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HISTOLOGIC REACTION OF ADULT RAT SKIN AFTER 13 MEV PROTON IRRADIATION AND ITS VIABILITY IN CELL CULTURE

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February 1968

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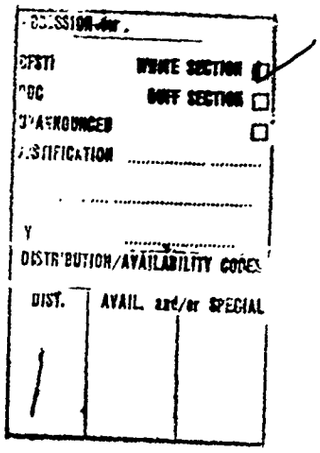

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FOREWORD

This study was conducted in the Biosciences Branch under task No. 775702 and was submitted as a thesis to the Graduate College of the Texas A&M University in partial fulfillment of the requirements for the degree of Master of Sciences in January 1966 by the senior author. The work was accomplished between May 1964 and February 1965. The paper was submitted for publication on 6 December 1967.

Radiation and dosimetry for this work were supplied under contracts AF 41(609)-1532 and AF 41(609)-2685, the latter being funded by the Commander, School of Aerospace Medicine. The work was accomplished by L. D. England, Texas Nuclear Corp., Austin, Tex..

The authors thank Dr. Stuart W. Lippincott and Major Harold W. Casey, for advice in making histologic diagnosis, and Llewellyn H. Mori, Airman First Class Franklin F. Wilson, and Airman First Class Paul A. Hughy, for their invaluable assistance in the laboratory.

The animals involved in this study were maintained in accordance with the "Guide for Laboratory Animal Facilities and Care" as published by the National Academy of Sciences–National Research Council.

This report has been reviewed and is approved.

GEORGE E. SCHAFER
Colonel, USAF, MC
Commander

ABSTRACT

The carcinogenic effects of low energy (13 Mev) protons on adult rat skin were studied in two phases: Phase 1—A pathologic study in which the oncogenesis of proton irradiation was determined eight months postirradiation. Phase 2—Cell culture experiments based on the hypothesis that growth and viability of adult skin cells irradiated in vivo and later cultured in vitro would reveal carcinoma potential before actual tumor formation could be detected.

In phase 1, one animal followed the anticipated pattern nine months postirradiation. It was biopsied for culture six times and in each instance gave a consistent growth in vitro. This animal later developed a basal cell carcinoma.

In phase 2, no animals provided viable cells in culture at intervals of 5, 30, or 60 days postirradiation. The interval between 60 days and eight months postirradiation unfortunately could not be followed in this study.

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I. INTRODUCTION

One of the most biologically unpredictable ionizing energies in the aerospace environment is that of the proton. These particles have a finite, energy-dependent range of penetration in tissue. Protons of low energy are of concern to man because of their absorption by tissues with release of energy at the terminal point of travel; thus biologic effects of this type of ionizing energy will be closely related to the malfunction of that tissue of the body in which it is deposited.

Lippincott et al. have published several articles in a series dealing with the field of particulate irradiation. They started with a general view of the accomplishments in the field beginning in 1947 (3). The review covered the therapeutic as well as basic tissue reactions. Their second article (4) was a detailed evaluation of carcinogenesis resulting from local proton irradiation of mouse skin. They pointed out that 10 Mev protons most often affected the epidermis, frequently causing epithelial hyperplasia.

The studies of proton effects by Lippincott et al. (3, 4) and a similar study undertaken by Harris (2) indicated that the amount of tissue alteration resulting from total skin proton irradiation appeared to be dose-related. Harris observed skin effects after 13 Mev proton irradiation as low as 200 rads with subsequent tumor formation. Scarring from ulceration and necrosis of the dermis with tumor formation were seen at 1,000 rads. The amount of tissue alteration appeared to be dose-dependent. A hypothesis was subsequently developed that the growth and viability of skin cells irradiated in vivo, removed by biopsy, and placed in cell culture would reveal this dose relationship at some point in time before neoplasms developed.

The ultimate objective of this study was to develop a cell culture technic for use as a biodosimeter in assessing neoplastic changes in adult skin after proton irradiation.

II. MATERIALS AND METHODS

Phase 1

At the termination of the Harris study in 1963 (2), Caraway evaluated the neoplasms from surviving rats receiving 13 Mev proton irradiations. All but one of these rats were sacrificed; rat 857 from the 200-rad group, having no visible tumors, was saved for cell culture studies.

Harris (2), using rats of the Sprague-Dawley strain, irradiated them simultaneously in groups of 10 in a "Ferris wheel" configuration around a cylindrical proton source. The flux varied along each rat by less than 10%. All irradiations were conducted in the Nuclear Physics Laboratory of the Texas Nuclear Corporation. Details of proton source, irradiation configuration, dose rate, and monitoring procedures are described in the appendix. Out of a total of 130 subjects, 32 survived to be sacrificed eight months later, as outlined in table I.

Histologic evaluations of neoplasms were made after routine formalin fixation of the specimens. The sections were stained with hematoxylin and eosin.

Phase 2

Subjects utilized in this series were 40 male Sprague-Dawley rats weighing approximately 270 gm. each. Individuals were randomly assigned to four groups of 10 each. Two rats from each group served as nonirradiated controls.

TABLE I

Harris rats irradiated with 13 Mev protons

Group	Dose in rads	Number surviving eight months postirradiation	Rats with tumor
I	101	1	None
II	202	2	One rat, basal cell carcinoma; the other, sebaceous gland adenoma.
XIII	300	3	None
III	406	2	None
XII	500	3	Squamous cell carcinoma
V	607	2	
XI	700	5	
VI	800	1	
X	900	3	
IV	1,001	2	
IX	1,100	1	
VII	1,200	5	
VIII	1,400	0	None
Control	None	2	None

Total dose for individuals in each group was as follows:

Group	*Dose in rads*
I	1,300
II	700
III	400
IV	200

Phase 2 followed the same irradiation configuration as phase 1.

Biopsy technic. A biopsy was performed on all subjects at 5, 30, and 60 days postirradiation. All subjects were anesthetized with a 1% solution of sodium pentobarbital administered intraperitoneally. The area of skin to be dissected was shaved and prepared using three 95% alcohol scrubs, allowing the skin to dry between each scrub. A 1-inch-square area of skin was dissected free and placed in Ringer's solution. Precut templates of adhesive tape were used to outline this area. The wound was then closed with number 0 silk.

The portion of skin in the sterile Ringer's balanced salt solution was vigorously shaken for about 1 minute. The wash solution was decanted and replaced with fresh solution. This washing was repeated with clean solution for a total of three times, effectively removing blood and debris and enhancing the chances for obtaining a sterile cell suspension.

Enzyme solution of bacterial collagenase. The enzyme solution was prepared in Krebs-Ringer phosphate solution free of calcium and magnesium. The solution was compounded in the laboratory using 500 ml. of 0.9% NaCl (commercial source), 0.23 gm. of KCl dissolved in 20 ml. of distilled H_2O, and 0.71 mg. of Na_2HPO_4 (anhydrous) dissolved in 0.6 ml. of 1 *N* HCl and then diluted to 100 ml. with distilled H_2O. The total volume thus obtained was 620 ml. The pH was 7.4. The powdered bacterial collagenase (300 mg.) was dissolved in 60 ml. of the above solution, giving a concentration of 5 mg./ml. After dialysis against the solvent for 2 hours at the room temperature, the preparation was sterilized by pressure filtration through a 0.45 μ Millipore filter. The final solution was placed in 10 ml. bottles, frozen, and stored until used.

Preparation of cell suspension. The preparation of cell suspension from whole skin was a modification of the Smith technic (7). Enzymatic removal of cells from the bioptic tissue

was accomplished by placing the *intact* bioptic material, epidermis down, in a sterile petri dish containing 10 ml. of enzyme solution and by incubating at 37° C. for 45 minutes. The skin was then turned over, and most of the cornified nonviable epidermal cells scraped off, using a rubber policeman. These cornified epithelial cells were removed from the enzyme by centrifugation and the enzyme returned to the bioptic material in the petri dish. A second incubation at 37° C. for one hour effectively loosened the germinal epithelial cells for removal with a rubber policeman. This cell suspension (in the enzyme) was removed with a pipet and filtered through nylon net filters of fine mesh to remove debris. The cells were then removed from suspension by centrifugation and washed twice in Smith's chick heart growth media.

Growth media. Chicken plasma was obtained by centrifuging unclotted chilled chicken blood obtained from the large wing vein without benefit of an anticoagulant.

Chick embryo extract was made using 18 eleven-day-old embryos. The embryos were forced through the tip of a sterile 50 cc. syringe into a sterile screw-capped bottle. An equal amount of 1× Hanks solution was added, mixed, and then quickly frozen in Dry Ice and acetone. After 24 hours, the mixture was rapidly thawed and then centrifuged in sterile tubes at 1,400 r.p.m. for 1 hour at 2° to 4° C. The supernatant was transferred to 10 cc. vials, refrozen, and stored for use as needed.

Smith's medium (7) was compounded in the laboratory as follows:

Parker medium 199 1×	40 cc.
Horse serum	5 cc.
Chick embryo extract	5 cc.
Hanks solution (commercial source)	50 cc.
Penicillin	400,000 units
Streptomycin	0.25 gm.

The pH of the medium was adjusted to 7.2, using $NaHCO_3$, and was sterilized by pressure filtration through a 0.45 μ Millipore filter.

Culture chamber. An exploded view of a Rose chamber (6) is presented in figure 1. This device holds 1 cc. of growth medium.

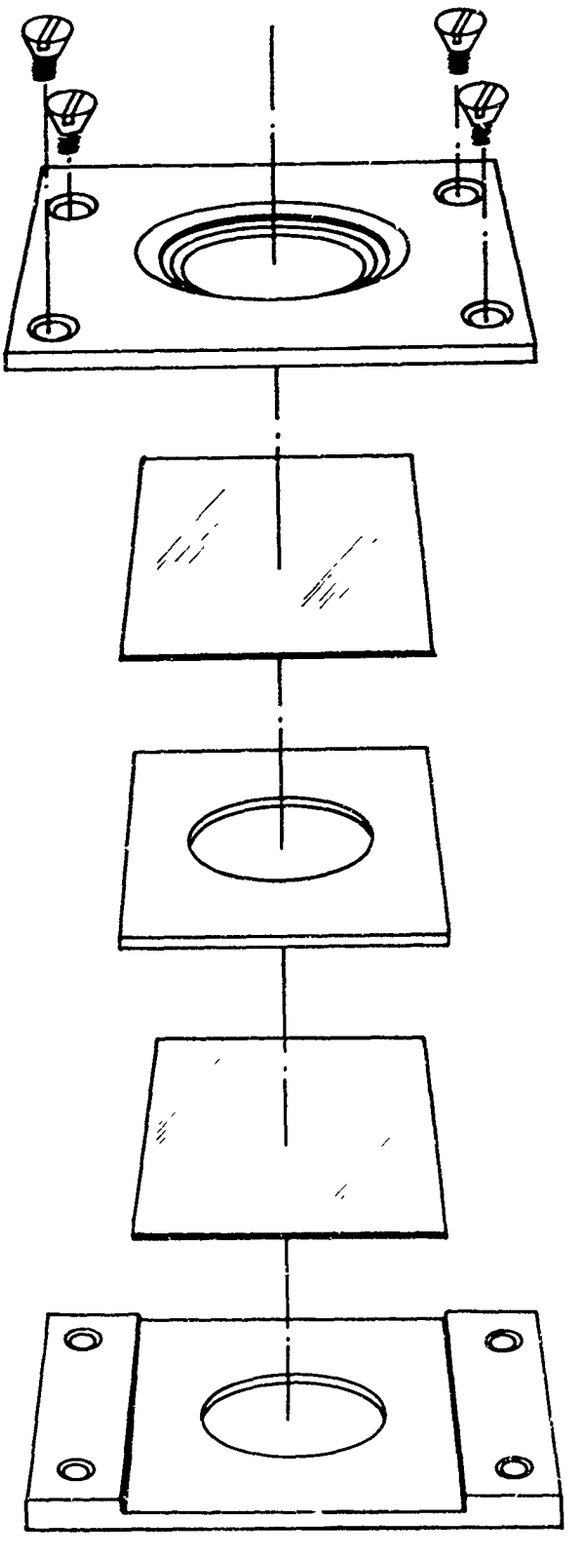

FIGURE 1
Fulton-modified Rose chamber.

Determination of cell viability. Viability was based on the observation of cells using time-lapse phase cinephotomicrography. This method was ideal for observing cell morphology during migration and mitosis.

Staining cells in a monolayer. Monolayers of skin cells, attached to the glass windows of the culture chambers, were fixed and stained, using the technic of Papanicolaou (5), which was developed for differentiation of epithelial cells found in vaginal smears.

III. RESULTS

Phase 1

The gross and microscopic appearance of rats from each dose group at the time of sacri-

FIGURE 2
Rat 822. A. *Gross appearance.* B. *Skin section.*

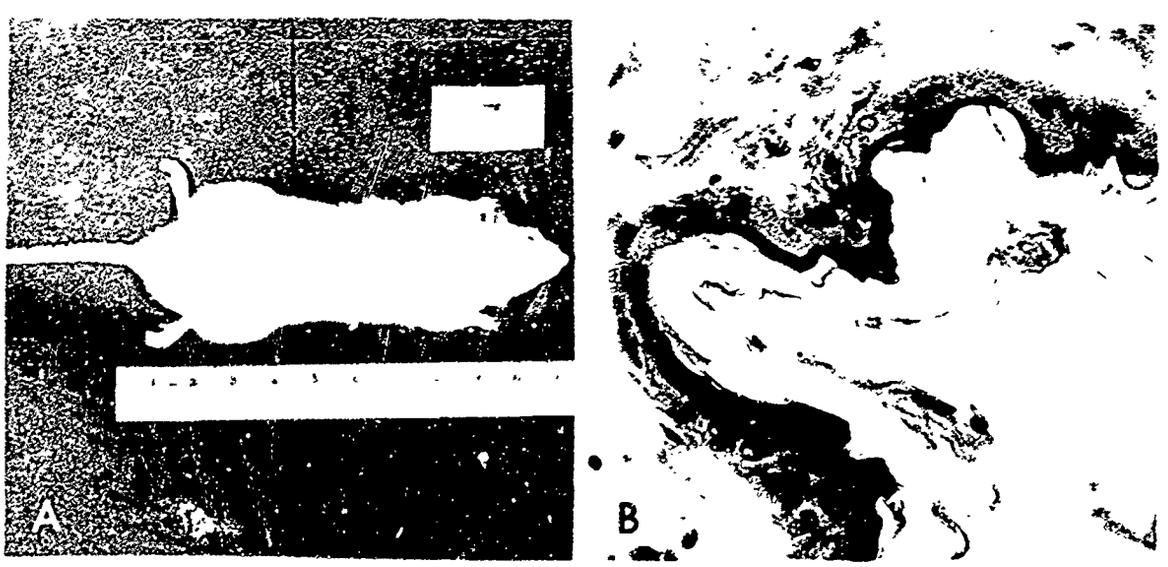

FIGURE 3
Rat 771. A. *Gross appearance.* B. *Skin section.*

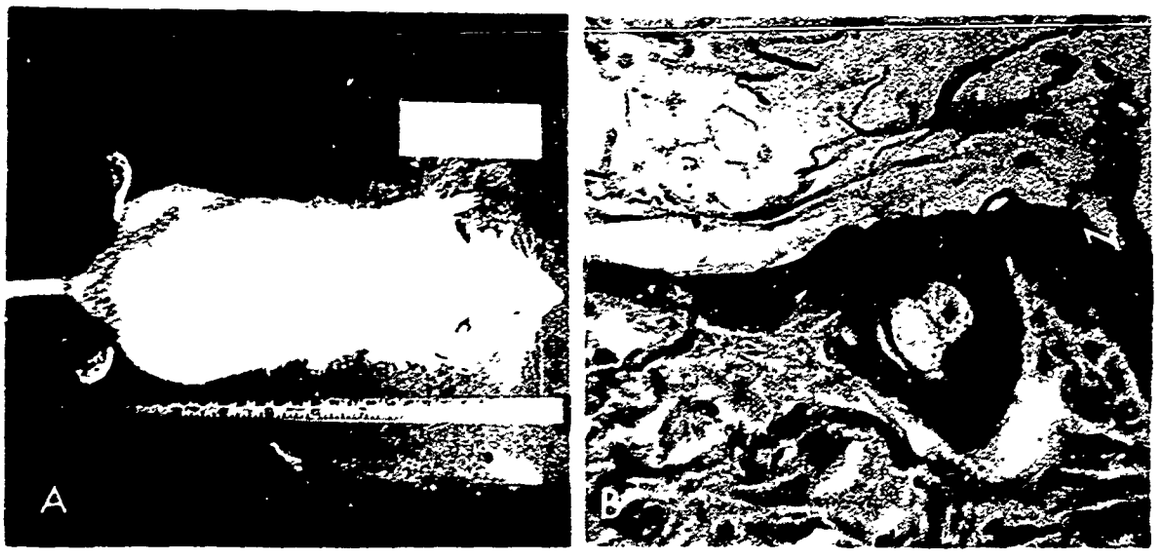

FIGURE 4

Rat 835. **A.** *Gross appearance.* **B.** *Skin section.*

FIGURE 5

Rat 865. **A.** *Gross appearance.* **B.** *Skin section.*

fice is presented in figures 2 through 13. Only one representative rat from each dose group is shown. Table I gives the histologic classification of these neoplasms. Figure 14 shows a skin section from a normal control rat. The absence of rete pegs in rat skin is normal. In other species that have been used in studies of skin pathology, loss of rete pegs postirradiation was considered evidence of damage.

Cultures are shown in figures 15 and 16. In figure 15, cells emerging from a micro-explant are seen. Note the broad flagstone

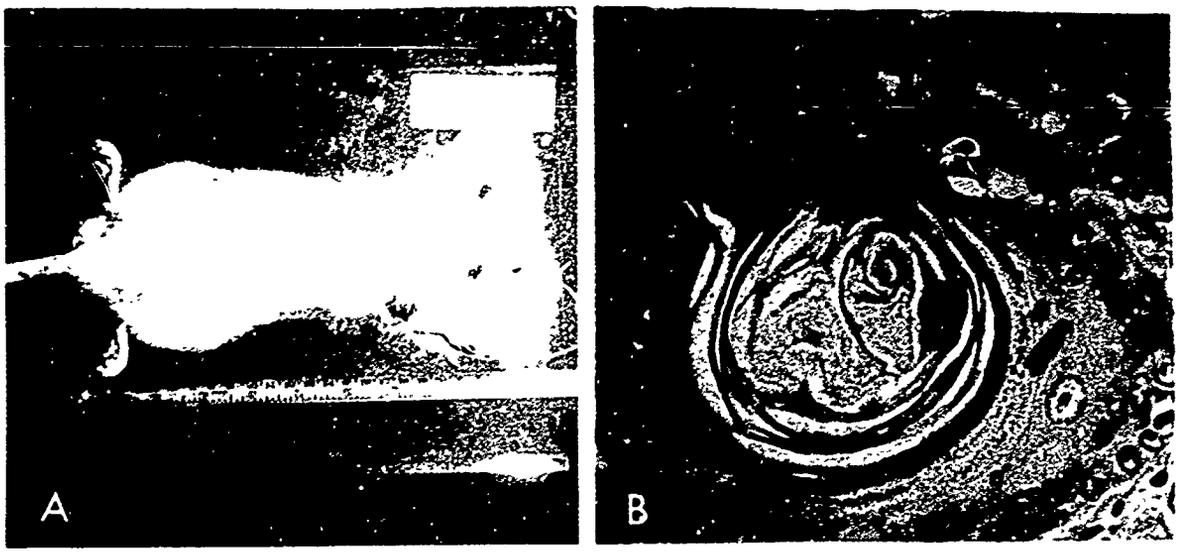

FIGURE 6

Rat 846. **A.** *Gross appearance.* **B.** *Skin section.*

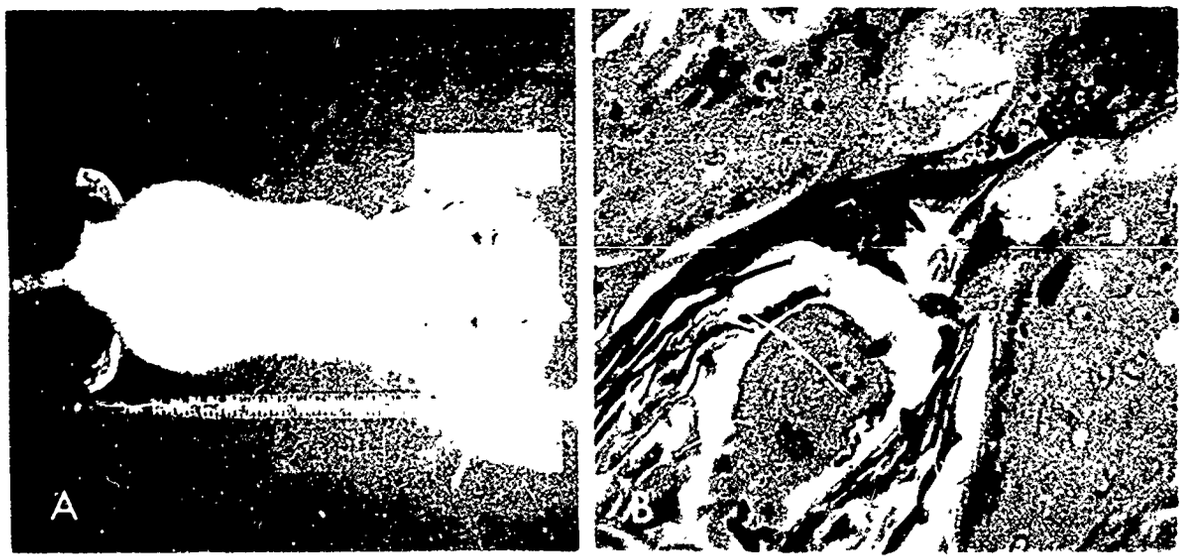

FIGURE 7

Rat 739. **A.** *Gross appearance.* **B.** *Skin section.*

appearance and multiple pseudopodia of the cells. The nucleus is well-defined and contains several clearly visible nucleoli. The nucleus and nucleoli stand out in stark relief under phase microscopy. In figure 16 individual cells are shown. By use of a skin section from a normal rat (fig. 14) as a reference, note the characteristic appearance of the nucleus of epidermal cells *in situ* as well as in culture. The growing culture was fixed and stained, using the technic of Papanicolaou (5). Cornification was demonstrated in the microexplant from which the broad cells emerged. The origin of the cells in this photograph appears to be from epithelial tissues, as indicated by the "Pap" stain.

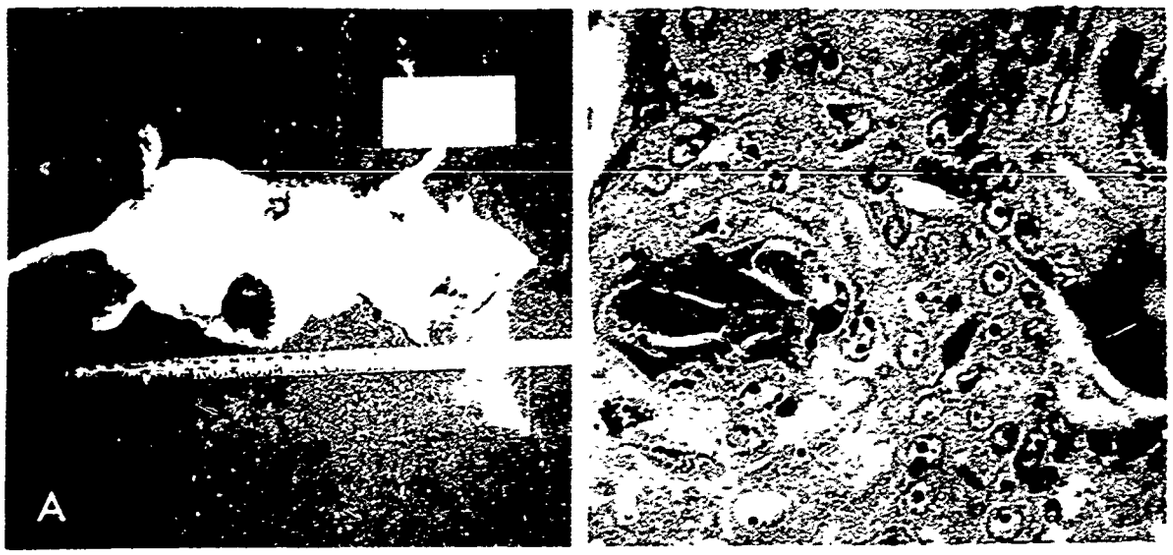

FIGURE 8

Rat 716. **A.** *Gross appearance.* **B.** *Skin section.*

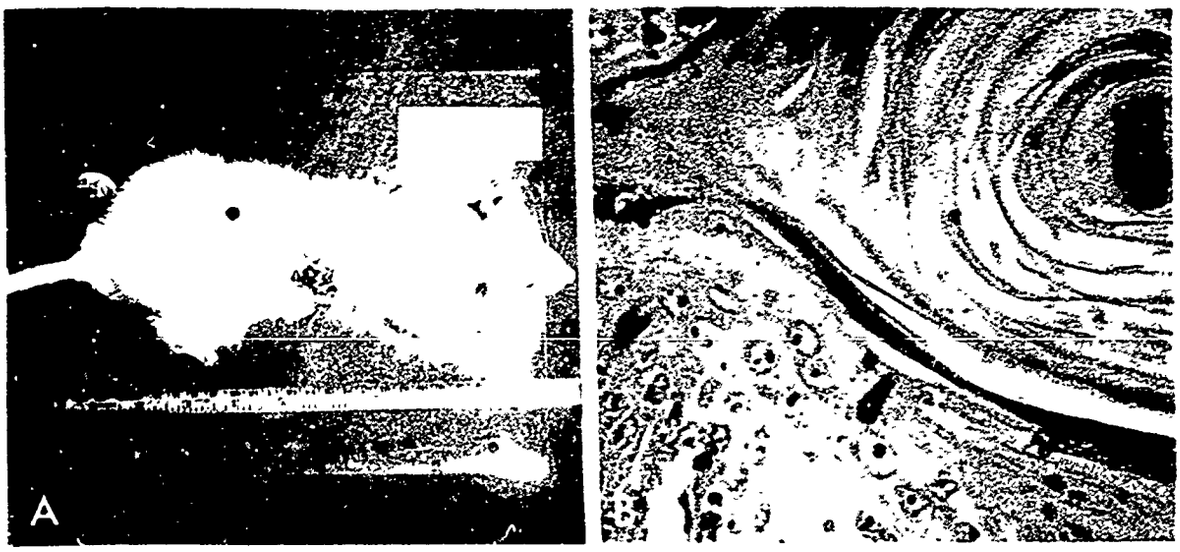

FIGURE 9

Rat 750. **A.** *Gross appearance.* **B.** *Skin section.*

Phase 2

Five-day biopsy. After a 6-day incubation period, no viability was observed in either the control subjects (nonirradiated) or the experimentals in any of the four irradiated groups. Chick embryo heart tissue placed in culture and serving as a media quality control demonstrated excellent viability. No changes were noted in skin sections examined histologically.

Thirty-day biopsy. Thirty days postirradiation, no viability was observed. Control chick embroy heart tissue demonstrated good

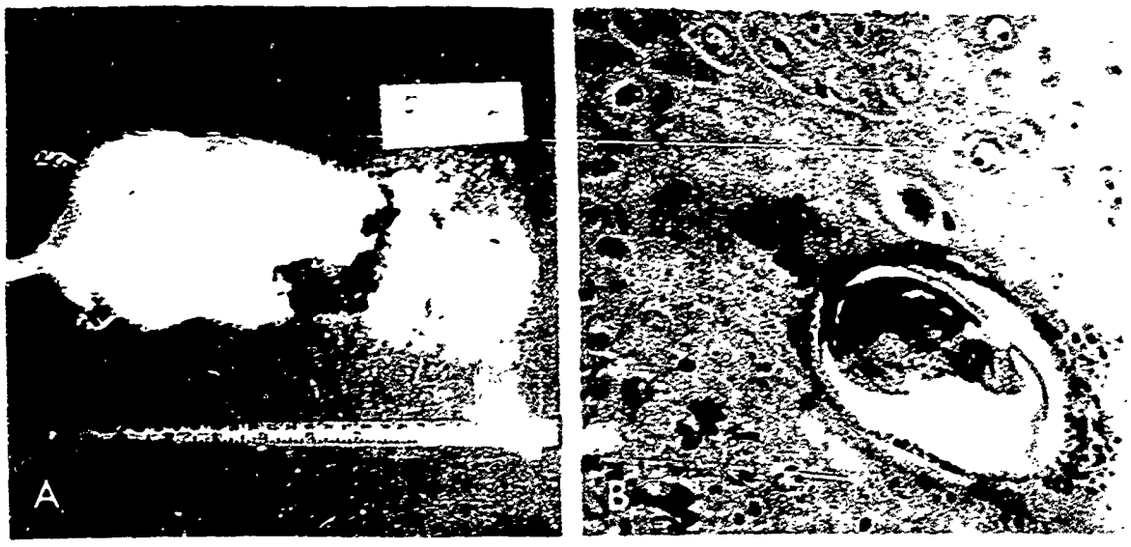

FIGURE 10

Rat 854. A. Gross appearance. B. Skin section.

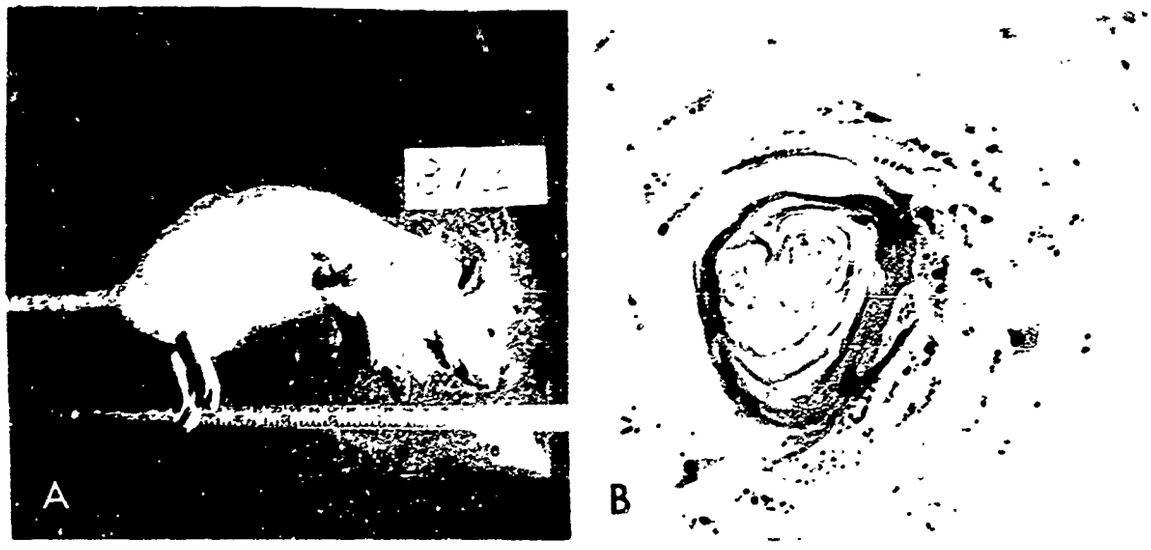

FIGURE 11

Rat 812. A. Gross appearance. B. Skin section.

growth. No changes were noted in skin sections.

Sixty-day biopsy. Skin cells failed to demonstrate any viability by 60 days postirradiation. Control chick heart tissue demonstrated excellent growth.

IV. DISCUSSION

Phase I

The results of this study and those of Lippincott et al. (4) indicate that the amount of

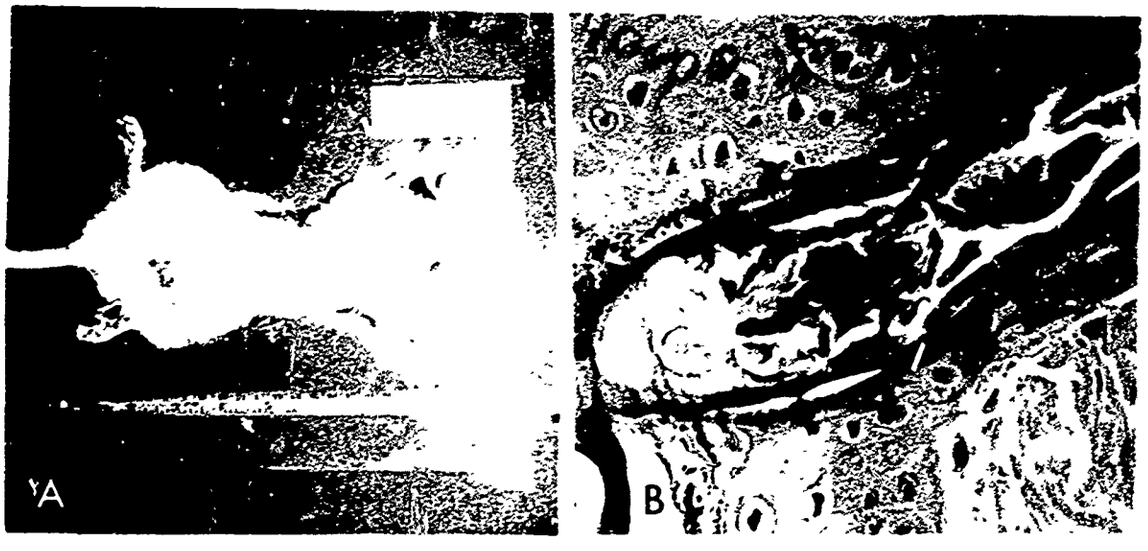

FIGURE 12

Rat 724. A. Gross appearance. B. Skin section.

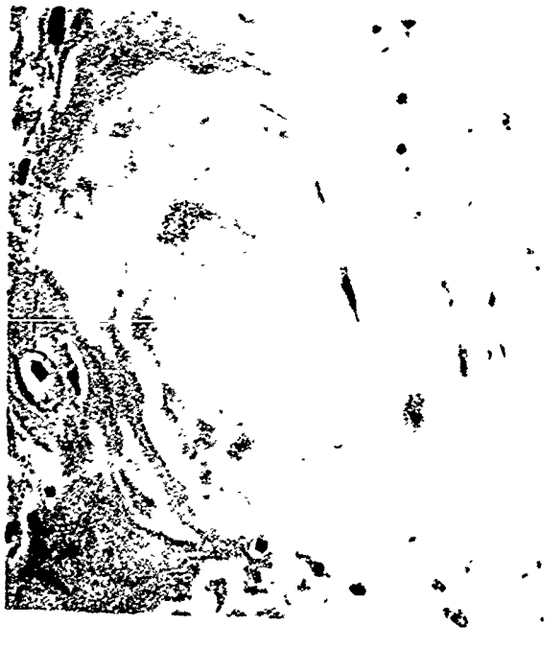

FIGURE 13

Rat 803. Skin section.

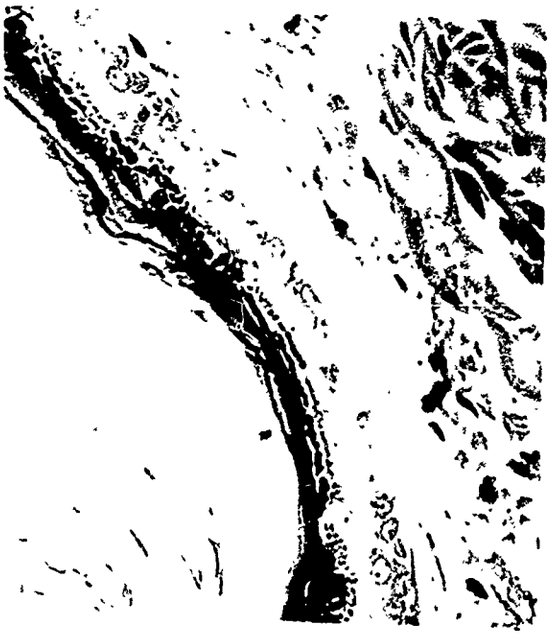

FIGURE 14

Rat 710. Skin section.

tissue damage resulting from proton irradiation may be dose-related. The fact that carcinoma appeared in surviving rats receiving 200 rads of proton irradiation may have been incidental; however, it did occur in this study and the possibility of space travelers developing carcinoma from exposures to low doses of proton irradiation does exist. After finding neoplasms at 200 rads, one wonders why no lesions were seen in the 300-rad dose group.

FIGURE 15

Viable cells.

FIGURE 16

Viable cells.

The three surviving rats developed no significant abnormalities. Epilation was the most prominent finding grossly in the 400-rad groups, with hyperplasia providing the most significant changes microscopically, showing an increase from the normal count of one to two cell-layers thick to an obvious count of four to five cell-layers thick. This observation coincides with that of Lippincott et al. (4).

Carcinoma was present in all groups surviving eight months which received an exposure dose of 500 rads or greater. The dermis was not involved except as a supporting structure for the invading epidermis. Extensive scar tissue formation was present at the site where sloughing had occurred in the groups given 1,000, 1,100, and 1,200 rads, respectively.

The degree of carcinogenesis was difficult to stipulate. The fact that neoplasms were present in those rats receiving more than 100 rads may be the only acceptable end point for this type of study. Death occurred within 30 days in all subjects receiving 1,400 rads.

The most striking feature of the neoplasms was the occurrence predominantly in one of three locations: posterior to the scapula, over the spinous processes between the scapulae, or over the spinous processes of the thoracic region. These locations were over bony prominences where the skin was stressed continually by movement. Lack of neoplasms over heavily muscled areas where less stress was applied to the irradiated skin seemed to add weight to a hypothesis of an accelerated rate of breakdown over the rate of repair in these bony areas. The concept of chronic irradiation as a prerequisite for carcinogenesis is not new and often is incriminated along with other stresses in combination as being carcinogenic.

In phase 1, skin biopsies from one irradiated rat consistently demonstrated good adaptability to life in a Rose chamber. Rat 857 never failed to provide viable cells. This rat was sacrificed two months after the last biopsy. A small ulcerating tumor had subsequently developed undetected in the fold of the flank and was removed for histologic evaluation along with three small tumors developing at the site of the last biopsy incision. A diagnosis of basal cell carcinoma was made on the tumor from the flank, and the other three

tumors were a mixture of a stitch-type inflammatory reaction and areas of epithelial cysts.

Russian investigators have shown a definite flux pattern in the mitotic index of not only bone marrow cells (red and white elements) but also lymph node cells after x-irradiation (1). An initial inhibition is followed by a return to normal and sometimes by an increase in the mitotic index well above that of controls. Rats, in particular, demonstrated this phenomenon. Many workers have already documented the ability of ionizing radiation to promote a neoplastic type of cell development in vivo. Neoplastic growth may be a sustained supercompensation reflecting previous trauma.

Phase 2

By use of cell culture technics, an attempt was made to find the point in time postirradiation that skin cells from irradiated subjects will develop neoplastic characteristics. Although culture of skin taken at intervals of 5, 30, and 60 days postirradiation did not grow, a long-term project of culturing irradiated skin at 30-day intervals for periods up to one year might reveal the point in time after which cells irradiated in vivo and then placed in culture would consistently grow.

In comparison, rat 857, from phase 1, consistently provided skin cells of good viability in culture. It contributed viable cells over a five-month period. The point in time at which this characteristic developed is still in question.

If nothing else, this study pointed out the possibility that rat 857 may be typical of subjects receiving this type of radiation. If this possibility does indeed exist, then it behooves the prudent space biologist to pursue this type of study with vigor.

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APPENDIX

13 MEV PROTON IRRADIATION OF RATS

14 January 1963

Radiation source

The protons for the irradiations described were produced with the Texas Nuclear Corporation 3.2 Mev Van de Graaf positive ion accelerator using the prolific He^3 (d,p) He^4 nuclear reaction. This reaction produces an 18.3 Mev proton; however, the net energy of the protons emerging from the accelerator was very nearly 13 Mev.

Irradiation geometry

The experiment was so designed that 10 rats would be rotated about the proton source at a fixed source to rat center distance. The rats were placed in thin-mesh aluminum screen cylinders which were freely suspended at one side of the cylinder wall. The wire mesh aluminum screen was 10 mils thick with 18 wires per inch so that 32.74% of the area was covered. The entire assembly was then rotated at 1 r.p.m. so that the whole surface of each rat was irradiated. The distance maintained from the center of the reaction diameter to the center of the rat was 12 cm. The variation of the proton flux along the length of the rats was about 10%.

Proton dosimetry

The general dose expression used in these computations was:

$$D\ (\text{rads}) = 1.6 \times 10^{-8} \left(\frac{\text{rad-gm.}}{\text{Mev}}\right) \frac{E_p}{R_p} \left(\frac{\text{Mev-cm.}^2}{\text{gm.-proton}}\right) \phi \left(\frac{\text{protons}}{\text{cm.}^2}\right)$$

where 1.6×10^{-8} results from the definition of the rad: 1 rad = 100 ergs/gm. = 6.24×10^7 Mev/gm.

E_p = Energy of incident protons in Mev/proton.
R_p = Range of incident protons in gm./cm.^{2}
ϕ = Integrated flux of protons per cm.^{2}

In our case:

E_p = 13 Mev (mean incident energy)
R_p = 0.19 gm./cm.^{2}

So that:

$$D\ (\text{rads}) = 1.094 \times 10^{-6} \left(\frac{\text{rad-cm.}^2}{\text{proton}}\right) \phi \left(\frac{\text{protons}}{\text{cm.}^2}\right).$$

The determination of the absorbed radiation dose resulting from the proton bombardment required careful monitoring during irradiation. The proton detector was a shielded CsI crystal optically coupled to a 6342A photomultiplier tube whose output was fed into an amplifier, pulse discriminator, and scaler.

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13 ABSTRACT

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