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SUMMARY UPDATE OF THE BROOKHAVEN TRITIUM TOXICITY PROGRAM WITH EMPHASIS ON RECENT CYTOGENETIC AND LIFETIME-SHORTENING STUDIES

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ABSTRACT: A number of years ago a multiparameter program to evaluate the toxicity of tritiated water (HTO) was undertaken in the Medical Department at Brookhaven National Laboratory. The results of most of these studies have been published and will receive brief attention. Emphasis will be placed on the unpublished studies involving the induction of sister chromatid exchanges (SCE's) in bone marrow of mice, new biochemical information, and preliminary results on lifetime-shortening and carcinogenesis. In brief, male Hale-Stoner Brookhaven (HSB) mice maintained on HTO concentrations ranging from 3.0 to 30.0 μCi/ml exhibited essentially the same number of SCE's per cell throughout their lifetime. Control mice showed a decrease in number of SCE's with age. The lack of a dose-response effect and the constant level of SCE's in HTO mice as compared to controls will be discussed. In the carcinogenesis study C57BL/6J male mice received various x-ray or HTO regimens. Mortality data from these and other studies in which CBA/Ca/BNL mice received single x-ray exposures or equivalent integrated dose exposures by single HTO injections will be discussed.

INTRODUCTION

The justification for evaluating biological effects and the related health physics of tritium has been well-documented in the general scientific literature and at the several workshops dedicated to this subject, including the last two in Chiba (1,2). In considering future studies it should be noted that much of the descriptive (dose-effect) studies have been done and it is the more detailed and mechanistic evaluations of tritium interactions in biological systems that still remain of interest and present some unanswered questions.

In the early 1970's, a program was begun in the Medical Department at Brookhaven National Laboratory to investigate tritium toxicity (TRITOX). What started as a proposed evaluation of the induction of dominant lethal mutations (DLM) in mice maintained on tritiated water (HTO), grew to a multiparameter program spanning many years. The scope of this program was widened through the energetic contributions of a number of collaborators including three Japanese colleagues who made significant contributions. Space limitations do not allow

descriptive details concerning all aspects of this work. Therefore, where work has been published, references describing materials and methods are cited and the results summarized. References are also cited next to the program outline presented on this page.

MATERIALS AND METHODS

Mice Breeding and Maintenance

With the exception of some lifetime-shortening and carcinogenesis studies, all animals used were of the Hale-Stoner-Brookhaven (HSB) strain. This is an albino strain which has been maintained in a single colony in the Medical Department at Brookhaven National Laboratory for more than 25 years. Animals are fed Purina laboratory chow (Ralston-Purina Company, St. Louis, Missouri) ad libitum and given tap water acidified to pH 2.4. In an attempt to reduce possible variations related to litter order, or to a specific female, only first litter animals from each breeding pair were used for the TRITOX studies. Animals are removed from the breeding colony at four weeks of age and placed in smaller animal rooms and divided into experimental groups. The tritium facility consists of two large rooms with high air exchange rates and a continuous monitoring system for measuring tritium in the room air. To insure that no cross-contamination took place between animals maintained on tritium and their controls, some control animals were maintained in the tritium facility while identical littermate control animals were maintained in separate animal rooms. No difference was noted between the control groups in different rooms and barely detectable amounts of tritium could be measured in the blood or urine of control animals maintained in the tritium facility.

BROOKHAVEN TRITIUM TOXICITY PROGRAM

I. GENETIC AND REPRODUCTIVE EFFICIENCY
- A. Dominant Lethal Mutation Rate (3-6, 12, 18, 21)
- B. Cytogenetic Studies (8, 27)
- C. Examination of Ova and Early Embryos (24)

II. SOMATIC EFFECTS
- A. Growth (Body Weight) (12)
- B. Nonspecific Lifetime Shortening (2)
- C. Bone Marrow Cellularity and CFU-S Content (10-12)

III. RELATIVE BIOLOGICAL EFFECTIVENESS (RBE)
- A. Comparison of HTO and ^{137}Cs Effects (12, 17, 18, 20)

IV. BIOCHEMISTRY AND MICRODOSIMETRY STUDIES
- A. Rate of Tritium Incorporation (13-16, 20, 24)
- B. Site of Tritium Incorporation (13-16, 20, 24)
- C. Rate of Tritium Disappearance - Enhancement (2)
- D. Histone and DNA Turnover Studies (15)
- E. Cellular Turnover Studies (2)
- F. Microdosimetry (22)

V. CARCINOGENESIS
- A. Induction of Leukemia

(References to subject listed in bibliography)

GENETIC, CYTOGENETIC, AND REPRODUCTIVE EFFICIENCY STUDIES

Dominant Lethal Mutations (DLM) Rate Studies

Experimental groups were maintained on 0.0, 0.3, 1.0 and 3.0 μCi/ml HTO. The testing methods and statistical analysis of data are described in ref. 3-6.

Cytogenetic Studies

Liver Cytogenetic Studies

The regenerating liver technique was used to measure the possible induction of chromosome aberrations by HTO in animals maintained on 0.0,0.3, or 3.0 μCi/ml HTO. Animals were evaluated by A. Brooks after approximately 90, 330,500-560, and 700 days exposure (7).

Induction of Sister Chromatid Exchanges (SCEs) in Bone Marrow Cells

In the initial studies carried out by Ikushima (9), male HSB mice were maintained on 0.0,and 3.0 μCi/ml HTO and their bone marrow evaluated at selected times for the induction of SCEs, using the method of Schneider et al. (8). In subsequent studies carried out under the direction of Tezuka, using the same methods, male mice were maintained on 0.0,3.0,7.5,15.0 and 30.0 μCi/ml HTO and evaluated at various times throughout their lifespan.

Micronuclei Evaluation in Red Blood Cells

Male HSB animals were given HTO (0.0,3.0,7.5,15.0 and 30.0 μCi/ml) beginning at 3 weeks of age. After 5-6 weeks blood samples were drawn and the red blood cells evaluated for micronuclei. These studies were carried out in collaboration with R. Tice.

SOMATIC EFFECTS

Growth (Body Weight)

Six groups of 20 male and 20 female animals were selected from the breeding colony at weaning. One male group and one female group were placed on the 3.0 μCi/ml HTO regimen while identical groups were placed on tap water in the tritium facility. The remaining two groups on tap water were placed in a separate animal room. Animals were weighed monthly and comparisons made among groups.

Non-Specific Lifetime Shortening

Initial studies were done on groups of 200 HSB mice maintained on 3.0 μCi/ml HTO together with age-matched controls. Cages were checked daily for deaths and animals examined individually weekly. Gross appearance and time of death were recorded on all animals.

Bone Marrow Cellularity and Colony-Forming Unit-Spleen (CFU-S) Content

Methodology for these determinations has been described in ref. 10-12.

Relative Biological Effectiveness (RBE) Studies

Because of the considerable debate concerning the assignment of a correct RBE or "Q" factor for tritium exposure in the form of HTO, portions of the TRITOX study involved a comparison of the effectiveness of HTO ingestion and equivalent exposures from external ^{137}Cs gamma rays. To accomplish this, the geometry of a gamma-ray radiation facility was arranged so that the depth-dose within the peritoneal cavity of exposed mice, as measured by implanted thermoluminescent dosimeters, was equal to the average daily dose rate to soft tissue in animals maintained on either 0.3 or 3.0 μCi/ml HTO for the same periods. DLM's were then measured.

In addition to the studies on continuous lifetime-exposed animals, one group of CBA/Ca/J mice received a single injection of HTO, which resulted in an integrated dose of 250 rad, and was compared to a group which received a single exposure of 250 rad of 250 kVp x-rays. These animals were observed throughout their lifetimes.

BIOCHEMISTRY AND MICRODOSIMETRY STUDIES

Rate of Tritium Incorporation and Disappearance

Methods for determinations of tritium content of tissues, cells and subcellular constituents have been previously described by Commerford et al. (2,13-15).

METHODS:

For comparison with the Commerford results (2), in recent studies data were obtained by repeated sampling (0.1 ml) of blood from individual mice following injection of sufficient HTO to produce comparable levels of ^{3}H in body fluids. Globin was prepared from the samples by the acid-acetone technique of Anson (25). The acetone was removed by methanol wash. The globin was dissolved in a small amount of water and detergent-type scintillation solvent added for ^{3}H determination.

Tritium Excretion Enhancement Studies

These studies involved the measurement of tritium excretion in animals maintained first on HTO and then given tap water or a variety of solutions containing artificial sweetners to stimulate the animals to greater ingestion of fluid. Details on the techniques used can be found in ref. 2.

LIFETIME-SHORTENING AND CARCINOGENESIS STUDIES

A well-documented late effect in mammals following exposure to ionizing radiation is the development of leukemia and other neoplasms. The two most common mouse strains used for studies of this type are the C57BL in which the induction of lymphomas predominate, and the CBA mouse in which acute myelogeneous leukemia together with hepatomas are common findings. Studies using both strains of mice have been undertaken in the TRITOX program and in a collaborative leukemogenesis program with E. P. Cronkite and V. P. Bond (Brookhaven National Laboratory). In the first of these studies, male CBA/Ca/BNL mice received either a single 250 rad, 250 kVp x-ray whole body exposure at a dose rate of approximately 90 rad/min. Matching animals received single i.v. HTO injection to give the same integrated dose. The treated animals together with age and sex-matched controls were then observed a minimum of twice daily throughout their lifetimes. When an animal died or was killed in a moribund state, the entire carcass was fixed in 10% buffered formalin for later detailed autopsy and histopathologic evaluation.

In the second study, C57BL/6J male mice were exposed to various regimens of HTO by injection or ingestion or to different regimens of x-ray exposure. For animals receiving three HTO injections, the dose was calculated on the basis of the integrated dose, but for the animals ingesting HTO it was calculated on the basis of the equilibrium reached by constant ingestion of HTO. X-ray exposures were at a dose rate of approximately 90 rad/min. Dose regimens are given in the Results section and Figures 2 and 3.

RESULTS

GENETIC, CYTOGENETIC AND REPRODUCTIVE STUDIES

Dominant Lethal Mutation Studies

All of the DLM studies (0.3,1.0,3.0 μCi/ml) are completed and published (2,12,16). Effects were seen in animals maintained on 1.0 and 3.0 μCi/ml HTO.

Cytogenetic Studies

In the regenerating liver studies, animals maintained on 3.0 μCi/ml HTO for 100,330 and 500-560 days exhibited a significant increase in the number of abnormal cells in the regenerating liver as compared to controls. Similar effects could not be measured in animals maintained on 0.3 μCi/ml. Details of this work have been published (7).

Bone Marrow SCEs

The SCE levels in femoral bone marrow cells of male mice maintained on 3.0 μCi/ml of HTO for 28 to 261 days were always higher than those in the age-matched control groups. In this work carried out by Ikushima (9), using a one-way analysis of variance and co-variance, the probability that the mean of all the control data is not different from that of the exposed animals is less than 0.0001. Ikushima also measured the average generation time for the bone marrow cells and found no difference between the exposed and controls.

In the subsequent study, in which males were exposed to 0.0,3.0, 7.5,15.0 and 30.0 μCi/ml, similar results were found. In all cases the animals on HTO showed a higher number of SCEs than controls; however, the various dose groups did not differ significantly from each other. Also, the control data were found to be best fitted by a curve showing a relatively sharp decrease in number of SCEs before the age of puberty followed by a continued but lower rate of decrease (Figure 1). In subsequent studies not yet complete, female animals have been maintained on 0.0,3.0,7.5,15.0 and 30.0 μCi/ml HTO in a manner similar to the previously examined males. The results of these experiments should give an indication of whether hormonal changes at puberty have an effect on SCE number, particularly in the control group. In addition, males have been maintained on 0.0,0.3,1.0 and 100 μCi/ml HTO to determine whether or not a difference in dose response might be obtained at these levels. Figure 1 shows the average of the SCEs for all animals maintained on 3.0 to 30.0 μCi/ml HTO and of their controls.

In none of the groups was there noted a significant effect of HTO exposure on cell generation times which is in agreement with Ikushima (9).

In another study in which mice were maintained on 3.0 μCi/ml of HTO for six months and then placed on tap water, the number of SCE/cells showed a moderate decrease following the termination of HTO ingestion (2).

Micronuclei Studies in Red Blood Cells

No further work has been done on the micronuclei cytogenetic studies other than the pilot study reported at the previous workshop in Chiba (2) which showed increases in micronuclei in mice maintained on 15.0 and 30.0 μCi/ml. These studies still need verification using larger groups of animals.

SOMATIC EFFECTS

The major portion of this work has been published (12). To summarize:

Growth and Body Weight

Continuous lifetime ingestion of 3.0 μCi/ml of HTO or equivalent external gamma exposure caused no measurable effect on growth as measured by body weight.

Lifetime Shortening

There was no difference between the animals ingesting 3.0 μCi/ml HTO and those receiving an equivalent external gamma exposure, nor was there any measurable effect on lifetime shortening in either experimental group as compared to control animals maintained on tap water.

Data is now available on CBA/CaJ mice which received either a single injection of HTO giving an integrated dose of 250 rad or a single x-ray exposure of 250 rad. As seen in Figure 2, there was no significant difference between the two exposures in terms of mortality. However, both groups differed from the control animals as would be expected from a single radiation exposure of this magnitude. These animals were exposed as part of the carcinogenesis study (see below).

Similarly, all of the more than 3000 animals in the C57BL/6J mouse carcinogenesis study have now died and the mortality data is available. As shown in Fig. 3, our results agree with the previous work of Kaplan (18) showing that three x-ray exposures of 175 rad, 8 days apart, were the most effective in inducing lifetime shortening. The next most effective regimen was the 3 HTO injections at 8-day intervals each giving an integrated dose equivalent to 175 rad, but at an obviously different dose rate than the x-ray exposure which was given at a rate of approximately 90 rad/min. The third most effective regimen was the single x-ray exposure of 525 rad followed by the least effective treatment, tritium ingestion at a concentration which gives a dose of 525 rad over 100 days (5.25 rad/day).

Bone Marrow Cellularity and CFU-S Content

Details of these studies have been previously published (12,19), showing decrease in marrow stem cells, but normal total cellularity.

Other unpublished studies in this laboratory have indicated that sublethal doses of ionizing radiation, capable of killing stem cells, stimulate more of the resting CFU-S pool into proliferation and thus maintain the normal cellularity within the marrow with a reduced total number of CFU-S.

Relative Biological Effectiveness (RBE)

In all studies completed to date where the radiation dose rate of exposure was equivalent, no significant difference ($p > 0.01$) was seen between ^{137}Cs gamma rays and internal tritium. However, there were some differences, as noted in Figure 3, in early mortality between x-rayed animals receiving the three 175-rad exposures at 8-day intervals and the animals receiving equivalent integrated dose injections of HTO.

BIOCHEMISTRY AND MICRODOSIMETRY STUDIES

The major information on amount and site of incorporation and turnover have been published (13,14,16,20,22,24).

RESULTS:

The biochemical data previously reported (2) were the result of a pilot study. The bizarre two-step disappearance curve of Hughes (2) could not be repeated. More reliable data are shown in Figure 4. Because of the small sample size, the data are less precise than those of Commerford (2), but, nevertheless, provide additional information to aid in interpreting his results. First, the discovery of a reversibly labeled component explained the initial rapid loss of label, where no loss was expected for the first three days during which time labeled cells continued to enter the blood at constant specific activity. Thereafter, cells should enter with continuously decreasing activity since they received label from a pool of tissue proteins which was rapidly turning over. The long tail in Commerford's data which fits an exponential with a $T_{1/2}$ of 11-13 days is best explained by the death of cells labeled after HTO had left the animal. Broken lines on the graph suggest how these factors would affect the true disappearance curve. The curve for single injection has been fitted to the points with these factors in mind. Unfortunately, there is insufficient data to determine the true life span, so the usually accepted value of 40 days has been assumed, although this is obviously at variance with the value of 25 to 28 days indicated in Commerford's data.

Carcinogenesis

The studies on the induction of leukemia and other neoplasms are complete to the extent that all animals have now died. The details on the mortality patterns have been discussed in the previous section under the heading of Lifetime Shortening. The histopathology has not yet been done and therefore

no statement may be made at this time concerning the cause of death or the relative effectiveness of tritiated water in causing leukemia, other neoplasms, or specific organ damage leading to death.

DISCUSSION

It appears from the information gathered to date that it is possible to detect somatic, cytogenetic and genetic effects resulting from exposures at concentrations as low as 1.0 and 3.0 μCi/ml which are 33 and 100 times the MPC's for HTO. Similar effects can be induced from exposure to external ^{137}Cs gamma rays at equivalent doses and dose rates.

The results of the SCE experiments are of particular interest. They indicate that, while ionizing radiation has been shown not to be an efficient inducer of SCEs, even at very low dose rates some as yet unknown mechanism causes a significant effect on the number of SCEs in animals maintained on 3.0-30.0 μCi/ml of HTO as compared with animals maintained on tap water. In this case the effect is one of maintaining the constant level of SCEs with age by the ingestion of HTO as opposed to the reduction in the control animals. The lack of a dose response over a factor of 10 in concentration is also fascinating and is as yet not understood.

It is possible that there are two mechanisms involved in the production of SCEs, one of which may be effective at very low doses where the radiation inhibits a repair mechanism, which is normally involved in reducing the number of SCEs with age, while the second is sensitive to very large doses of radiation (10s to 100s of rad), causing sufficient DNA disruption.

The marked difference in the mortality curves for three 175 rad exposures at 8-day intervals and three HTO injections at 8-day intervals giving the same integrated dose demonstrate what one might expect on the basis of a dose-rate effect. However, until the histopathology is completed and the cause of death verified for differing treatments, including the single 250 rad exposures, it is unwise to suggest possible mechanistic differences in effect resulting from the different types of exposure.

The reduction of bone marrow stem cells in animals maintaining normal total marrow cellularity levels is of interest since it demonstrates the animal's reaction to an effect at the primitive cell level by recruiting cells from the G_o resting state to compensate for the initial effect. As predicted on the basis of established principles of radiobiology, exposure to tritium

beta rays from HTO ingestion results in measurable effects on several animal systems.

The biochemical data indicate the need for additional studies to determine whether the short life span of red cells in mice ingesting HTO is an effect of chronic irradiation or is a peculiarity of this strain (HSB) of mice. The data also suggest a method for obtaining more quantitative measurement on the retention of tritium in humans since the technique is readily adaptable to human blood and could be used to estimate the retention in a person accidentally ingesting as little as one millicurie of HTO. The dynamic relationship between tritium metabolites in tissues and hemoglobin could be further elucidated using small amounts of HTO in a large primate and comparing levels of bound tritium in the hemoglobin with biopsy samples of selected tissues.

For further discussion of the overall results of the TRITOX program (and tritium effects in general) see the previously published references 20-23.

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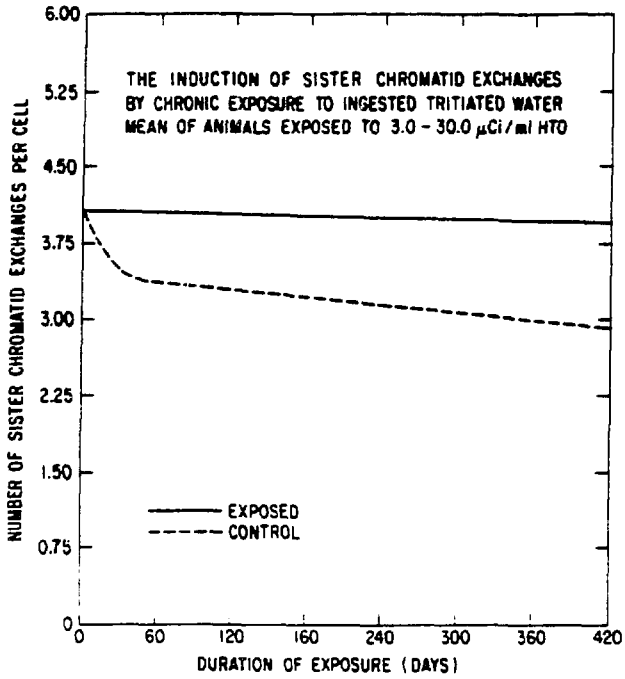


FIGURE 1

FIGURE 2

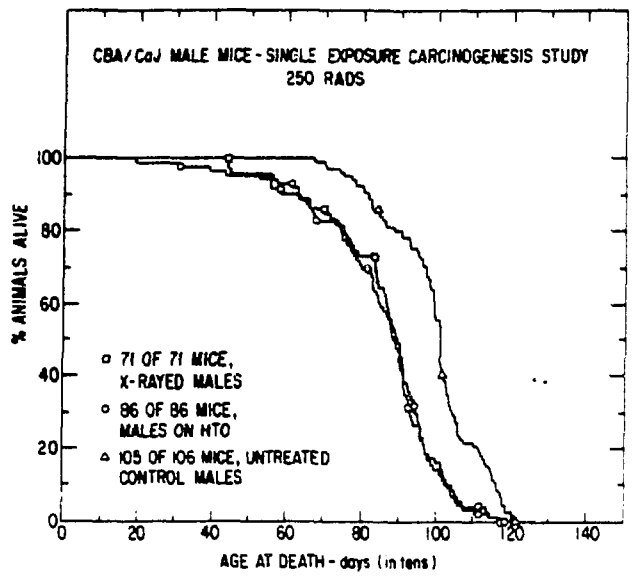
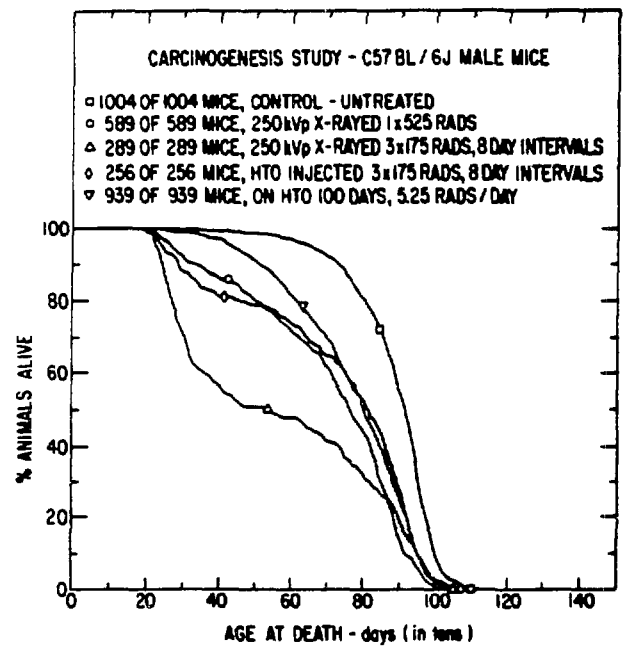


FIGURE 3



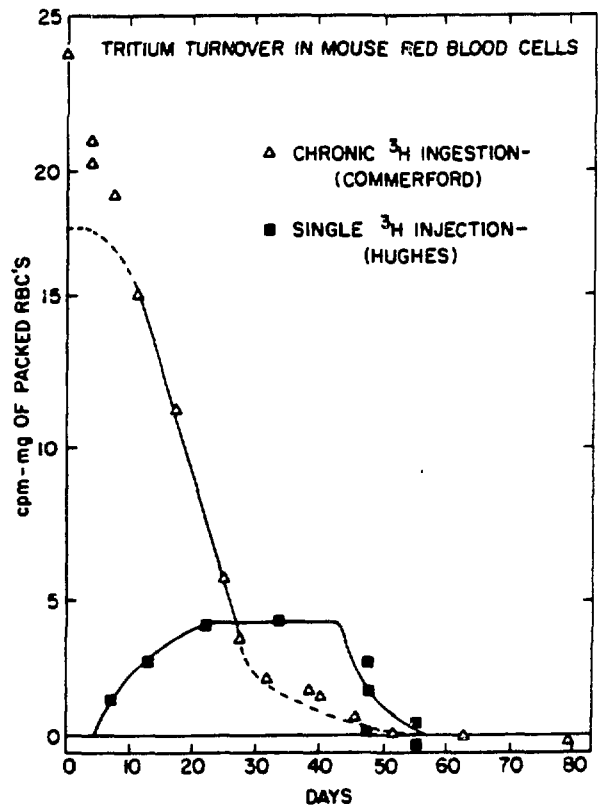


FIGURE 4