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THE TRITIUM TOXICITY PROGRAM IN THE MEDICAL DEPARTMENT

BROOKHAVEN NATIONAL LABORATORY

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MASTER

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INTRODUCTION

It is most apropos that during this symposium honoring Dr. E.P. Cronkite, some brief mention should be made of the tritium toxicity (TRITOX) program which developed at Brookhaven during his chairmanship of the Medical Department. In addition to being a staunch supporter of the TRITOX program, and an active participant in its planning, Dr. Cronkite served on the NCRP Committee (1) which addressed directly the problem of the possible genetic hazards to man from tritium compounds.

The development of the TRITOX program as related to worldwide fission and fusion energy programs has been previously reviewed (2,3) and it should suffice to say that the world is becoming more dependent upon nuclear energy as a source of electric power and with this reliance comes the possibility of introducing into the environment increasing amounts of HTO.

On the next few pages will be briefly outlined the program which began in 1971 and is still active.

The initial concept in establishing this program was to examine primarily one particular question; "what might be the genetic hazard (as measured by the dominant lethal mutation test) of continued exposure to tritiated water (HTO) at levels approximately 100 times the maximum permissible concentration (3.0 $\mu\text{Ci/ml}$)?". In order to answer this question, it was necessary to establish a rather large monitored tritium facility and a mouse colony for breeding and maintaining a large number of animals on the HTO regimen. It also became evident that there were many unanswered questions concerning the many possible effects of HTO ingestion. Therefore, over the ensuing years a multiparameter examination of somatic, genetic and cytogenetic effects as well as questions related to biochemistry and dosimetry resulting from continuous maintenance on an HTO regimen was undertaken. Since much of the information obtained from

this program has been published (4-12), only a brief summary of the entire program as outlined in Figure 1 will be presented at this time.

MATERIALS AND METHODS

Mice Breeding and Maintenance:

With the exception of studies on induction of leukemia, all animals used in these studies were of the Hale-Stoner-Brookhaven (HSB) strain. This is an albino strain which has been maintained in a single colony for more than 20 years in the Medical Department, Brookhaven National Laboratory. The food used is Purina Laboratory Chow, Ralston Purina Co., St. Louis, Mo. Animals receive this regimen ad lib. and tap water acidified to pH 2.4. Breeding partners are established by random selection from animals born during the same week without attention to litter mate selection. These animals remain together throughout their reproductive lifetime. To reduce possible litter variation, only first litter animals from each breeding pair were used for the TRITOX studies.

At 4 weeks of age, animals were removed from the mouse colony and divided into experimental groups. Depending upon the studies involved, the animals were maintained on HTO concentrations ranging from 0.3 to 30.0 $\mu\text{Ci/ml}$ HTO or acidified tap water. For longterm studies, half of the control animals was maintained in the tritium facility while the other half was removed to a similar room which contained only mice on tap water. (This was to determine whether animals maintained on tap water in the tritium room as controls, would receive a significant inhalation exposure to HTO due to air contamination. When the first generation animals reached 8 weeks of age, breedings were done within the various experimental groups resulting in second generation animal who together with their parents, had been maintained on either HTO or tap

water. Groups of male and female animals from both HTO and control groups were then maintained for longterm observation. From these larger groups, 20 male and 20 female animals were randomly selected and put aside for monthly weighing. At the time of weighing these animals were closely examined for any apparent differences in appearance. Methods for other determinations are as follows:

I. GENETIC, CYTOGENETIC, AND REPRODUCTIVE EFFICIENCY STUDIES

A. Dominant Lethal Mutation (DLM) Rate Studies

When second generation animals assigned to the study reached 8 weeks of age they were divided into 4 groups for DLM testing (13). The 4 groups were as follows: Group 1 - Males and females maintained on HTO, Group 2 - Females on HTO, males on tap water, Group 3 - Males on HTO, females on tap water, Group 4 - Males and females on tap water (controls). Breeding groups were arranged with one male placed with 5 females for a 5 day breeding period. Fifteen days after the midpoint of this breeding period, the females were sacrificed, the number of pregnant females noted and the ovaries and uterine contents examined. Ovaries were evaluated for the number of corpora lutea (CL) and the uterine contents classified as to number of viable embryos (VIA), early embryonic deaths (ED), and late embryonic deaths (LD). Early embryonic deaths were characterized by a dark "mole" or resorption site interpreted as death occurring between implantation and approximately 10 days, whereas LD's were characterized by a formed but dead embryo. From the four parameters measured, CL, ED, LD, and VIA, the preimplantation loss (PRE) from each mating was calculated:

$$PRE = CL - (VIA + ED + LD).$$

New breeding groups were started each week so that a continuing program of data accumulation took place in all four experimental groups, thus allowing for the detection of possible temporal shifts in the colony.

Results of the DLM evaluations were compared using 3 statistical tests: (1) students 't' test (14), a parametric test which assumes normal distribution of the data. In these evaluations, the analysis makes use of the pooled errors of all groups. (2) The rank test developed by Kruskal and Wallis (15). This is a non-parametric test described as being applicable for a complete random design with any number of populations in which the final analysis is made using a (χ^2) test. (3) The final statistical test involves an arcsine transformation which normalizes the data, computes the mutation index for each treatment, which are compared using a χ^2 test (16).

B. Cytogenetic Studies

1. Liver Cytogenetic Studies

In the adult mouse the liver is not a very mitotically active organ. Therefore, individual cells will tend to accumulate injury during continuous radiation exposure. This damage will become visible as chromosome aberrations (CA) when the cells are stimulated to division by partial hepatectomy. To make the CA evaluations, animals were maintained on 0.3, 3.0 $\mu\text{Ci/ml}$ HTO and acidified tap water beginning at weaning and continuing until sacrifice. After approximately 90, 330, 500 - 560, and 700 days, the animals underwent partial hepatectomy followed after 54 hours by chromosome analysis using previously published methods (4).

2. Bone Marrow Cell Evaluation for Induction of Sister Chromatid Exchanges (SEC's).

Before and at selected times of continuous HTO ingestion (3.0 $\mu\text{Ci/ml}$) mice were evaluated for the induction of SEC's in their bone marrow. Selected animals were given continuous BrdUrd infusions for 24 hours using the technique as described by Schneider et al. (17).

Two hours previous to sacrifice by CO_2 inhalation the animals received an injection of colchicine. Bone marrow cells from the femur and tibia were

harvested and evaluated for the induction of SCE's as previously described (18).

3. Micronuclei Evaluation in Red Cells

The induction of micronuclei in red blood cells is accepted as a sensitive measure for evaluating cytogenetic effects of various agents. Preliminary studies have been done on animals maintained on HTO (3.0, 7.5, 15.0, and 30.0 $\mu\text{Ci/ml}$) for periods of 5 to 6 weeks, with the ingestions started at approximately 3 weeks of age. At the end of the ingestion period blood samples were taken and the red blood cells evaluated for micronuclei as described by Tice (18).

II. SOMATIC EFFECTS

A. Nonspecific Lifetime Shortening

Two hundred animals maintained on 3.0 $\mu\text{Ci/ml}$ and age matched controls were followed throughout their lifetime. Animals were examined weekly for gross changes in appearance. Cages were checked daily for deaths. Any observed changes and time of death were noted.

B. Growth (Body Weight).

From the animals described in the previous paragraph, 20 animals were randomly selected from each group. These animals were weighed monthly and comparisons made between groups.

C. Bone Marrow Cellularity and CFU-S Content.

The leg bone marrow (femur and tibia) of animals on HTO and tap water were analyzed for total cellularity, relative number of hematopoietic stem cells (CFU-S) and total number of CFU-S. Harvesting of the marrow was done using the quantitative grinding technique of Stoner and Bond (20). The stem cells evaluation was made using the spleen colony assay as described by Till McCulloch, (21). In this evaluation a known number of bone marrow cells

are injected (i.v.) into recipient mice which previously received a lethal (750 rads) whole body 250 kvp x-ray whole body exposure. After seven days the recipients are killed, their spleens fixed in Bouin's solution and 24 hours later the number of surface colonies counted. Details of this technique used in these studies have been previously published (7).

III. RELATIVE BIOLOGICAL EFFECTIVENESS (RBE) STUDIES

Over the past many years there has been considerable debate concerning the assignment of the correct RBE or 'Q' for tritium exposure in the form of HTO. To examine this question, comparisons have been made between animals maintained on HTO and those receiving a continuous (22 hours/da) exposure to ^{137}Cs gamma rays. The geometry of the gamma ray radiation facility was arranged so that the depth dose within the peritoneal cavity of exposed mice, as measured by implanted thermoluminescence dosimeters, was equal in dose rate to the exposure resulting from the average soft tissue dose in animals maintained on HTO for extended periods.

The measured values for tritium content of soft tissue varied depending upon water content. Taking an average value for several soft tissues, it was found that a value of 0.69 rads per day would be a reasonable estimate of soft tissue absorbed dose for animals maintained on 3.0 $\mu\text{Ci/ml}$. The gamma radiation facility was thus constructed so that the initial dose rate was 0.69 rads per 22 hour day at the beginning of the longterm experiments. Lifetime shortening, induction of DLM's, growth, bone marrow cellularity, CFU-S content and induction of bone marrow SCE's were compared between the two experimental groups. Two comparative studies were made. In the first of these, animals received ^{137}Cs gamma exposures equivalent to 3.0 $\mu\text{Ci/ml}$ of HTO. In the second study, a smaller source was used to give a dose equivalent to the continuous ingestion of 0.3 $\mu\text{Ci/ml}$ of HTO.

IV. BIOCHEMISTRY AND MICRODOSIMETRY STUDIES

A. Rate of Tritium Incorporation

A number of determinations were made to determine the radiation dose delivered to tissues of interest on an activity/gram basis and also on the basis of tritium incorporated into specific subcellular fractions. The pattern of tritium incorporation was determined by analysis of fresh tissue. Animals were sacrificed by cervical dislocation, and tissues placed immediately into weighed counting bottles containing a tissue solvent - scintillator fluid. Tissue weights were then determined and the tissue samples counted using a well type scintillation counter. The total tritium content was calculated on the basis of wet tissue weight. For determinations of tritium content in subcellular constituents techniques as previously described by Commerford (8). Briefly, chromatin was isolated using conditions designed to minimize degradation. This involved homogenization, pelleting through 2.2 M sucrose and extraction from nuclear fragments with EDTA followed by lyophilization and resolution in 2 M NaCl. Material obtained in this way represents a complex of DNA, RNA, histones and residual protein in ratios of approximately 30%, 3%, 30% and 37% of the total weight. These components were extracted in sequence, with the histones removed first on the basis of their solubility in 0.2 M HCL, followed by RNA which becomes acid soluble when heated 18 hours at 37°C in 0.3 M KOH (22) and finally DNA, which becomes acid soluble when heated 30 minutes at 90° in 1 M HClO₄. The remaining acid soluble material represents residual protein. Tritium content from all fractions is determined using standard liquid scintillation techniques. All counts were corrected for background chemiluminescence.

The rate of tritium disappearance from tissue, cellular and subcellular components was determined in the same manner on animals which had been maintained on HTO for approximately 6 months followed by maintenance on acidified tap water.

V. CARCINOGENESIS (INDUCTION OF LEUKEMIA)

A known effect of ionizing radiation exposure in mammals is the development of leukemia. This question is being investigated in mice of the CBA strain. This strain was chosen because it has a low incidence of spontaneous acute myelocytic leukemia and a high incidence of the same disease after irradiation. Animals have received single whole body x-ray exposures of 50, 100, 200 or 300 rads (250 kVp, 100 rad per minute) at 3 and 9 months of age. In addition, other animals have received external whole body ^{137}Cs gamma exposures at dose rates of 1.2 or 1.8 rad per day (5 days/wk) until they have accumulated total doses of 300 rads. Additional animals receive fractionated (250 kVp x-ray) exposures (1-3 times/wk) to accumulated doses of 50-300 rads.

For comparison, equivalent aged animals have received either a single injection of HTO or continuous ingestion of 3.0 $\mu\text{Ci/ml}$ which would result in an integrated whole body doses equal to the x or γ exposures. All animals are examined daily for the first 6 months following exposure and twice daily thereafter to determine their health status. Sick animals which appear to be near death are sacrificed following peripheral blood counts (RBC, WBC and differential). Animals are then autopsied and microscopic evaluation made of liver, kidney, lung, spleen, mesenteric lymph nodes, femoral bone marrow, and sternum, including surrounding muscle. This project is part of an overall leukemia study being done in collaboration with E.P. Cronkite.

RESULTS

Due to the relatively long history of the TRITOX Program, much of the results have already been published. Therefore whenever such information is available, only brief comments will be made with the original reference being cited.

I. GENETIC, CYTOGENETIC AND REPRODUCTIVE STUDIES

A. Dominant Lethal Mutation Studies

Studies have been completed on animals maintained on 3.0, 1.0 and 0.3 $\mu\text{Ci/ml}$ HTO together with equivalent dose external gamma exposed animals. Summaries of data for 3.0 $\mu\text{Ci/ml}$ (7) and the 1.0 $\mu\text{Ci/ml}$ (23) animals have been published.

In summary, when both the male and female breeding partners are maintained on 3.0 $\mu\text{Ci/ml}$, a significant reduction in viable embryos ($p < .0001$) and a significant increase in early deaths ($p < .01$) is observed. Similarly, when only the female is maintained on 3.0 $\mu\text{Ci/ml}$, a significant reduction ($p < .01$) in viable embryos is seen.

If both breeding partners are maintained on 1.0 $\mu\text{Ci/ml}$, a significant ($p < .01$) reduction in viable embryos is noted. In all other cases for 3.0, 1.0 or 0.3 $\mu\text{Ci/ml}$, no significant effects are observed.

B. Cytogenetic Studies

(1) Regenerating Liver Studies

Animals maintained on 3.0 $\mu\text{Ci/ml}$ for 100, 330 and 500-560 days exhibited a significant increase in the number of abnormal cells in the regenerating livers as compared to animals maintained on acidified tap water. Details of this study have been previously published (4). Similar effects were not noted in animals maintained on 0.3 $\mu\text{Ci/ml}$.

(2) Bone Marrow Cells

The SCE levels in femoral bone marrow cells of mice maintained on 3.0 $\mu\text{Ci/ml}$ HTO for 28 to 261 days were always higher than those in age-matched control groups. In mice drinking HTO the number of SCE's per cell range from 2.00 to 4.03 while comparable figures for control animals were 1.70 to 2.81. Significantly (1.0%) higher numbers of SCE's were seen in HTO animals on 81, 163, 192, 247, and 261 days after continuous ingestion of HTO. At 72, 86, and 227 days, the difference was significant at the 5% level. Using a one-way analysis of variance and covariance, the probability that the mean of all the control data is not different from that of the exposed animals is less than 0.0001. Details of this study are reported elsewhere (18).

(3) Micronuclei Studies in Red Blood Cells

Results of preliminary studies on the induction of micronuclei in animals maintained continuously on HTO for 5 to 6 weeks beginning at age 3 weeks, indicate that for animals drinking 30.0 $\mu\text{Ci/ml}$ HTO, a significant increase in micronuclei was found ($p < .01$). A slight increase was noted in animals maintained on 15.0 $\mu\text{Ci/ml}$, however, no effect was seen at 3.0 or 7.5 $\mu\text{Ci/ml}$. It should be noted that these are preliminary results and the studies are being repeated.

II. SOMATIC EFFECTS

A. Growth (Body Weight)

Continuous ingestion of 3.0 $\mu\text{Ci/ml}$ of HTO or equivalent external gamma exposure caused no measurable effect on growth as measured by body weight.

B. Nonspecific Lifetime Shortening

Continuous ingestion of 3.0 $\mu\text{Ci/ml}$ of HTO or equivalent external gamma exposure caused no measureable effect on nonspecific lifetime shortening. By gross appearance, it was impossible to identify the HTO, gamma ray exposed or control animals.

C. Bone Marrow Cellularity and CFUS Content

There was no effect on the total number of leg bone marrow cells in any of the animals maintained on HTO or receiving continuous external gamma ray exposures. In contrast, reductions in number of bone marrow stem cells (CFU-S) were noted as early as 8-12 weeks in the 3.0 $\mu\text{Ci/ml}$ mice and by 24 weeks in the 1.0 $\mu\text{Ci/ml}$ animals. In both groups, the stem cell depression continued with some variability throughout the lifetime observation. No effect was measurable in the 0.3 $\mu\text{Ci/ml}$ animals, other than a somewhat greater than normal variability in number of CFU-S per leg. Details of this study have been previously published (7,9).

III. RELATIVE BIOLOGICAL EFFECTIVENESS (RBE)

In all studies completed to date, there is no significant difference ($p < .01$) between animals ingesting HTO and animals receiving equivalent external gamma ray exposures. However, for several of the parameters measured, the effects were somewhat greater for the HTO, although not significantly so. This might be interpreted as an indication that the RBE or Q value for HTO may be slightly greater than 1 but less than 2. (It should be noted that in all cases, the reference radiation must be strictly defined).

IV. BIOCHEMISTRY AND MICRODOSIMETRY STUDIES

When animals are placed on an HTO regimen, tritium concentrations in body water and soft tissues rapidly approach equilibrium levels (5). When removed from the HTO regimen the tritium level in tissue dropped rapidly from 2.02 $\mu\text{Ci/ml}$ before withdrawal to 0.07, 0.01 and 0.001 $\mu\text{Ci/ml}$ at 7, 14 and 28 days later. The rate at which nonexchangeable tritium disappears from brain and liver histones indicates a half-life of 117 days (95% confidence limits of 85 to 138 days) for liver histone and 159 days (95% confidence limits of 129 to 208 days) for brain. The tritium activity in liver and brain indicate that the brain data points fit a straight line with a slope indicating a half-life of 593 days with 95% confidence limits of 376 to 1406 days. In contrast, the curve for liver shows a pronounced curvature indicating the presence in the liver of two cell populations with distinctly different turnover times. These two liver cell populations exhibit half-lives of 12 and 318 days representing 23 and 77% of the total DNA, respectively. The initial specific activity in liver DNA of 0.90 dpm per microgram, as we have reported (23) is in good agreement with an expected value of 0.89, which can be calculated on the basis of previous studies (24). Further details of these studies have been previously published (8,11,12).

V. CARCINOGENESIS

Studies on the induction of leukemia are still in progress with no definitive results as yet available. On the basis of routine examination, no other malignancies have been found.

SUMMARY

A summary of all the findings to date are shown in Figure 2.

It appears from this information that it is possible to detect somatic, cytogenetic and genetic effects resulting from exposures at 33 to 100 times the mpc's for HTO. Similar effects are also seen for exposure to

external gamma rays at an equivalent dose.

The reduction in bone marrow cells in animals maintaining normal total cellularity are of interest since they demonstrate both the presence of an effect at the primitive cell level as well as the animal's ability to compensate for this effect by recruiting stem cells from the G_0 resting state. This evidence of damage together with the observed cytogenetic changes leads one to contemplate the possible importance of radiation exposures at these levels for the induction of leukemia or other blood dyscrasias. Studies to investigate this question are now underway.

As predicted on the basis of established principles of radiobiology, exposure to tritium beta rays from HTO ingestion results in measureable effects on several animal systems.

The importance of position of incorporation of H into molecules of biological importance has not been well defined, nor has the low dose portions of the dose response curve for several effects of interest. Answers to these questions together with measurement of H turnover data as a means for analysis of cell kinetics in several systems are now underway.

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FIGURES

Figure 1

Outline of Brookhaven TRITOX Program.

Figure 2

Summary of Results of Brookhaven TRITOX Program.

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BROOKHAVEN TRITIUM TOXICITY PROGRAM

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V. CARCINOGENESIS

- A. Induction of Leukemia**

1/1/84

TRITOX SUMMARY

	HTO CONCENTRATION		
	0.3 μ Cl/ml	1.0 μ Cl/ml	3.0 μ Cl/ml
SOMATIC EFFECTS			
Growth	0	0	0
Life-Time Shortening	0	0	0
Bone Marrow Cellularity	0	0	0
Bone Marrow CFU-S	0	+	++
GENETIC EFFECTS			
Dominant Lethal Mutations	0?	+	++
CYTOGENETIC EFFECTS			
Marrow Sister Chromatid Exchanges	0?	NA	+
Regenerating Liver Aberrations	0?	NA	+
Micronuclei in Erythroid Cells	NA	NA	+

NA — Not Available.

1/1/84