the 18 Gy broad beam has been much more damaging to the brains

of the embryos than 160 Gy microbeam. From this comparison, it is evident that duck embryonic brain slices appear to tolerate radiation doses a factor of -10 higher from microbeams as compared with the broad beam modality. However, to compare the modalities directly, dose delivery from microbeams should be expressed as dose uniformly delivered to a unit volume of tissue (containing both the volumes of the microbeams and the interbeam volumes). For example, a dose of 160 Gy from the microbeam translates to about 45 Gy (i.e., 3.6-times lower) when expressed as dose delivered to a uniform volume of tissue. When the microbeam dose is normalized to the full volume, the duck embryo brains tolerate radiation doses -3 time higher from microbeams, as compared with broad beams.

Histopathology

No histopathological abnormalities were observed in the brains of ducks in the control group, in the lower dose microbeam group (40-160 Gy), and in the lower dose broad beam group (6- 12 Gy). Significant lesions, however, were found in animals in the 450 Gy microbeam dose group. Furthermore, two birds in the 18 Gy broad beam dose group had mild focal perivascular cuffing by lymphocytes in the cerebrum. Fig. 4a - 4c shows the brain lesions in the group irradiated with 450 Gy microbeams, all in the cerebrum. Fig. 4a shows severe focal perivascular cuffing by lymphocytes and mild scattered spongy change; Fig. 4b shows hyaline change in the vessel wall and swollen endothelial cells; and Fig. 4c shows vascular thrombosis of a vessel. Vascular pathology was found only at 450 Gy microbeams. All these vascular lesions were multifocal and severe; they did not have the spatial pattern of microbeams. Neuronal cell injury, characterized as vacuoles in the cytoplasm of neurons, also was observed in the brain of the bird given 450 Gy microbeams. Again, there was no geometrical pattern in the distribution of the damaged cells. A cerebral section of a control duck is shown in Fig. 5. Several ducks examined, including unirradiated controls, had multifocal pale areas of "cotton ball" appearance scattered throughout the brain (not present in Fig. 4 and 5), which we believe are probably artifacts due to the fixation or the processing methodsused,

DISCUSSION

The results of this study clearly indicate that duck embryonic brain tissue has a relatively high tolerance to microbeam irradiation, When the microbeam doses were normalized to allow for the spacing between the individual beams, the normalized doses indicate that duck embryo brain tolerates microbeam doses at least 3 times higher than broad beam doses. The primary biological end point used in the study was ataxia. Ataxia in the microbeam-irradiated ducks of 40, 80 and 160 Gy may have been the result of partial smearing of the microbeams due to minor movements of the embryos during irradiation (a typical irradiation time for the entire array of 160 Gy microbeams was about two min). The fact that a significant proportion of animals developed this neurological symptom, without obvious signs of radiation induced brain damage, suggests that development of locomotor centers in the duck brain may have been adversely affected by irradiation at an early stage of development. Similar findings have been reported after conventional X-irradiation of rat embryos, where a single dose of 3 Gy impaired rat locomotor-y coordination, without evidence of radiation induced lesions in the brain (1); locomotor impairment was evident by 40 days after birth.

Long-term follow up of the microbeam irradiated ducks confirmed that ataxia would show up within -75 days of hatching. As indicated above, only two ducks died during the 76 to 250 day follow-up period, both of unknown causes, a duck in the 80 Gy microbeam dose subgroup at 137 days after hatching, and a duck in the control group at 183 days. Others appeared to behave normally without evidence of neurological impairment.

The biological basis for high normal tissue tolerance to microscopically thin beams of ionizing radiation was first discussed by Curtis and colleagues (3,4). These authors irradiated mouse brain with a 25 μm wide deuteron beam (22 MeV). The beam, administered through a small

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Fig. 4 Photornicrographs of the cerebrum of a duck irradiated with the microbeam at the dose 450 Gy, and euthaniid on day 23. a) Shows severe focal perivascular cuffing by lymphocytes (large arrow) and mild scattered spongy change in the cerebrum (small arrows); b) Shows hyaline change in the vessel wall (large arrow) and swollen endothelial cells (small arrows), and c) shows vascular thrombosis in a vessel.

Fig. 5 Photornicrograph of the cerebrum of an unirradiated duck

incision through the skull, had a tissue penetration depth of about 1.5 mm. The threshold dose for brain damage (24 days post-irradiation) was found to be in the region of 4000 Gy. When the beam width was increased to I mm, the threshold dose decreased to~140 Gy. Curtis suggested that the probable reason for this high tissue tolerance to microbeams, was related to regeneration of radiation damaged blood vessel segments by surviving vascular endothelial cells located outside of the path of the microbeam (4). It was conjectured by Slatkin *et al.* (14) that oligodendroglial cells derived from surviving progenitor cells may also contribute to this regeneration effect. The enhanced tissue sparing is probably related, in part, to a 'volume effect', although other processes appear to be involved. It is well established in mainstream radiobiology that the threshold dose for normal tissue damage increases as the volume of tissue irradiated decreases (17). Sparing of normal tissues has also been noted using grid radiotherapy (8), but the grid effect is considerably less pronounced than that observed using microbeam modalities. The difference is presumably related to the fact that the X-ray beams used in grid therapy are typically 1 mm or more in width, and have considembly broader edges than synchrotron-generated microbeams.

In a recent study at the ESRF on two week old suckling rats, hind brains were irradiated with a parallel array of 28 μm wide synchrotron generated X-ray microbeams (7). The center- to-center spacing between the beams was either 105 μm or 2 10 μm. The peak skin entrance doses used were 50 Gy and 150 Gy. Over a 15-month observation period, none of the irradiated rats died or had to be euthanized due to neurological dysfunction.

Studies on adult rat brain have also indicated a remarkably high tolerance to microbeam irradiation. Brain necrosis was not observed at up to 60 days after irradiation with parallel arrays of 27 μm wide X-ray microbeams (75 μm center to center spacing), at skin entrance doses in the range 312 to 5000 Gy (14). A longer term study on rat brain, using 27 μm wide microbeams with 50 - 100 μm beam spacings, indicated no discernable damage at up to 500 days after irradiation (5).

A tumoricidal effect has been observed in rats bearing intracerebral 9LGS tumors irradiated with unidirectional arrays of parallel microbeams (5,6,15), and with two orthogonally crossed arrays of parallel microbeams (6,15). In the first study, the microbeam width and spacing was 27 μm and 100 μm, respectively. Unidirectional microbeams at 625 Gy at skin-entrance doses resulted in cure rates of -36%, while cross-fired array microbeams at 3 12 Gy and 625 Gy skin-entrance doses resulted in -55% cure rate. Histopathological analysis revealed no significant brain damage in the case of the unidirectional beam, but there was evidence of loss of tissue structure in the orthogonally cross-irradiated zones of normal brain. In the second study (5) microbeam width was the same 27 μm, while the microbeam spacing varied from 50 to 100 μm. In this study unidirectional microbeams at a skin-entrance dose of 150 Gy resulted in long-term survival (>120 days) in about 35% of rats bearing intracranial 9LGS tumors, and strong palliation otherwise. The biological basis of the tnmoricidal effect of unidirectional microbeams is unknown but it may be due, at least in part, to radiation-induced failure of the vascular regeneration process in the tumor.

CONCLUSIONS

The first conclusion derived from the described studies is that embryonic brain of the duck exhibited a high tolerance to irradiation with unidirectional microplanar X-ray beam arrays. Tolerance doses appeared to be lower than those observed for suckling rats using similar microbeam arrays by Laissue *et al. (7).* As indicated above, the difference could have resulted from the movement of the duck embryos in the shell during the irradiation. Species differences and/or differences in the developmental stage of the brain in the two studies, may also have been contributory factors. A probable explanation for the high tolerance to microbeam irradiation is the regeneration of the microvasculature from the endothelial cells that survive between the individual microbeams (4,14). A second conclusion is that the brain damage threshold from microbeams seems to depend only on the valley dose and not the peak dose. A third conclusion derived is that the findings of the present study support the rationale that MRT could have potential value in the treatment of CNS neoplasms in infants.

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