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ARMY MEDICAL RESEARCH LABORATORY

FORT KNOX, KENTUCKY

REPORT NO. 110
12 January 1953

THE INFLUENCE OF IONIZING RADIATION ON MITOSIS IN THE CORNEAL EPITHELIUM OF THE RAT *

* Subtask under Biological and Medical Aspects of Ionizing Radiation, AMRL Project No. 6-59-08-012, Subtask, Effect of Ionizing Radiation.

MEDICAL RESEARCH AND DEVELOPMENT BOARD
OFFICE OF THE SURGEON GENERAL
DEPARTMENT OF THE ARMY

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THE INFLUENCE OF IONIZING RADIATION ON MITOSIS IN THE CORNEAL EPITHELIUM OF THE RAT

1. Morphological changes induced by x-irradiation and the effectiveness of BAL and cysteine in reducing the frequency of these changes*

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Project No. 6-59-08-014
Subtask AMRL, S-1
MEDEA

ABSTRACT

THE INFLUENCE OF IONIZING RADIATION ON MITOSIS IN THE CORNEAL EPITHELIUM OF THE RAT

1. Morphological changes induced by x-irradiation and the effectiveness of BAL and cysteine in reducing the frequency of these changes.

OBJECT

To study the effects of x-irradiation on the mitotic process in the corneal epithelium of the rat.

RESULTS AND CONCLUSIONS

X-rays produce mitotic aberrations in the corneal epithelial cells of the rat. Two effects were observed: 1) a "physiological effect" produced in those cells which were dividing at the time of exposure and characterized by chromosome stickiness resulting in bridges at anaphase and telophase; and 2) a "structural effect" produced in the interphase or early prophase cells but becoming apparent only at division and characterized by chromosome fragments and chromosome bridges in the anaphase and telophase. The "structural effect" was demonstrated in Wistar and Sprague-Dawley rats, in young and adult animals, in animals exposed to local and total body irradiation, and in animals exposed to hard as well as soft x-rays. Cysteine instillation was observed to reduce the frequency of the "structural effects", but the instillation of BAL proved ineffective.

RECOMMENDATIONS

The effectiveness of different types of ionizing radiations (alpha, beta, neutrons, deuterons) in producing aberrant mitoses in the cornea should be investigated. A dose-effect correlation should be established for each type of radiation.

It would be of interest to determine if the frequency of the aberrations is related to the intensity of the x-rays.

Studies should be made to determine if the "physiological effect" can be influenced by chemical means.

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THE INFLUENCE OF IONIZING RADIATION ON MITOSIS IN THE CORNEAL EPITHELIUM OF THE RAT

1. Morphological changes induced by x-irradiation and the effectiveness of BAL and cysteine in reducing the frequency of these changes.

1. INTRODUCTION

Proliferating cells suffer certain insults from ionizing radiation. Alberti and Politzer (1) described abnormal mitotic figures in the cornea of the salamander as a result of irradiation. Strangeways (2) observed such aberrations in the chick fibroblast. Sax (3) has made extensive studies with Tradescantia describing the chromosomal abnormalities resulting from irradiation. Von Sallmann (4) reported mitotic aberrations in the lens epithilium of the rabbit following x-ray exposure. Such radiation insults have been described in a wide variety of plants and animals.

To date aberrations have not been described in the proliferating cells of the cornea of the rat following x-ray exposure. Von Sallmann (4) referred to the Friedenwald, Buschke, and Moses (5) paper in which these authors reported nuclear fragmentation in the cells of the basal epithelial layer of the rat cornea after exposure to various doses of ultraviolet radiation and after exposure to nitrogen mustard, but they did not observe such mitotic abnormalities as described by Lea (6) after exposing corneas to x-rays over the dose range from 50 to 2000 r.

The present report will describe the aberrant mitotic figures seen in the cornea of the rat following x-ray exposures and the effectiveness of BAL and cysteine in reducing the frequency of these aberrations.

II. EXPERIMENTAL

A. Apparatus and Methods

The cornea is a feasible tissue with which to work, and it affords an excellent source of somatic mitoses. The ease with which the cornea may be excised, subsequently stained and mounted bestows an advantage over many other tissues. The cells of the corneal epithelium are oriented in four or five layers which overlay the much thicker substantia propria. The two basal epithelial layers are mitotically active under normal conditions and the rate of mitosis is remarkably steady (7). The distribution of mitoses appears rather uniform with a slight excess in the periphery as opposed to the central area of the cornea. For most of the preparations flat mounts of whole corneas were used.

The histological procedure adopted for counting purposes was a modification of that described by Gay and Kaufmann (8). The fixative employed was a mixture of 3 parts glacial acetic acid and 11 parts 95% alcohol. Satisfactory fixation was obtained within a few hours. Since the limbus was more easily recognized in the unstained eye, the cornea was excised while still in the fixative by cutting circumferentially near the limbus. A small scleral flange was left attached so that the cornea could be manipulated to avoid damage to the epithelium. The cornea was freed of any adhering conjunctiva, iris or ciliary body tissue and transferred to 60% acetic acid for 3 minutes. It was then placed in a saturated solution of synthetic orcein in 60% acetic acid for 3 minutes, removed, rinsed in 60% acetic to remove the excess stain and cleared in 95% alcohol. Four or five radial incisions were made in the cornea to facilitate flattening. The cornea was then mounted as a flat preparation in a gum sandarac medium which was miscible with 95% alcohol.

For photographic purposes a single cell layer was desirable. Corneas were subjected to the Feulgen reaction, then squash preparations were made and mounted in Canada balsam.

The animals used for most of the study were adult Sprague-Dawley male rats weighing from 200 to 275 grams. One group of adult male Wistar rats of the same weight range was used to check any possible difference in response due to genetic variables. One group of 6 week old Sprague-Dawley rats was used to determine the possible effect of age. The rats were maintained on identical diets and, in general, were subjected to the same environmental factors and handling routines.

All the animals were sacrificed by decapitation in the early afternoon. This was followed quickly by enucleation of the eyes. The eyes were rinsed briefly in saline and transferred immediately to the fixative. Since a maximum time of only 1/2 minute elapsed from the time of sacrifice until the eye reached the fixative there was little chance for the mitotic activity to be disturbed by excitation.

Most of the animals were exposed to hard x-rays. The radiation factors were: 200 kv, 6 ma, 1 mm Al + 1/2 mm Cu filtration, with an output of 40 r per minute at a 30 cm distance. Only one group of animals was exposed to soft x-rays. For the latter a thin beryllium window tube was used and operated at 10 ma and 50 kv with an output of about 500 r per minute at a 40 cm distance.

B. Results

1. Morphological Changes

Several animals were exposed to 300 r and sacrificed at intervals from 2 1/2 minutes to 90 minutes after the exposure. There was a steady decrease in mitoses from 2 1/2 minutes until 75 minutes

were observed to contain the bridges. In some of the bridges there appeared to be an uneven distribution of the chromosome substance, and many chromosomes appeared to be stretched. In some cells "lagging" chromosomes were observed; in others there appeared a "ball" of chromosome substance, or Feulgen-positive material, attached to its pole only by a thin strand. These early effects of the radiation will be termed "physiological effects" (6).

Characteristic morphological changes were observed in many of the dividing cells 24 hours after irradiation, recognized as chromosome fragments and chromosome bridges in the anaphase and telophase stages of division (Fig. 2). No "stickiness" characteristic of the "physiological effect" was observed this late after exposure. These late effects of the radiation will be termed "structural effects" (6).

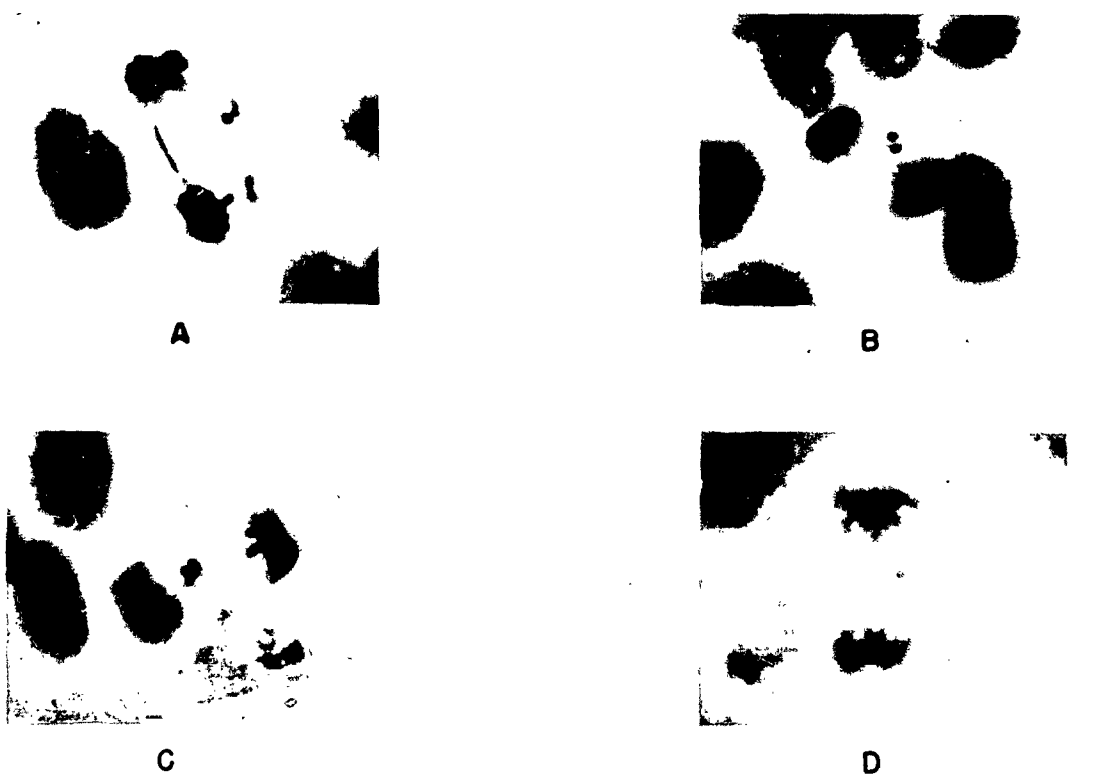

FIG. 2 - MORPHOLOGICAL CHANGES INDUCED BY X-RAYS IN THE CORNEAL EPITHELIUM OF THE RAT. THE 24 HOUR EFFECT ("STRUCTURAL")

(a) Telophase with bridge and acentric fragments.

(b) Telophase with acentric fragments.

(c) Telophase with acentric fragments.

(d) Telophase with acentric fragments and a "lagging" chromosome.

following the irradiation, after which there was an absence of dividing cells. Sixty minutes after exposure only a few telophases were visible. Corneas examined within the 60 minute period (15, 35, and 45 minutes after exposure) contained dividing cells which exhibited morphological changes (Fig. 1). The chromosomes of these cells appeared agglutinated and "sticky" which in many cases resulted in bridge formations at anaphase. Several anaphase and telophase stages

FIG. 1 - MORPHOLOGICAL CHANGES INDUCED BY X-RAYS IN THE CORNEAL EPITHELIUM OF THE RAT. THE EARLY EFFECT ("PHYSIOLOGICAL")

(a) Telophase with two bridges. The chromosome substance of the bridges appears evenly distributed.

(b) Telophase with uneven distribution of the chromosome substance along the bridge. To the left of the bridge is a "ball" of chromosome substance connected to the main mass by a thin strand.

(c) Telophase similar to (b) with an unevenly distributed bridge and a "ball" of chromosome substance.

(d) Telophase with a "lagging" chromosome.

The "structural effects" were demonstrated over the dose range of 100 to 1000 r. Some animals were exposed to hard x-rays and some to soft x-rays. All animals were sacrificed 24 hours later. In both groups the aberrant mitoses were obvious. The effect was manifested in young animals as well as in adult animals. To test the possibility of the existence of a difference in strains of animals in the production of these anomalies, a group of Wistar and a group of Sprague-Dawley rats each received 300 r. The animals were sacrificed 24 hours later and in both groups aberrant mitoses were present.

The possibility of the production of some substance(s) during total body irradiation capable of invading the cornea and executing "structural effects" led to the conception of the following experiments. In one group of animals the body was shielded and the head exposed to 300 r. The reverse experiment also was performed, shielding the head and exposing the body. In another experiment only one eye was irradiated, receiving 500 r; the second eye served as a control. In all the experiments the animals were sacrificed 24 hours later. Mitotic aberrations were noted in the exposed eye, whereas the unexposed eye appeared normal. The aberrations were pronounced in the animals whose bodies were shielded and heads exposed but there was no observable effect in those whose heads were shielded.

2. BAL and Cysteine

Both BAL and cysteine have been used in attempts to "protect" against radiation. These agents were employed to determine whether they were effective in reducing the frequency of "structural effects" produced in the cornea by x-irradiation.

One drop of a 10% solution of BAL* in peanut oil, pH 7.0, was instilled into each eye of several rats, followed in 5 minutes by a second instillation. The animals received 200 r immediately after the second instillation. Untreated irradiated, untreated non-irradiated and treated non-irradiated controls were included in each experiment. All animals were sacrificed 24 hours later. The mitotic counts were based on the percentage of morphological aberrations per total telophases counted. In most cases 200 telophases were counted per cornea but in some instances there were not 200 telophases to be found in the cornea (Table 1). Only the treated and untreated irradiated groups are included in Table 1, for mitotic aberrations were not observed in the non-irradiated groups. Under the conditions of this experiment BAL afforded no significant decrease in the number of morphological changes produced by the irradiation.

* Delta Chemical Works, 23 W. 60th St., New York 23, N. Y.

TABLE 1

THE INFLUENCE OF BAL ON THE FREQUENCY OF 'STRUCTURAL EFFECTS' INDUCED BY X-RAYS IN THE CORNEA OF THE RAT

All animals received 200 r and were sacrificed 24 hours after exposure.

Total Telophases Counted		Number Abnormal		Percent Abnormal	
A*	B**	A	B	A	B
184	200	61	62	33.2	31.0
200	200	56	72	28.0	36.0
158	200	58	78	36.7	39.0
125	200	42	73	33.6	36.5
Mean				32.88	35.63
Standard Deviation				±3.12	±2.90

P >.1 t = 1.117

*A - with BAL

**B - without BAL

The cysteine was instilled into the eyes from a stock 200 mg/cc solution in distilled water adjusted to pH 7.0. The instillations and the exposures were performed in the same manner as with BAL, using comparable controls. All the animals were sacrificed 24 hours later. However, before the cysteine could be instilled, it was necessary to employ a wetting agent; a 1% solution of methyl cellulose in distilled water, pH 7.0, sufficed. The counts were based on the percentage of morphological changes per total telophases counted (Table 2). As in the BAL experiments, no aberrations were observed in the non-irradiated control groups so these groups are not included in Table 2. The data reveal a significant decrease in the frequency of aberrant mitoses in those animals treated with cysteine prior to the irradiation.

TABLE 2

THE INFLUENCE OF CYSTEINE ON THE FREQUENCY OF 'STRUCTURAL EFFECTS' INDUCED BY X-RAYS IN THE CORNEA OF THE RAT

All animals received 200 r and were sacrificed 24 hours after exposure.

Total Telophases Counted		Number Abnormal		Percent Abnormal	
A*	B**	A	B	A	B
200	200	49	81	24.5	40.5
200	175	67	63	33.5	36.0
200	200	77	66	38.5	33.0
200	200	59	95	29.5	47.5
200	200	47	94	23.5	47.0
200	200	41	101	20.5	50.5
200	200	57	110	28.5	55.0
200	200	61	98	30.5	49.0
Mean				28.62	44.81
Standard Deviation				±8.14	±7.08

P <.01 t = 3.976

*A - with cysteine

**B - without cysteine

III. DISCUSSION

In agreement with Friedenwald's observations (9), sixty minutes after irradiation only a few telophases were observable in the cornea. Seventy-five minutes after exposure there were no mitoses, indicating the onset of a mitosis-free interval. This mitosis-free interval resulted from the fact that the interphase or early prophase cell, upon irradiation, suffered a delay and could not enter division for some hours or days later, the length of the delay being dependent upon the dose. On the other hand those cells which had passed the "critical" stage continued through mitosis and could form two daughter cells. The measured time after irradiation until the absolute disappearance of mitosis should give some indication of the mitotic time. Buschke, Friedenwald, and Fleischmann (7) determined the mitotic time in the cornea of the rat, using mitotic "poisons", to be of the order of 70 minutes. The results reported here are in practical agreement. The exposure time was 7 1/2 minutes and at 60 minutes after irradiation only a few telophases were visible, just 67 1/2 minutes from the beginning of the exposure until the virtual disappearance of mitosis. The mitoses examined during the 60 minute period following irradiation had already entered division at the time of the irradiation. Many of these figures suffered a disturbance characterized by an apparent stickiness of the chromosomes resulting in bridges at anaphase and telophase (Fig. 1a, 1b, & 1c). Lea (6) states that cells in division at the time of irradiation suffer a "physiological" change, characterized by a "stickiness" of the chromosomes so that they adhere where they happen to touch; furthermore, only those cells in division at the time of irradiation display this "physiological" disturbance. Fragmentation has been reported in cells examined at anaphase in experiments in which only the "physiological effect" of radiation is expected. Most authors attribute these fragments to the mechanical breakage of chromosomes proceeding to opposite poles at anaphase, but stuck together as a result of the "physiological effect". Very rarely were chromosome fragments observed in the cornea during the 60 minute period after exposure. What often appeared to be a fragment was probably a manifestation of the uneven distribution of the chromosome substance, a "ball" of chromosome substance still attached to a thin strand (Fig. 1b & 1c). The distribution of the chromosome substance appeared uneven in many of the anaphase and telophase bridges, perhaps an effect of the stress exerted by the respective poles resulting in a "stretching" of the chromosome (Fig. 1b & 1c). A "lagging" chromosome may be due to the mechanical breakage of a bridge (Fig. 1d).

Friedenwald (9) has shown that in 24 hours after a 200 r exposure the corneal epithelium had recovered from the mitosis-free interval. Dividing cells studied 24 hours after irradiation were in all probability in the interphase or early prophase at the time of irradiation. Aberrations observed in these figures were probably the result of radiation insults to the interphase or early prophase cell. Corneas examined 24 hours after exposure contained abnormal mitoses characterized by chromosome fragments and bridges in the anaphase and telophase configurations, an effect, presumably, quite distinct from the "physiological effect". These anomalies could have resulted from chromosome or chromatid breaks inflicted upon the interphase or early prophase cell by the radiation. Lea (6) has termed this effect a "structural change". Extensive work with favorable cytological tissues (Sax, K., allium root tips (10); Carlson, J. G., Chortophaga neuroblasts (11); Marquardt, H., Bellevalia romana pollen grains (12))has indicated that only those cells which were in the interphase or early prophase at the time of irradiation suffered "structural effects".

X-rays induced "structural effects" in the corneas of both Wistar and Sprague-Dawley rats. This effect was manifest whether young or adult animals were exposed, whether the rats were irradiated with hard or soft x-rays or whether total body or local irradiation was employed.

BAL has been used successfully by some investigators (13, 14) to prolong the survival of animals after exposure to radiation. Friedenwald (9) showed BAL to be effective in reducing the length of the mitosis-free interval suffered by the cornea after x-ray exposure. Von Sallmann studied the influence of BAL on x-ray cataract and reported that BAL was totally ineffective in increasing the radio-resistance of sensitive elements of the lens (15). In the present investigations BAL was ineffective in reducing the frequency of "structural effects" brought about by irradiation. The counts were scored on the percentage of abnormal telophases per total telophases counted. In most cases 200 telophases were counted per cornea but in three corneas 200 telophases were not to be found. To what this decrease in total number of mitotic figures might have been attributed can only be conjectured. The animals were exposed to the same experimental conditions. Perhaps it was due to individual variation in metabolism or viability. It would not seem to have been an effect of the BAL, for the treated non-irradiated controls did not display a decrease in mitosis.

Cysteine, on the other hand, proved effective in reducing the frequency of "structural effects". Von Sallmann (4) reported cysteine

to be effective in reducing the frequency of aberrant mitoses observed in the lens epithelium of the rabbit. The fact that cysteine does reduce the frequency of "structural effects" raises the question as to whether cysteine induces restitution of broken chromosomes or actually affords a degree of "protection" to the chromosome against breakage by radiation. This problem is of great interest and continued thorough investigations may elucidate the mechanism(s) of radiation-induced morphological changes in dividing cells.

IV. CONCLUSIONS

X-rays produce mitotic aberrations in the cells of the corneal epithelium of the rat. Two effects were observed: 1) a "physiological effect" produced in those cells which were dividing at the time of exposure and characterized by chromosome stickiness resulting in bridges at anaphase and telophase; and 2) a "structural effect" produced in the interphase or early prophase cells but becoming apparent only at division, and characterized by chromosome fragments and chromosome bridges in the anaphase and telophase. Investigations of the "structural effect" resulted in the demonstration of this effect in Wistar and Sprague-Dawley rats, in young and adult animals, whether the animals were exposed to local or total body irradiation, and whether exposed to hard or soft x-rays. Cysteine instillation was observed to reduce the frequency of the "structural effects", but the instillation of BAL proved ineffective.

V. RECOMMENDATIONS

The effectiveness of different types of ionizing radiations (alpha, beta, neutrons, deuterons) in producing aberrant mitoses in the cornea should be investigated. A dose-effect correlation should be established for each type of radiation.

It would be of interest to determine if the frequency of the aberrations is related to the intensity of the x-rays.

Studies should be made to determine if the "physiological effect" can be influenced by chemical means.

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