716453

Third Year
COMPREHENSIVE PROGRESS REPORT
AEC CONTRACT AT(45-1)-1781

March, 1966

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AEC Contract #AT(45-1)-1781

THE STUDY OF IRRADIATION EFFECTS ON THE HUMAN TESTIS: INCLUDING HISTOLOGIC, CHROMOSOMAL AND HORMONAL ASPECTS

Progress Report

This report covers the period from June 1963 to March 1966.

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I. Introduction

As requested for the third year progress report of our contract, the following summarizes the accomplishments and present day goals of our program. Details of our data along with pertinent reprints and a master's thesis are included in the main body and enclosure portion of our report.

A. Research Accomplishments - Special Reference to Original Aims

1. "To relate radiation dosage to changes in gonadal function." During the first three years of our study we have exposed the testes of thirty-one inmate volunteers to acute doses of x-ray and followed changes in their gonadal function. Our x-ray source was a G. E. Maximar 250 KV machine. The dosage varied from 7.5 to 400r to the midplane of the testes (little f factor = 0.95). Both spermatogenesis and hormonal function were studied.

For changes in spermatogenesis, testicular biopsy specimens obtained at varying intervals following x-ray exposure and serial seminal fluid specimens were examined. These observations were then compared with each subject's control data. We have found that 30r is the lowest dose of x-ray that results in azoospermia. With 15r a significant reduction in sperm count occurs circa 120 days following exposure. To date 7.5r has not significantly altered the sperm count, however not enough time has lapsed since exposure to be certain as to the results following this dose. With respect to recovery, more time is needed before final conclusions can be made, but as of now two of three men receiving 50r have demonstrated a return to control sperm count levels by 350 days following exposure. Recovery from doses of 100r and 400r will probably require an excess of 375 days. In general, as one increases the x-ray dose, the time required for "azoospermia" becomes shorter. Furthermore, the time required for recovery will probably be correspondingly longer.

Prior to our being able to adequately relate the x-ray dosage to changes in seminal fluid sperm concentration, we had to devise an improved method for estimating sperm counts. This was accomplished by means of the Coulter Counter and a new diluent for seminal fluid. A manuscript describing this technique was published in the Journal of Laboratory and Clinical Medicine, March, 1965.

During our examination of the seminal fluid, it became apparent that when a bilateral or unilateral testicular biopsy was perfonned, a certain percentage of the research subjects showed a significant drop in their sperm counts. This called for more extensive evaluation of the mechanism involved and a prolongation of our control period (control period being defined as that time required for hormonal, histologic and seminal fluid studies prior to x-ray administration and including that time required for the sperm count to return to pre-control biopsy levels if a "drop" ensued). We have concluded from our initial studies that the most likely mechanism to explain the sperm count drop is an antigen-antibody reaction. An account of our data was published in Fertility and Sterility, July-August, 1965.

Fortunately, in the majority of inmate volunteers who demonstrate this change following a biopsy, the drop is quite transient in nature. However, it can last up to circa 300-400 days. Thus we have changed our format. We now have two main groups of inmates--those that receive x-ray and have

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repeat testicular biopsies and those that receive x-ray and have only seminal fluid studies carried out. In the main, we have concentrated on the 100r dose with respect to evaluating histologic changes in the germinal epithelium consequent to x-ray exposure. The method of Oakberg for "scoring" the testis was followed, along with a slight modification. Oakberg quantitatively relates the specific germ cell types to the mean number of Sertoli cells present in the seminiferous tubules that are examined. This is done to correct for tubular shrinkage, particularly at higher doses. We have found that relating the specific germ cell types to the tubule unit removes the variable created by counting the Sertoli cells. Testicular biopsy specimens from the control period were scored, and these served as our frame of reference.

Biopsy specimens obtained 9, 15, 24, 27, 33 and 49 days following x-ray exposure were scored and compared to the control data. We found that as early as nine days there was a significant reduction in the numbers of A pale and B spermatogonia; also the preleptotene, leptotene, zygotene and pachytene spermatocytes were reduced in numbers. By counting back in time, using the duration of spermatogenesis as outlined by Clermont and Heller, it is concluded that at 100r the Ad, Ap and type B spermatogonia sustained immediate damage from the x-ray administered. Observations at the other time intervals led to the same conclusion with respect to the germ cell damage at the time of exposure.

Our preliminary observations on specimens four hours following exposure indicate that cellular metabolism is altered. For example, although the "scoring" of the testis was not altered from the control observations, germ cells, presumably spermatogonia, were distorted in shape and stained more heavily.

Plasma testosterone levels have been determined in our volunteers to assess Leydig-cell function. This has been accomplished with the collaboration of Dr. Hortense Gandy in the Department of Obstetrics and Gynecology, Cornell Medical College. A double isotope method was used for these determinations. Prior to irradiation, possible changes in testosterone levels due to a diurnal rhythm, as well as day-to-day variations in the same individual, were investigated. Our plasma testosterone values for normal adult males during the control period ranged from 0.26 to 1.5 μg%. These data compare favorably with those published by Hudson et al. and Lipsett et al. No diurnal rhythm could be detected. The day-to-day variation in testosterone levels for the same individual was noted to be as much as 50%. Following irradiation in the dose range from 15 to 400r, plasma testosterone levels did not change significantly. Measurements were determined on specimens obtained at 4, 24 and 72 hours and at 2 and 4 weeks following irradiation. In some instances, longer intervals up to several months were evaluated.

Urinary estrogen excretion was also studied in the early phases of our program to assess possible changes in Leydig-cell function. Determinations were performed before and following irradiation in

some twenty subjects. No significant alteration in estrogen excretion was noted.

When the testis is damaged by any means, including irradiation, urinary gonadotrophin excretion increases provided a normal pituitary-hypothalamic system remains intact. There are several methods for measuring urinary gonadotrophin titers. Three of these were utilized: first, the rat ovarian weight method, which measures all the gonadotrophins and is designated a general gonadotrophin assay; next, the chorionic gonadotrophin method of Steelman-Pohley, which measures only the Follicle-Stimulating-Hormone (FSH); and finally, the rat ventral prostate method of Greep, which measures only Interstitial-Cell-Stimulating-Hormone (ICSH).

General gonadotrophin titers were determined serially in fifteen volunteers who received doses of 15 - 400r. A significant elevation in gonadotrophin excretion was observed in eleven of these men. The rise in gonadotrophin titers did not occur until 30 to 135 days following irradiation, which was generally after the sperm count decreased. Since the general gonadotrophin assay measures both FSH and ICSH and since the germinal epithelium is theoretically related to only FSH levels, the delay in rise and the lack of rise in four of the subjects may reflect the relative insensitivity of this assay method. Therefore, we focused our attention on the specific FSH assay.

A preliminary survey in nine men was made with respect to changes in FSH excretion following irradiation. An increase in urinary FSH titers was demonstrated in all but one, and that individual received only 15r. His sperm count decreased but not to severe oligospermic levels. The time required for the FSH titers to rise varied from 55 to 123 days. Further studies will be necessary before a more precise relationship can be made with respect to sperm count changes and urinary FSH excretion levels.

ICSH assays were performed serially in fifteen males who received 15 to 400r. No significant elevation occurred following irradiation. This is not surprising since our assessment of Leydig-cell function by plasma testosterone measurements and urinary estrogen excretion assays failed to reveal any decrease in hormonal production.

Since chromosomal damage occurs as a result of irradiation exposure, we performed chromosomal analyses in some of our male subjects before and after they were exposed to x-ray. Short-term culture was carried out on peripheral blood specimens and testicular tissue before and at varying intervals following x-ray. The blood studies served as an additional control for our methodology, since it was unlikely that a significant volume of blood would be exposed to the x-ray directed to the testicular region. The criteria of Court-Brown and his associates were used for categorizing any detected abnormalities. Briefly, Group A relates to modal number, Group B relates to chromatid changes and Group C relates to chromosomal changes. In three control and eight post-irradiation blood samples, no significant chromatid or chromosomal changes to indicate damage were noted.

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Ten control testicular cultures were established and analyzed. Following irradiation at 100r, the testicular specimens were obtained and analyzed. In the control series 201 metaphase plates were karyotyped, and 90 metaphase plates were karyotyped in the post-irradiation specimens. There was no significant change in modality nor were there any chromatid changes due to x-ray irradiation. However, a significant increase in both stable and unstable chromosomal abnormalities was detected. For example, there were fourteen type C changes in the 90 karyotypes following x-ray as compared to six type C changes in the 201 karyotypes from the control specimens.

In addition to examining the possible chromatid and chromosomal aberrations secondary to irradiation, we studied the phenomenon of endoreduplication. Previous reports by other workers have documented an increase in chromosomal endoreduplication following either in vitro or in vivo irradiation. The test system used by them was peripheral blood lymphocytes in short-term culture. Since we were irradiating testicular tissue directly, it was felt that this tissue would be an ideal in vivo model for testing the radiation dose-effect with respect to endoreduplication. For our preliminary studies we analyzed the chromosomes of the testicular cultures of twenty-one inmates prior to irradiation. These data served as our control material. Following exposure to 100r, repeat testicular biopsies were performed four hours and fifteen to forty-two days later for similar analysis. The incidence of endoreduplication was increased in the "four-hour" specimens but returned to control levels in the "fifteen to forty-two day" specimens.

To recapitulate, then, we have explored and obtained data on the following dose-related parameters: 1) seminal fluid sperm concentration, 2) germinal cell quantitation, 3) urinary gonadotrophin excretion titers and 4) chromosomal changes. Also, we have demonstrated that Leydig-cell function remains intact up to and including exposure to 400r x-ray under the conditions employed.

2. "To utilize the radiation-induced gonadal changes as the means for studying anterior pituitary-testicular interrelationships." Since we have observed selective damage to spermatogenesis with retention of Leydig-cell function following x-ray exposure to 15 - 400r, we were able to confirm the specific relationship between the germinal epithelium and urinary FSH titers. Furthermore, the observations with respect to normal ICSH titers in face of the elevated FSH levels highlight the specificity of these gonadotrophin assay methods.

3. "To explore therapeutic and medical protective measures with respect to gonadal irradiation." Until we have clearly defined the dose response curves for the various parameters of gonadal damage due to irradiation, it will not be possible to evaluate proposed protective measures.

4. "To explore our current concepts concerning radiation dosage expressed in physical terms and their relation to biologic effects." Only after we obtain our neutron and beta data will we be able to evaluate this aspect of our research plan.

B. Present Objectives

1. In view of the sensitivity of human germinal epithelium to relatively low doses of acutely delivered x-ray, it will be necessary to concentrate on the lower portion of the dose response curve. Also, since the inmate volunteers exposed to 400r have not recovered completely to their pre-irradiation level of spermatogenesis by circa 375 - 400 days, it will not be necessary or desirable to further explore the higher dose levels. With respect to our observations at lower doses, i. e., 7.5r or lower, we will need to expand our control observations in order to nail down the significance of the seminal fluid changes in a very precise fashion. This is important when one considers the small x-ray doses employed and the inherent variability in seminal fluid sperm concentration.

2. Now that we have documented the effect of testicular biopsy on sperm counts, additional control groups are needed for proper delineation of causal factors responsible for seminal fluid changes in our studies. For example, we are examining the effect of just wearing the scrotal suspensory without concomitant biopsy. Also, we are examining two "no procedure" periods to properly evaluate what constitutes a significant depression in sperm count.

3. Although not strictly pertinent to this contract, we are pursuing the mechanisms involved in the sperm count drop following testicular biopsy. This is under our NIH research grant.

4. From our preliminary studies it is apparent that quantitation of germinal epithelial changes is feasible. We have selected the time intervals of 9, 15, 24, 33 and 49 days. One hundred r will remain the dose level for this phase of our study. We shall increase the number of observations per time interval for appropriate statistical analysis.

5. Since sperm concentration changes may not be the most sensitive parameter, we are planning a pilot study where careful examination of sperm viability and morphology will be carried out. The necessary comparisons between these data and sperm counts should give us important information as to early "damage" changes.

6. A very important phase of our investigation will be carried out this next year. That is the construction of the neutron dose-response curve. Both 2.5 and 14.5 MEV energy levels will be studied. Then the RBE for the testis can be calculated.

7. Urinary gonadotrophin excretion titers, particularly FSH, will be studied at appropriate time intervals following irradiation. These data will be correlated with the quantitative changes noted in the germinal epithelium. By this means, we should be able to determine whether or not a specific germ cell or germ cells regulate the level of endogenous FSH. Presently this information is not known, so the importance of these observations is self-evident.

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8. Our investigations with respect to chromosomal aberrations secondary to irradiation will continue. More points on the dose response curve need to be documented. For this aspect of our study we shall confine our observations to neutron exposure.

C. Graduate Students and Postdoctoral Fellows

Since our contract was activated, one graduate student received his Master's degree and four postdoctoral fellows have been trained. Mr. Kenneth L. Swinth received his Master's degree for his work on the neutron generator shielding. A copy of his Master's thesis accompanies this report (enclosure #1). Although they did not receive salary support from our project, Drs. A. de Souza, A. Barr, D. L. Gordon and G. Halling have worked on this project as part of their two year postdoctoral training. Currently Dr. de Souza is attached to the research staff at the University of Hawaii. Dr. A. Barr is continuing his histologic studies in the Department of Anatomy, McGill University, under Dr. Clermont. Dr. Gordon was appointed full-time Instructor in Medicine at the University of Washington and is on leave-of-absence while serving his two year obligation in the Air Force. Finally, Dr. Halling is completing his two year training period this June.

D. Bibliography

RLO-1781-1 Gordon, D. L., Moore, D. J., Thorslund, T. and Paulsen, C. A.: The Determination of Size and Concentration of Human Sperm with an Electronic Particle Counter. J. Lab. Clin. Med. 65:506, 1965.

RLO-1781-2 Gordon, D. L., Barr, A. B., Herrigel, J. E. and Paulsen, C. A.: Testicular Biopsy in Man. I. Effect Upon Sperm Concentration. Fert. and Ster. 16:522, 1965.

RLO-1781-3 Paulsen, C. A., Gordon, D. L., Halling, G., Barr, A., and Gandy, H. M.: Effect of X-Irradiation of Testis on Plasma Testosterone in Normal Male Volunteers (abstract). To be presented at Second International Congress on Hormonal Steroids, Milan, Italy, May 23-28, 1966.

RLO-1781-4 Paulsen, C. A.: The Effects of X-Ray Irradiation on the Human Testis (abstract). To be presented (by invitation) at the annual conference of the Society for the Study of Fertility, Cambridge, England, July 5-8, 1966.

RLO-1781-5 Gordon, D. L., Herrigel, J. E., Moore, D. J., and Paulsen, C. A.: Efficacy of Coulter Counter in Determining Low Sperm Concentrations. Am. J. of Clin. Path. (in press).

E. Present Status of Scientific Knowledge

Until we demonstrated the extreme sensitivity of the human testis to ionizing irradiation, there was little appreciation of this matter from the available animal data. Our findings relative to the minimal dose, i. e., 7.5 - 15r, that results in decreased spermatogenesis and the fact that complete recovery may not occur when doses of 400r are employed indicate the need for well-documented human data.

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For example, the MED in the mouse is in the range of 20r; while in dogs, complete recovery of spermatogenesis has been reported to occur within one year after exposure to 600r. Thus the animal data do not necessarily give us the answers we need relative to the human being.

Our studies with respect to the histologic and hormonal changes afford us a unique opportunity to learn more of the physiologic mechanisms involved in pituitary-gonadal function. Our present day concepts lack precision and finality. Therefore, the importance of our studies directed to this area is quite clear. Previously, we had to rely on steroid hormone administration studies to investigate pituitary physiology. Overlap in action led to uncertain results. The irradiation studies are not hampered by this, since isolated seminiferous tubule damage is produced.

One area we need to explore is the effect of irradiation on the epididymis, vas deferens and accessory gland functions. It may be that we are altering nutrient factors concerned with maintaining "healthy" sperm during transport from testis to the ejaculatory site. Our studies on motility, morphology, etc., are a beginning in this direction. Biochemical and other basic studies may be important to carry out in the future. Aside from absent seminal fluid fructose levels in total ductal block, there is almost nothing known with respect to specific alterations in the transport "vehicle" and specific defects in sperm morphology and viability.

Our proposed electron microscopy studies should enlarge our meager knowledge concerning the ultrastructure of the testis and its functional aspects. Dr. Vilar has studied the Sertoli cell quite extensively, but the germinal epithelium has been neglected.

Finally, we need the basic "damage" and "recovery" data so that we may devise means for protection. This is obviously important since the probability of gonadal irradiation is increasing with each decade. Aside from preventative measures, there is no known way to satisfactorily protect the gonad in a practical manner.

F. Federal Support - Overall Research Program

Total federal support in addition to funds supplied by this grant:

NIH AM 05436-05 END: Pituitary-Gonadal Interrelationship, $44,461, 9/1/65 to 8/31/66

NIH T1 AM 5161-08: Endocrinology and Metabolism Reproductive Physiology (Training Grant), $36,921, 7/1/66 to 6/30/67

The amounts listed above represent support for one year; continued support has been recommended by the granting agency.

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PROGRESS REPORT

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II. Seminal Fluid Analysis

A. Adaptation of Coulter Counter for Determination of Sperm Concentration

The most readily available means for serially evaluating testicular function is seminal fluid analysis. In this examination, the parameters most commonly analyzed are sperm concentration, volume, morphology, and motility. Conventionally, the measurement of sperm concentration has been performed by the hemocytometer method. However, a number of limitations inherent in this procedure have been demonstrated. (1)(2) These include unequal distribution of particles in the counting chamber and variability in the counting and identification of sperm by the examiner. In our laboratory, 200 paired sperm counts performed by two technicians using the hemocytometer method resulted in a deviation of greater than ±10 per cent of the mean in 20 per cent of the specimens. This large variance has also been noted when the hemocytometer method was used for counting red blood cells. (3) Also, with the large number of seminal fluid specimens that were to be examined, the problem of technician fatigue was of considerable importance.

An electronic counter has been used satisfactorily in counting red and white blood cells and platelets. (2)(4)(5)(6) Segal and Laurence (7) adapted this instrument so that human sperm cell volume and concentration could be determined. Unfortunately, there was a wide range of error between the results they obtained for hemocytometer and electronic counting. However, Glover and Phipps (8) reported a better correlation in their preliminary studies with bull sperm. Since the electronic counter would improve the evaluation of large numbers of seminal fluid samples by virtue of its rapidity, we investigated the accuracy of this counter for the determination of sperm concentration in both normal and oligospermic seminal fluids.

Materials and Methods

Seminal fluid specimens, collected into clean medicine glasses, were obtained from inmate volunteers at the Washington State Penitentiary. Following measurement of volume and estimation of motility, the seminal fluid samples were transferred to shell vials, frozen, and shipped to our laboratory in insulated containers.

In all, 417 seminal fluid specimens were compared by both the electronic and hemocytometer counting methods. The initial study consisted of 223 specimens (see reprint in enclosure section), most of which were in the normal range. The second study compared 194 specimens with concentrations below 10 million sperm per ml. In both studies the two procedures were performed independently by separate technicians.

Prior to electronic counting, the seminal fluid was thawed, thoroughly mixed, and 0.1 ml was diluted (1:1000) with 100 ml of a mixture of 10 ml of 1 per cent saponin in isotonic saline, 85 ml of isotonic saline, and 5 ml of glacial acetic acid. Counts were performed 60 ±15 minutes after dilution. If the sperm concentration was less than 10 million per milliliter, the count was redetermined with a 1:500

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dilution; if between 175 and 350 million/ml, the dilution was increased to 1:2000, and if the concentration was greater than 350 million/ml, a 1:4000 dilution was utilized.

The electronic counter used was a Model B Coulter Counter with a 50μ aperture tube. The counter was calibrated with particles of a known volume, i. e., ragweed pollen with a mean corpuscular volume (MCV) of 3,884μ^3 and human red blood cells, MCV of 86μ^3. The settings for the aperture current, amplification, and internal gain trim were kept constant at 1/2, 1 and 65, respectively. With the use of a 50λ mercury manometer, 0.05 ml of diluted seminal fluid was caused to pass from the sample beaker through the 50μ orifice into the aperture tube. The presence of an electrode in both the diluted sample and the aperture tube creates a constricted electric current path at the orifice. Particles such as sperm that pass through the orifice have less conductivity than the electrolytic diluent; this causes an increased resistance in the circuit and an increase in voltage that is in direct proportion to the volume of the particle. The voltage pulses produced are amplified and registered on the decade counter. (3)

A particle size distribution plotter was adjusted to the Model B counter. This plotter progresses through 25 cycles or "windows" and measures the concentration of particles of progressively increasing size. Thus, the sperm population of a seminal fluid specimen was distinguished from another population of smaller particles representing debris. The "trough" between the debris and sperm populations was localized and considered to be the lower size border of the sperm population (Figure 1). The upper border was determined by finding the point at which the sperm population most clearly matched the "trough" in height. On the plotter graph, these points occur at a window number; each window represents 4 threshold divisions on the Model B counter. Therefore, once the borders of the sperm population were delineated by the plotter graph, these points could be transposed to the counter and all particles within this size range could be counted.

After disconnecting the plotter, a mean of five counts was obtained. A coincidence factor was added to correct for the simultaneous passage of 2 or more particles through the aperture that would be registered as a single particle. The frequency with which this occurs is directly proportional to the concentration of particles being counted. (2)(9) To correct for particle contamination of the reagents and glassware, a solution of diluent without seminal fluid was counted five times at the same threshold settings as the sample. The mean result, which represents background, was subtracted from the coincidence-corrected count. Then the dilution factors were taken into account to obtain the final concentration of sperm per milliliter (Table I).

The hemocytometer count was performed initially using a standard red blood cell pipette (1:200 dilution). Sperm counts under 20 million per milliliter were recounted using a white blood cell pipette (1:20 dilution). The same diluent and time factor after dilution were used for both methods of counting. Only intact sperm, heads with attached middle pieces or middle pieces with attached tails were counted, since the differentiation of the isolated sperm head from debris was considered to be too variable. Each sperm observed in two large diagonally-situated

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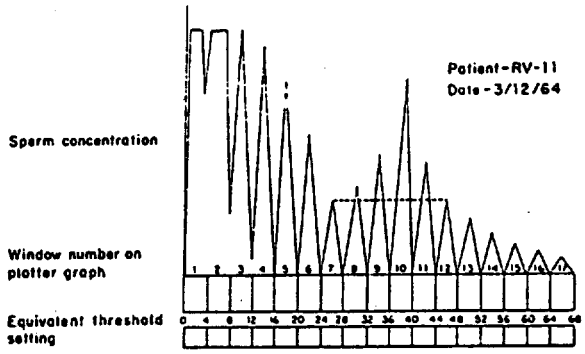

Fig. 1. Diagrammatic representation of sperm size distribution by plotter graph. This is one example of a sperm size distribution obtained. As is indicated in Table II, the curve is usually skewed to the right. However, the upper threshold is determined in the same manner for all curves, regardless of shape.

Table I. Example of estimation of sperm concentration by electronic counter, utilizing plotter graph in Fig. 1

Example	*Window*	*Threshold*	*Estimation*
Lower border of sperm population	7	24	
Upper border of sperm population	12	48	
Uncorrected count between thresholds 24 and 48			1,140
Coincidence factor equals 0.4% or			+ 5
Coincidence corrected count			1,145
Diluent blank at thresholds 24 and 48			− 31
Corrected count minus diluent blank			1,114
Multiplication factor (1:1,00 dilution and 0.05 ml. aliquot counted)			× 20,000
			22,280,000
Electronic count	22 million sperm/ml. seminal fluid		
Hemocytometer count	21 million sperm/ml. seminal fluid		

Patient, RV-11.
Date, 3-12-64.

squares of a Neubauer chamber, representing 0.1 cubic millimeter each, is equivalent to 1 million sperm/ml of seminal fluid for a 1:200 dilution or 100,000 sperm/ml of seminal fluid for a 1:20 dilution. Two to four hemocytometer counts were determined and the mean was accepted as the hemocytometer count, expressed in millions of sperm per milliliter of seminal fluid.

The reproducibility of the hemocytometer and electronic methods was compared by counting two seminal fluid specimens eight times by each method. Fresh dilutions were prepared for each determination. The results were then analyzed for variance and validity.

Results

The values obtained by calibrating the electronic counter with red blood cells and ragweed pollen were in close agreement, $0.38\mu^3$ and $0.39\mu^3$ per threshold division, respectively; therefore, each threshold division measured a particle volume of $0.4\mu^3$.

The size range of sperm present in 100 seminal fluid specimens is depicted in Table II. The mean values for the lower and upper borders of the population were 24 (range 16-40) and 56 (range 40-68), respectively. These threshold settings represented a mean population range from 10 to $22\mu^3$, since each threshold division measured a particle volume of $0.4\mu^3$. The mode of this population was $15\mu^3$ and remained constant with varying sperm concentrations.

The sperm concentration for the first 223 seminal fluid specimens in this study ranged from 1.4 to 471 million per milliliter. The values obtained by the two methods are plotted against each other in Figure 2 and are analyzed for variance in Table III. The calculated slope is 0.99843 with a correlation coefficient of 0.99921.

For the one hundred and ninety-four specimens with sperm concentrations of less than 10 million/ml of seminal fluid, the values for the two methods of determining sperm counts are compared in Table IV. For sperm counts of less than 1.1 million/ml, the correlation between the two procedures is less precise. Seventeen sperm counts between 1.1 and 10 million/ml of seminal fluid by the hemocytometer method were undeterminable by the electronic method because the debris could not be distinguished from the sperm population. Forty-seven seminal fluid specimens contained sperm concentrations between 1.1 and 10 million/ml of seminal fluid as determined by both techniques. These counts are plotted against each other in Figure 3. The calculated slope is 0.98843 and the correlation coefficient is 0.93269. An analysis of variance is depicted in Table V.

Two seminal fluid specimens were counted 8 times by both procedures to determine the variability of each procedure. While there was no statistically significant difference in counting the specimen with a low sperm concentration, this was not true of the specimen with a medium sperm concentration. The variance for the hemocytometer and

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Table II. Size distribution range for sperm in 100 seminal fluid specimens

	Window on plotter graph with equivalent threshold divisions (in parentheses below)*													*Total No. of specimen*
	5 (16-20)	6 (20-24)	7 (24-28)	8 (28-32)	9 (32-36)	10 (36-40)	11 (40-44)	12 (44-48)	13 (48-52)	14 (52-56)	15 (56-60)	16 (60-64)	17 (64-68)	
Lower threshold	7	14	40†	31	5	2	1							100
Mode				2	10	61†	19	1						93‡
Upper threshold						3	6	15	21	20†	20	12	3	100

*Multiply by $0.4\mu^3$ to obtain volume.
†Mean.
‡Specimens analyzed for mode, 93.

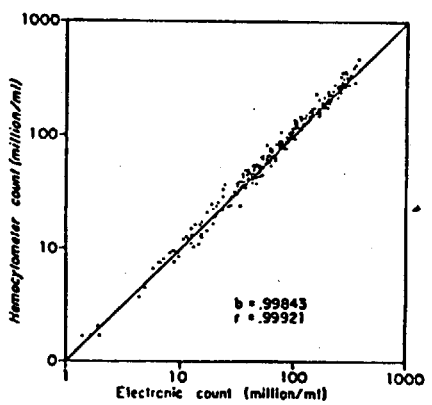

Fig. 2. Comparison of sperm concentration determined by electronic and hemocytometer methods.

Table III. Analysis of variance for slope plotted in Fig. 2

Source	*Degrees of freedom*	*Sum of squares*	*Mean square*
Slope (b)	1	53.48,331	53.48,331
Error	221	0.71,226	0.00,322
Total	222	54.19,560	

Hemocytometer Count (million sperm/ml of seminal fluid)	Electronic Count (million sperm/ml of seminal fluid)										
	No Sperm Population Delineated	0.1-1.0	1.1-2.0	2.1-3.0	3.1-4.0	4.1-5.0	5.1-6.0	6.1-7.0	7.1-8.0	8.1-9.1	9.1-10.0
No Sperm Count	65	4	1	0	0	0	0	0	0	0	0
0.1-1.0	50	9	0	0	0	0	0	0	0	0	0
1.1-2.0	5	1	8	0	0	1	0	0	0	0	0
2.1-3.0	4	0	1	3	1	0	0	0	0	0	0
3.1-4.0	3	0	0	2	1	3	0	0	0	0	0
4.1-5.0	0	0	0	0	0	2	3	0	1	0	0
5.1-6.0	1	0	0	0	0	0	0	1	0	1	0
6.1-7.0	2	0	0	0	0	1	1	2	1	0	1
7.1-8.0	0	0	0	0	0	0	0	2	0	1	0
8.1-9.0	1	0	0	0	1	0	0	0	1	1	1
9.1-10.0	1	0	0	0	0	0	0	0	0	3	3

TABLE IV

Comparison of 194 Sperm Counts Between 0 and 10 Million Determined by the Hemocytometer and Electronic Methods

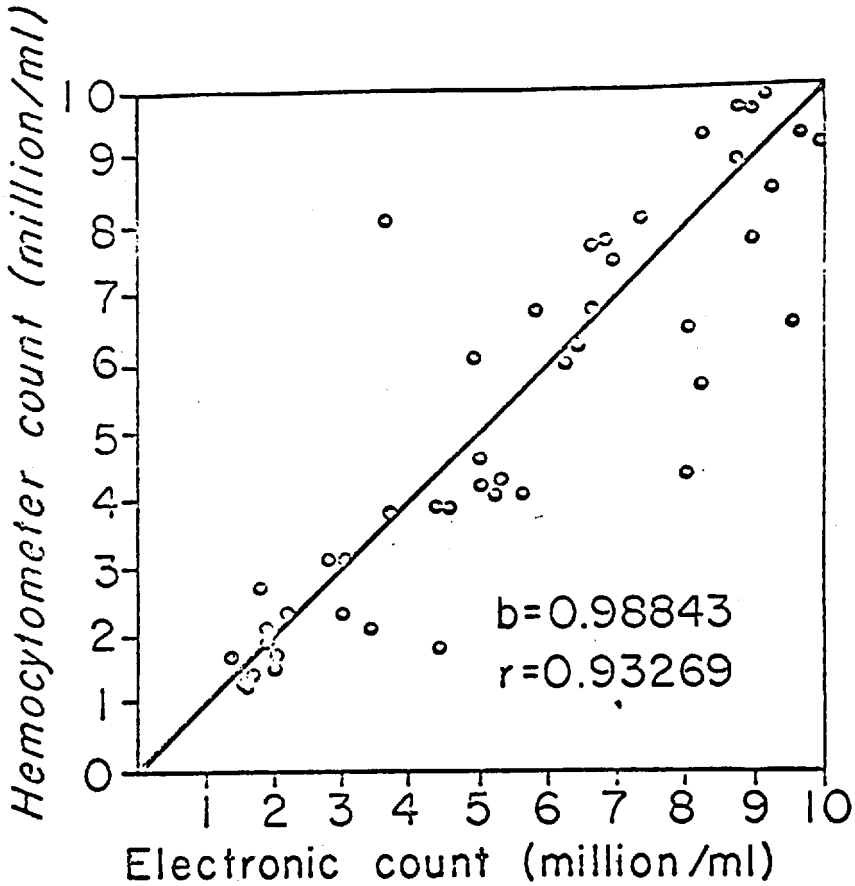

FIGURE 3

Comparison of 47 Sperm Counts Between 1.1 and 10 Million Determined by the Hemocytometer and Electronic Methods

Source	Degrees of Freedom	Sum of Squares	Mean Square
Slope (b)	1	3.32741	3.32741
Error	45	0.40963	0.00910
Total	46	3.73704	

TABLE V

Analysis of Variance for Slope Plotted in Figure 3

electronic methods was 52.19 and 2.203, respectively, the electronic procedure being significantly less variable (Table VI).

Discussion

The electronic method for the determination of sperm concentration has been compared with the established hemocytometer method, and it has been shown that similar estimates could be obtained by each technique. For the first 223 paired seminal fluid specimens the comparison gave a slope of 0.99843 and a correlation coefficient of 0.99921, which did not significantly differ from 1.0 ($t = -0.203$), nor did the intercept vary from zero ($t = 0.418$). Similarly, in the comparison of the 47 specimens that had hemocytometer and electronic counts between 1.1 and 10 million sperm/ml of seminal fluid the slope did not significantly differ from 1.0 ($t = -0.203$) and the intercept did not vary from zero ($t = -0.100$). Because of this high degree of correlation the two procedures are considered to be interchangeable.

Other investigators had been relatively unsuccessful in correlating the electronic and hemocytometer counts (7)(8)(10). The closer degree of correlation in this study appears to be due to two modifications in the electronic procedure: (a) the use of an improved diluent, and (b) the use of variable threshold settings to correspond to variations in individual sperm populations.

In addition to spermatozoa, raw seminal fluid contains varying amounts of crystalline matter, white and red blood cells, degenerating epithelial cells, and other debris. All particles that pass through the 50 micron orifice are registered on the electronic counter; therefore, a good diluent should remove debris without altering the sperm.

The combination of glacial acetic acid, 1 per cent saponin and isotonic saline in a 5:10:85 ml ratio effectively lyses most red cells within 15 minutes and most white cells and other debris within 45 minutes. The saponin also acts as a declumping agent. Therefore, at 60 ± 15 minutes only a small amount of debris remains in relation to the large numbers of sperm. A population of particles is thus obtained on the plotter graph that is distinct by its size distribution pattern, and these particles are counted as the sperm population.

If one reduces the number of sperm present in a given sample, it becomes harder to distinguish these particles from the remaining debris. At sperm concentrations below 1.1 million/ml of seminal fluid, and in specimens which contain an unusually large amount of debris, sperm populations are not delineated. Therefore, when the ratio of debris to sperm makes a consistent plotter graph unobtainable, or when the count is below 1.1 million sperm per ml, the count should be reestimated by the hemocytometer method.

In analyzing specimens of high sperm concentration it is important not to exceed the capacity of the Coulter counter. If more than 8,500 particles are counted in a determination an appropriate dilution must be made.

The electronic counter does not replace the microscope for motility or morphology studies, but it has been shown to be most

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Table VI. Analysis of variance for repeated counting of 2 seminal fluid samples by hemocytometer and electronic methods*

	Sperm concentration (million/ml.)		
	Hemocytometer procedure	*Electronic procedure*	*Test for t*
Specimen A (low sperm concentration)			
Mean ($\bar{x}$)	13.92	13.61	Not significant
Variance (S^2)	1.210	0.432	Not significant
Specimen B (medium sperm concentration)			
Mean (x)	83.2	81.9	Not significant
Variance (S^2)	52.19	2.203	< 0.001

*Eight determinations by each procedure.

valuable as a fast and reliable means of processing large numbers of seminal fluid specimens. Finally, the greater accuracy of this method, as judged by its reproducibility, has led us to use the Coulter counter exclusively for the studies as outlined in this project.

B. Effect of Testicular Biopsy and Related Procedures

All experimental design should include adequate controls so that results may be as precise and specific as is possible. Since one of the parameters of our investigations was histologic changes in spermatogenesis which necessitated performing serial testicular biopsies, we re-evaluated our concepts with respect to the effect this procedure had on sperm production. Other workers had reported that a testicular biopsy produced germinal epithelial damage in the bull. (11)(12)(13) Usually this resulted in a temporary sperm count depression. (11)(14)

Our initial study of seminal fluid changes following the control bilateral testicular biopsy revealed that nine of twenty (45%) research subjects demonstrated a significant drop in sperm count ($p = <0.05$). (14a) Since 19 of the 20 biopsies were bilateral, we needed to enlarge our observations to include an evaluation of the effects of unilateral testicular biopsy. Two other factors that might influence the sperm count also needed evaluation. These included the possible effects of a psychologic reaction to the biopsy and thermal changes due to the scrotal suspensory which is worn following the biopsy.

In an attempt to control these variables, each inmate passes through four periods of time prior to irradiation and following the initial control period. During the initial control period a minimum of twelve seminal fluid specimens is examined. Then the inmate volunteer enters the four "periods of time" in random fashion with respect to chronology. The periods of time are labeled as follows: 1) unilateral testicular biopsy, 2) unilateral sham biopsy, 3) wearing a suspensory for two weeks and 4) a no procedure period. During each period, which lasts 84 days, seminal fluid specimens are collected for examination weekly or every other week. The sperm count data for each period are subdivided into four intervals of twenty-one days. Then the Mann-Whitney statistical technique is used to compare the data obtained during these subdivisions with the sperm count data obtained during the original control period. In this fashion we shall be able to define more clearly what constitutes a significant alteration in sperm count following irradiation. Also, it will afford us data to more clearly understand the observed reduction in sperm count following testicular biopsy.

Our studies have not been completed as yet, but to date a significant reduction in sperm count has been noted following unilateral testicular biopsy. When such a decrease occurs, it does so during the same subdivision previously noted for bilateral testicular biopsy (see reprint in enclosure section of this report).

C. Effect of X-ray Irradiation on Sperm Count

Table VII tabulates the time required for the irradiation testicular damage to be reflected in the seminal fluid. The actual initial drop

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TABLE VII. EFFECT OF X-RAY IRRADIATION ON SEMINAL FLUID

RV #	Sperm Count Mean (range)	X-Ray Dose	Days Post-Irradiation: Last Normal Count	First Persistent Low Count	First Persistent Count <1.0 Million	No. Days Below 1.0 Million	First Persistent Return to >1.0 Million	First Persistent Return to Control Level
14	59 (33-79)	400r	31	45	59	420	479	>500
26	71 (14-159)	"	52	66	66	489	555	>514
22	113 (62-185)	"	12	19	76	204	280	>280
5	141 (54-220)	"	49	64	70	>143	>203	>203
23	302 (195-410)	"	15	29	81	440	521	>521
3	157 (81-343)	100r	47	54	131	124	255	>517
4	67 (16-229)	"	39	53	74	<356	<430	>437
19	13 (10-17)	"	31	38	80			
16	56 (32-84)	"	41	48	99	374	473	>585
6	59 (38-89)	"	40	47	110	439	549	>556
27	62 (39-105)	"	-	20	99	>459	>558	>558
38	122 (22-210)	"	51	65	107	> 91	>198	>198
28	42 (8-89)	"	50	57	57	>140	>197	>197
32	75 (39-128)	"	14	31	98	>105	>203	>203
15	225 (135-366)	50r	55	62	142	172	314	>433
17	60 (12-186)	"	42	56	167	188	355	355
8	23 (5-52)	"	87	94	115	138	253	344
21	69 (26-125)	30r	55	67	118	23	141	-
30	174 (76-300)	"	49	56	187	28	215	-
29	19 (4-51)	"	46	60	60	252	312	>263
34	55 (24-79)	"	60	67	234	-	-	-
20	211 (95-358)	15r	81	87	-	-	-	374
18	163 (82-286)	"	144	193	-	-	-	294
31	220 (80-416)	"	127	134	-	-	-	148
41	324 (199-652)	"	50	82	-	-	-	-
10	31 (9-55)	7.5r	>180	-				
13	29 (4-87)	"	49	76				136
63	120 (45-239)	sham	> 79	-				
66	112 (54-162)	"	43	85				
72	169 (78-274)	"	> 79	-				
19	13 (10-17)	"	46	60				158

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in sperm count lies somewhere between the last normal count and the first persistent low count observed. The mean number of days in this table, where completed, may be summarized as follows:

X-Ray Dose	Last Normal Count	First Persistent Low Count	First Count Below 1 Million	No. Days Below 1 Million	First Return to Over 1 Million	Return to Control Levels
400r	32	45	70			
100r	35	46	95			
50r	61	71	141	166	307	
30r	53	63	150	101	223	
15r	101	124	none	none		272
7.5r		no consistent reduction				

The day on which the first persistent low count was observed was fairly uniform, with the exception of the 15 and 7.5r doses where azo- or severe oligospermia was not achieved. The time required for sperm production to drop below one million per ml appears to demonstrate an inverse dose-response relationship. This should prove to be a useful parameter for assessing the magnitude of radiation exposure to the testes.

The data with respect to recovery of sperm production are incomplete at this time.

III. Alterations in Spermatogenesis Following X-ray Irradiation - A Quantitative Histologic Study

Prior to irradiation control observations were made on each subject. These included seminal fluid examinations, urinary gonadotrophin assays and a bilateral testicular biopsy. Since the biopsy procedure, per se, can affect spermatogenesis, x-ray was not administered until we could determine by a minimum of three consecutive sperm counts that the post-biopsy period was comparable to the pre-biopsy control period.

A 250 KV G. E. Maximar machine was used to deliver a mid-organ dose of 100r (95 rads) to both testes. The subject was positioned in the supine attitude with the penis taped to the lower abdominal wall. The legs were slightly spread and a plastic-contained bolus of sugar supported the testes. A 10 x 10 cm cone was used and the F. S. D. was 75 cm. The x-ray beam was collimated toward the testes but also included a small portion of the upper thighs. The AP diameter of the testes was measured, which served as the basis for the calculations of mid-organ exposure.

Post-irradiation testicular biopsy specimens were obtained from six subjects. Each subject represented a different time interval following x-ray exposure. In this fashion the intervals of 9, 15, 24, 27, 33 and 49 days post-irradiation were studied. Testicular biopsy specimens obtained from 9 subjects served as our control frame of reference. Each biopsy specimen was obtained under local anesthesia with demerol, atropine and secobarbital premedication. After procurement, the specimens were immediately immersed in freshly-prepared Cleland's or Zenker-formol fixative. The paraplast-embedded specimens were sectioned at 5 micra and stained with iron hematoxylin, hematoxylin and eosin, or by the hematoxylin-periodic acid-Schiff technique.

For analysis the various types of germinal cells were identified by the criteria of Clermont using the following abbreviations: Ad, Ap and B are A-dark, A-pale and B spermatogonia, respectively; R, L, Z and P are resting or preleptotene, leptotene, zygotene and pachytene primary spermatocytes, respectively; while D II refers to diakineses, reductional divisions and secondary spermatocytes. The spermatids were labeled Sa through Sd_2 in order of maturation. For uniformity all quantitative studies were based on Cleland-fixed material.

The counting procedure followed the same pattern for all material. A low-power photomicrograph was made of an entire representative section and all circular (transverse) tubules were numbered for identification. All cells in ten of these tubules were counted for each specimen by each of two observers. To compare the observers, identical tubules as well as different sets of tubules were counted. This was done to determine if the two observers agreed in classification and to determine if different sets of ten tubules gave reproducible results. The Sertoli cells were counted only if a nucleolus was clearly visible. All other cell types which could reasonably be identified, even though only a piece of the nucleus could be seen, were scored. Some closely-related cell types were combined for ease and uniformity of classification. For example, L-Z, Sa-Sb, and Sc-Sd were scored together. In addition to the "scoring" all sections were carefully screened to ascertain whether the quantitative data on ten tubules was consistent with gross changes.

The approximate life span of each germ-cell type, as constructed from the data of Heller and Clermont (15) was utilized in calculating back to the most likely target cell precursors for any cell found missing or reduced in numbers at the various time intervals following irradiation. Briefly, according to Clermont (16) the normal germinal epithelium is maintained by a stem-cell line of A-dark spermatogonia. This cell replicates itself every 16 days at Stage V of the germinal epithelial cycle. At Stage V of the following cycle half of these daughter A-dark cells presumably divide to form A-pale spermatogonia, while the other half replenish the basic stem-cell line. Each of the A-pale cells, so formed, divides one cycle later (again in Stage V) to form two B spermatogonia. Although each A-pale spermatogonium is capable of giving rise to two B-type spermatogonia, their numbers to not appear to be proportionately greater since the life span of the B spermatogonium is only circa 9 days. It divides during Stage II to produce the preleptotene primary spermatocyte (R). The time required for the primary spermatocytes to progress through the meiotic prophase and maturation divisions and emerge as the Sa spermatid in Stage I is 24-25 days. Then 24 days after the first appearance of the newly-formed spermatid it is released as a mature spermatozoon into the lumen and rapidly transported through the ductal system. Thus the approximate time required for spermatogenesis, exclusive of transport, is 74 days.

The first analysis of the control cell population compared the two observers' data. As seen in Table VIII, observers AB and DM counted the same total cell population for the given sets of tubules. Internal differences varied with the particular cell type. For example, there was less precise agreement in distinguishing between A-pale and B spermatogonia than in classifying the more primitive A-dark type.

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TABLE VIII

CHI SQUARE TEST FOR RANDOM VARIATION BETWEEN TWO COUNTERS

Cell Type	Number of Cells Counted dm	ab	Total	Chi Square	Probability of Random Variation
S	141.2	143.5	284.7	.01	90%
Ad	54.6	57.5	112.1	.02	90%
Ap	118.0	105.2	223.2	1.147	20-30
B	84.4	73.5	157.9	1.104	20-30
R	27.1	27.1	54.2	.01	90
LZ	80.9	73.1	154.0	.649	30-50
P	364.0	354.7	718.7	.524	30-50
DII	13.9	14.8	28.7	.006	90
Sab	445.9	508.3	954.2	2.517	10-20
Scd	283.7	299.6	583.3	.104	70-80
Totals	1613.7 49.3%	1657.3 50.7%	3271.0	6.09 DF=9	70-80

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Similarly in meiotic prophase, leptotene primary spermatocytes were found to proceed too imperceptibly into the short zygotene stage to make a reliable distinction, and these two types were combined into one group (LZ). The meiotic metaphase plates and the secondary spermatocytes were combined (D II) because they both occur so infrequently and are usually seen together. It is not possible, without the aid of special acrosomal system stains, to consistently distinguish between the Type Sa spermatids in Stage II and the early Type Sb spermatids in Stage III; the differences between Types Sc and Sd spermatids are also frequently subtle. By grouping the spermatid series into two groups (Sab and Scd) the variation between observers was minimized to the extent that all control observations could be validly combined.

The mean number of cells counted per 10 tubules, the life span of each cell type or group, and the individual coefficients of variation for the control population are shown in Table IX. In general, the CV was less than 30%, which, while high, permits a satisfactory basis for quantitation. As would be expected from the short life spans involved, the preleptotene (R), the metaphase and secondary spermatocytes (D II) are infrequent and highly variable. The CV for these groups indicates that any type of statistical validity would probably require analysis of more tubules than are usually available per specimen. It was not necessary, however, to rely on these cells to pinpoint the initial site of damage since the time intervals chosen following irradiation would reflect changes in the less variable cell types.

Aside from certain changes in the numbers of germ cells, there was no reduction in Sertoli cells nor was there any alteration in the basement membrane or tunica propria. Furthermore, there was no damage to the rich network of capillaries and small vessels.

The cell counts per 10 tubules for the various time intervals following 100r are shown in Table X with the comparison to the control mean cell counts.

In the spermatogonial series, no decrease in cell population was noted for the more primitive A-dark spermatogonia at the 9, 15, or 27-day intervals, but definite reductions were seen at 24, 33 and 49 days. The A-pale cells were reduced, but to a lesser degree, at the 9, 27, and 49-day intervals. No significant change was noted at 15, 24, or 33 days. The B spermatogonia were decreased at all time intervals, most markedly at 9 days.

A few preleptotene spermatocytes (R) could be found in all specimens except at the 9-day interval. While none were "scored" for the 49-day period, a scan of the entire section revealed an occasional R cell; this was not so for the 9-day specimen.

Leptotene-zygotene primary spermatocytes were present at each interval, but in greatly reduced numbers except at the 24-day period, where only a moderate decrease was seen. It might be noted that the ancestors of the L-Z cells, 24 days previously, were A-pale spermatogonia.

TABLE IX

Cell Type		Approximate Life Span	Mean	C.V.
Sertoli		---	146	21.8
Spermatogonia				
	Ad	Indef.	56	28.5
	Ap	16	112	12.5
	B	9	78	23.5
Spermatocytes				
	R	1	27	44.4
	LZ	7	77	28.0
	P	16	360	13.0
	DII	1	15	84.3
Spermatids				
	Sab	10	477	26.7
	Scd	14	290	20.4

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TABLE X

Cell Type	Control Mean ± S.D.	Days following Irradiation					
		9	15	24	27	33	49
Sertoli	146 ± 31.8	162	173	177	126	181	175
Spermatogonia							
Ad	56 ± 16.0	54	71	10	43	19	17
Ap	112 ± 13.9	65	97	73	63	90	52
B	78 ± 18.4	12	40	24	34	28	23
Spermatocytes							
R	27 ± 11.8	0	9	8	1	6	--
LZ	77 ± 21.6	10	22	43	16	26	2
P	360 ± 46.6	251	205	126	22	90	52
DII	15 ± 12.3	9	10	4	1	7	2
Spermatids							
Sab	477 ± 127.3	595	326	708	262	281	53
Scd	290 ± 68.8	226	394	428	112	364	188

The first striking decrease in the pachytene population was detected at 24 days and this decrease became more pronounced with succeeding lapses of time.

A few cells in reductional divisions, including occasional secondary spermatocytes (D II) were seen in every case.

Changes in spermatid numbers were evident at 49 days for both Sab and Scd groups. This does not reflect prior damage to spermatogonia since these spermatids were at the LZ or early pachytene stage of meiosis at the time of irradiation. Since this subject also had a testicular biopsy four hours following irradiation, we believe that this may have produced the early reduction in spermatids. However, by 49 days, the Sab spermatids were nearly depleted and the more mature Scd spermatids were beginning to show a decrease.

In evaluating the changes in cell counts following irradiation, the life span of each cell group as well as the ancestral precursor of that cell type at the time of exposure must be kept in mind.

Therefore our preliminary data point to the fact that the B spermatogonia were directly and severely damaged. This conclusion is based on several observations. The drastic reduction in these cells and in the preleptotene through zygotene spermatocytes at the 9-day interval covers the possible progression of all viable B spermatogonia through this time interval. This is confirmed by the subsequent decrease in pachytene spermatocytes, which first became marked at 24 days and approached extinction by 49 days. Thus the span from the earliest B spermatogonia at the time of exposure to the termination of the pachytene stage of maturation is covered.

While the life span of the B spermatogonia is only 8-9 days, that of the A-pale cells is about 16 days. Therefore, the decrease in numbers of B cells at 9 and 15 days, and the decrease in LZ spermatocytes at 15 days, reflects the direct damage sustained by the A-pale spermatogonia. The presence of B, R. and LZ at both 15 and 24 days suggests that the damage to these A-pale cells was not as extensive as that sustained by the B spermatogonia. The reduction in pachytene spermatocytes at 27 days also reflects primary damage to A-pale spermatogonia as well as to the B spermatogonia.

Considering that more than one generation of A-dark spermatogonia exists, it is more difficult to evaluate primary irradiation damage to these cells. However, since the mitotic peaks for both A-pale and A-dark spermatogonia occur during Stage V of the cycle, it may be presumed that the A-dark generations adhere to the rhythmic 16 ± 1-day cycle. Assuming this to be the case, the initial depletion in A-pale spermatogonia at 9 days probably reflects target damage to the A-dark spermatogonia, while the restoration in numbers of A-pale at 15 days would suggest repopulation by the less damaged generation of A-dark cells immediately preceding it. In any event, the lack of a uniformly severe reduction in A-pale spermatogonia in view of a sustained and marked reduction in more mature cell types suggests that much of the damage to the A-dark spermatogonia is reflected by delayed cell death. This would account for the virtual depletion of primary spermatocytes by the 49th day. The long delay of 24 to 33 days before marked reduction in A-dark spermatogonia, themselves, could also be explained by this phenomenon.

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In summary, these data suggest that 100r of x-ray produces severe and immediate damage to types B and A-pale spermatogonia. This damage results in death, either immediately or prior to cell division. In contrast, the manifestation of damage to the A-dark cells is delayed; therefore one or more divisions may occur before eventual death ensues.

IV. Evaluation of Pituitary Gonadotrophin and Plasma Testosterone Levels

A. Urinary Gonadotrophin Titers

A reciprocal relationship occurs between gonadal function and uarinary gonadotrophin levels. Thus when primary testicular damage is produced, urinary gonadotrophin titers become elevated. This increase is theoretically proportional to the degree of gonadal damage.

Measurement of serial urinary gonadotrophin titers before and following irradiation were performed: 1) to determine radiation dose necessary to produce elevated gonadotrophins, 2) to determine the time lapse following irradiation for gonadotrophin elevation to occur, 3) to relate the degree of gonadotrophin elevation to irradiation dosage, and 4) to determine which gonadotrophin(s) (FSH and/or ICSH) were responsible for the elevation.

Urine specimens were collected during a twelve hour overnight period (7:00 p.m. - 7:00 a.m.) for three or four consecutive nights and pooled for extraction. The urine was stored at 4°C before shipment in ice to our laboratory. There it was stored at 4°C until processed. The Kaolin-acetone extraction method of Albert (17) was used in which the protein material with the gonadotrophin activity was adsorbed on kaolin at pH 4.5. Then elution with NH_4OH was carried out and the eluate precipitated with acetone. The precipitate was dried and stored at 4°C until assayed.

General gonadotrophin assays (GGA), which measure both FSH and ICSH activity, were performed by the ovarian weight method in immature female rats (18). Increases in ovarian weight above uninjected control levels is gonadotrophin dose related. The response of the unknown was compared to a two-dose response curve of a known standard. The relative potency was then determined by parallel line analysis (19). Pergonal-25 was used as our reference standard until July, 1965. Thereafter, a menopausal gonadotrophin standard, UPM-1, supplied by the NIH, has been used. All data for GGA is expressed in terms of that standard in mg-eq. UPM-1 per 24 hours.

The HCG-augmentation assay of Steelman-Pohley (20) which measures primarily FSH activity is now being used instead of the GGA since our preliminary data indicate that urinary FSH levels are related more specifically to changes in the germinal epithelium. The principle of this assay is to "flood" the assay animal with an ICSH preparation, in this case HCG. By this means, a plateaued ovarian weight response is obtained. Then when a urine extract or standard preparation containing FSH is injected along with the HCG into the assay animal, a "steep" ovarian weight dose response curve is obtained. The assay design was 3 + 2 and the potency of the unknown computed by slope

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ratio analysis (21) in terms of the Second International Reference Standard (I. U./24 hours).

ICSH activity was assayed by the ventral prostate method in hypophysectomized immature male rats (22). The assay animals were not pretreated with cortisone but received orange juice, bread, carrots and dried milk in addition to Purina laboratory chow. The assay design was 2 + 1. The potency of the unknown was computed by parallel line analysis in terms of the Second International Reference Standard (I. U./24 hours). All subjects had a minimum of three pre-irradiation assays of GGA titers or, after April 1965, FSH titers. Three assays of ICSH titers were also performed in those subjects studied by this parameter. Using the statistical technique described by Finney, the weighted mean and standard deviation was computed for all control assays excluding those assays in subjects with a sperm count below 60 million/cc at the time of urine collection or for a two month period following the control urine specimen collection period. Our normal population had the following levels of urinary gonadotrophin hormones per 24 hours:

GGA - mean 1.1 mg-eq. UPM-1/24 hours
upper limit = 2.3 mg-eq. UPM-1/24 hours

FSH - mean 6.0 I. U./24 hours
upper limit = 13.6 I. U./24 hours

ICSH - mean 6.8 I. U./24 hours
upper limit = 19.8 I. U./24 hours

In view of the rather wide confidence limits for these bioassays, the criteria for determining significant post-irradiation elevation were as follows:

1. lower 95% confidence limit of any post-irradiation assay had to be above 2 S. D. of the weighted mean for the control population (i. e., upper limit indicated in previous tabulation);

2. lower 95% confidence limit of any post-irradiation assay had to be above the 95% confidence limit of the individual's own control level.

The second criterion was necessary for those subjects who had sperm counts of less than 60 million/cc during the pre-irradiation control period. Some of these individuals had control gonadotrophin titers which were above our normal population's control titers.

Urinary ICSH activity was measured in fourteen subjects who received 15 - 400r (Table XI). Due to the wide confidence limits for this assay, small changes in ICSH titers can not be detected. However, no statistically significant elevation in ICSH levels was noted following exposure to x-ray in the doses indicated.

Urinary estrogen excretion titers were measured by the immature rat uterine weight method (23). These titers failed to show any decrease during the post-irradiation period.

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TABLE XI

ICSH TITERS FOLLOWING IRRADIATION

Radiation Volunteer Number	X-ray Dose	Highest Control Titer (I.U./24 hrs.)	Highest Post-Irradiation Titer (I.U./24 hrs.)
18	15r	2.0*+	2.4*+
20	15r	18.6+	5.1 (3.5 - 7.4)
21	30r	9.2 (4.3 - 19.6)	11.92*+
30	30r	5.7 (2.7 - 12.0)	5.7 (3.8 - 8.7)
8	50r	6.9 (3.3 - 14.5)	7.5 (3.0 - 18.4)
15	50r	2.5 (1.2 - 5.2)	8.4 (4.0 - 17.9)
17	50r	10.6 (4.9 - 22.8	9.2 (4.3 - 19.8)
3	100r	2.0*+	12.4 (4.9 - 31.4)
6	100r	7.7 (3.7 - 16.4)	15.5 (6.93 - 34.71)
16	100r	9.72 (4.5 - 20.9)	12.23 (5.6 - 26.7)
28	100r	23.07 (10.3 - 51.6)	25.9 (14.1 - 47.6)
14	400r	2.0*+	11.43 (5.3 - 24.8)
23	400r	2.0*+	8.78 (4.1 - 14.9)
26	400r	8.14 (3.8 - 17.3)	10.4+

* In some assays, a definite titer was not obtained due to limited amount of urine extract. The numbers listed then indicate the maximum titer that could be present.

\+ Values so listed are approximation of mean titers, data were insufficient to calculate 95% confidence limits.

The urinary ICSH and estrogen data correlate well with more direct measurements of Leydig-cell function, namely plasma testosterone levels. Furthermore, no subject developed clinical evidence of androgen deficiency. The results of plasma testosterone determinations will be reported elsewhere in this report. There was no indication, therefore, that doses of x-ray up to 400r produce any significant alteration in Leydig-cell hormonogenesis.

Data with respect to GGA and FSH titers from all subjects receiving 15 - 400r are presented in the following paragraphs. Two volunteers received 7.5r, but their data is not complete at this time.

400r (Table XII): Five subjects were studied at this dosage. All showed prompt drops in sperm count to azoospermic levels by 75 days. Four inmates showed significant elevation in GGA titers within 60 days while the fifth subject demonstrated elevated titers between 46 to 103 days following irradiation. At this high dose of x-ray, the GGA elevation was more rapid and consistent than at any of the lower dosage levels. One inmate studied by assay of FSH titers showed the same response pattern as those in which GGA titers were measured.

100r (Table XIII): Nine subjects received 100r; five were evaluated with GGA assays and four were evaluated with FSH assays. In the group studied with GGA, four of the inmates showed significant elevation in post-irradiation gonadotrophin levels. One volunteer, RV 3, maintained normal GGA titers up to 345 days even though his sperm count dropped below control levels at the expected time. Furthermore, he became azoospermic at 131 days. Urine collections were reliable and he remained in good general health. It may be that some gonadotrophin inhibiting substance was present in his urine to explain the lack of rise in GGA titers. No further studies have been carried out to clarify this finding. In contrast to the findings in men receiving 400r, the time lapse for GGA titers to increase was quite variable. The increased titers were noted during the first, second, third and fourth months, respectively. On the other hand, the sperm count drop was quite uniform at circa 81 days. The "timing" for the peak elevation to appear was also variable and was not related to the onset of elevation.

FSH assays were carried out in four subjects. A rather consistent elevation in FSH titers was noted 60-90 days following irradiation. The elevation in FSH levels correlated more reliably with the time when the sperm count was decreasing to azoospermic levels.

50r (Table XIV): GGA titers were measured serially in three subjects who received 50r. Two men showed increased titers, one between 78-108 days and one between 67-89 days. The remaining subject did not shown an increase by 365 days. All men demonstrated the same pattern of sperm count drop reaching azoospermic levels by an average of 141 days. The increased gonadotrophin titers in this small sample resembled the pattern observed at 100r. However, the sperm count drop in the 50r group was significantly delayed beyond that for the 100r group.

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TABLE XII: GONADOTROPHIN TITERS FOLLOWING 400r

GENERAL GONADOTROPHIN LEVELS						
RV No.	Last Normal Gonadotrophin*	Initial Elevated Gonadotrophin*	"Peak" Elevation*	Highest Titer (mg-eq UPM-1/24 hr)	Sperm Count	
					Onset of Initial Drop*	Onset of Azoospermia*
5	-	57	90	6.5	64	70
14	28	56	165	5.4	45	59
23	-	35	200	7.8	29	81
26	46	103	190	5.3	66	66
FSH LEVELS				(I.U./24 hr)		
22	25	55	163	.65	19	76

*The numbers under these columns indicate the days following x-ray exposure.

TABLE XIII: GONADOTROPHIN TITERS FOLLOWING 100r

GENERAL GONADOTROPHIN LEVELS					Sperm Count	
RV No.	Last Normal Gonadotrophin*	Initial Elevated Gonadotrophin*	"Peak" Elevation*	Highest Titer (mg-eq UPM-1/24 hr)	Onset of Initial Drop*	Onset of Azoospermia*
3	345	-	-	-	54	131
4	20	68	115	4.8	53	74
6	90	120	252	7.3	47	110
16	28	30	58	12.8	48	99
27	56	96	108	7.3	20	99
FSH LEVELS				(I.U./24 hr)		
28	64	104	104	100	57	57
32	-	63	118	58	31	98
38	64	84	84	20	65	107
74	56	83	83	20	+	

*The numbers under these columns indicate the days following x-ray exposure.

+Vasectomized before sufficient observations could be made.

3002661

19-B

TABLE XIV: GENERAL GONADOTROPHIN TITERS FOLLOWING 50r

RV No.	Last Normal Gonadotrophin*	Initial Elevated Gonadotrophin*	"Peak" Elevation*	Highest Titer (mg-eq UPM-1/24 hr)	Sperm Count	
					Onset of Initial Drop*	Onset of Azoospermia*
8	78	108	163	4.0	94	115
15	67	89	89	6.5	62	142
17	365	-	-	-	56	167

*The numbers under these columns indicate the days following x-ray exposure.

30r (Table XV): GGA titers were studied in two subjects and FSH titers were studied in two subjects who received 30r. There was no significant rise in GGA titers by 156 and 197 days whereas FSH titers rose significantly between 81 and 123 days. Each of the four subjects developed azoospermia. At this low x-ray dose, the FSH assay appears to be a more sensitive indicator of germinal cell damage.

15r (Table XVI): At this dose two subjects were studied by assaying GGA and two were studied by assaying FSH titers. In the GGA group, one man developed elevated levels between 108-135 days, while the other subject had no elevation at 135 days. No further studies were possible in this subject. In the FSH group, one subject demonstrated increased titers 69-81 days post-irradiation while the other subject showed no elevation by 246 days. Each of the four men became oligospermic but did not develop azoospermia.

Conclusions:

1. The elevation in GGA titers is due to an increase in the FSH moiety and is a reflection of the germinal-cell damage produced by x-ray irradiation.

2. ICSH titers remain normal, which is consistent with other data indicating that Leydig-cell function is not altered by the doses of x-ray we employed.

3. Specific "FSH" titers were more often elevated than GGA at the lower doses where germinal epithelial destruction was less marked. Therefore, measurement of FSH titers appears to be a more sensitive indicator of spermatogenetic function than GGA levels.

B. Plasma Testosterone Levels

These studies were carried out in collaboration with Dr. H. Gandy in the Department of Obstetrics and Gynecology at Cornell Medical School. A double isotope method was used for measuring plasma testosterone.

Initially we explored the possibility of a diurnal variation in plasma testosterone levels. For this purpose plasma samples were drawn at 0800 and 2000 in five subjects. 17-hydroxysteroids were measured in the same specimens. In contrast to the reduction in plasma corticoids at 2000, there was no significant decrease in testosterone levels in the evening. Individual testosterone levels varied between 0.3 and 0.88 μg %.

Next we evaluated day-to-day variations in the same individual. For this purpose, plasma specimens were drawn at 0900 for 6-7 consecutive days in three subjects. The lowest and highest testosterone levels were 0.62→0.90, 0.62→0.86, and 0.3→0.52 μg % for each of the three men so studied.

Finally, plasma testosterone levels were studied in ten men before and at varying intervals following exposure to 15, 30, 100 and 400r. Although considerable variation in levels was noted, there was no definite pattern to indicate a significant reduction in testosterone production by the Leydig cells.

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TABLE XV: GONADOTROPHIN TITERS FOLLOWING 30r

GENERAL GONADOTROPHIN LEVELS						
RV No.	Last Normal Gonadotrophin*	Initial Elevated Gonadotrophin*	"Peak" Elevation*	Highest Titer (mg-eq UPM-1/24 hr)	Sperm Count	
					Onset of Initial Drop*	Onset of Azoospermia*
21	156	-	-	-	67	118
30	197	-	-	-	56	187
FSH LEVELS				(I.U./24 hr)		
29	84	123	150	60	67	234
34	81	102	102	55	60	60

*The numbers under these columns indicate the days following x-ray exposure.

TABLE XVI: GONADOTROPHIN TITERS FOLLOWING 15r

RV No.	Last Normal Gonadotrophin*	Initial Elevated Gonadotrophin*	"Peak" Elevation*	Highest Titer (mg-eq UPM-1/24 hr)	Sperm Count: Onset of Initial Drop*	Sperm Count: Onset of Azoospermia
GENERAL GONADOTROPHIN LEVELS						
20	108	135	204	8.0	87	-
FSH LEVELS				(I.U./24 hr)		
31	246	-	-	-	134	-
41	69	81	180	30	82	-

*The numbers under these columns indicate the days following x-ray exposure.

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Finally, plasma testosterone levels were studied in ten men before and at varying intervals following exposure to 15, 30, 100 and 400r. Although considerable variation in levels was noted, there was no definite pattern to indicate a significant reduction in testosterone production by the Leydig cells. (See Table XVII below.)

TABLE XVII

Dose	No. Pts.	Control	4 hrs.	72 hrs.	2 wks.	2 mos.
		(plasma testosterone µg %)				
15r	2	.71 1.03	.62 .92	.81 .88	.86 -	- -
30r	2	.65 1.44	.52 1.06	.30 .56	.50 -	- -
100r	3	1.11 .47 .52	.98 .53 .62	.82 .67 .55	.99 .70 .55	- - -
400r	3	.37 .81 .75	.53 - .35	.50 - .40	.42 .84 .32	.44 1.00 .77

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V. Chromosomal Studies

Numerous reports have documented chromosomal damage following in vitro irradiation. (24)(25)(26)(27)(28) Similar changes have been reported after in vivo irradiation. (29)(30)(31) The purpose of our study was to explore the dose-response relationship of such damage following in vivo exposure. Testicular tissue grown in short-term monolayer culture was analyzed for chromosomal changes before and at four hours and fifteen days post-irradiation. One hundred r was selected as the single dose for our preliminary investigation. As further control, chromosomal analyses of peripheral blood lymphocytes were carried out on specimens collected during the control period and at 30 minute, 4 hour and 15 day intervals following exposure to x-ray.

The blood was cultured by a modification of the method of Moorehead (32). Phytohemagglutinin-P was added to the heparinized blood. This mixture was allowed to stand in an ice bath for twenty-five minutes after which it was centrifuged at 25 g's for five minutes. Two to three ml of the cell-rich plasma were added to a two ounce medicine bottle containing TC 199, penicillin (100 units/ml) streptomycin (100 units/ml) and mycostatin (30 units/ml); then the material was incubated at 37°C under 5 % CO_2 tension for 72 hours. When the cells were "harvested," colcemid (0.2 μg/ml) was added to the culture for four hours. The cells were placed into a hypotonic solution for fifteen minutes and then fixed in acetic acid-methanol (1:3) for thirty minutes. After fixation the cells were suspended in the fixative and tripped onto a cold wet slide, allowed to air dry, then stained with tetrachrome.

Testicular tissue was cultured by the method of Steinberger and Steinberger, as described by Paulsen (33). Using histochemical techniques which identified 3-β-ol dehydrogenase, the Steinbergers demonstrated that the majority of cells grown from testicular tissue in monolayer culture were Leydig cells rather than germinal cells.(34)

For culture, the tissue was minced in Hank's Balanced Salt Solution, placed in 0.25% trypsin for fifteen minutes at 31°C. A suspension of one million cells per ml was made in the nutrient media, which contained TC 199, 20% fetal calf serum and antibiotics (penicillin 100 units/ml and streptomycin 100 μg/ml). The cell suspension was plated onto petri dishes and incubated at 31°C for a period of 10-14 days. The culture was then harvested in the same fashion as the lymphocyte cultures.

Each slide was scanned for the purpose of locating 25 suitable metaphase plates. These were then photographed, karyotyped and analyzed. The criteria of Court-Brown and associates (35) for categorizing irradiation-induced abnormalities was used in the analysis. In this method the number of cells with damaged chromosomes are counted, not the total number of abnormalities. They classified the chromosomal damage into three groups, A, B and C.

Group A cells relate to the modality. The normal modal number of 46 chromosomes per cell serves as the reference point. Abnormalities, then, are shifts from normal modality.

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Group B cells include breaks, gaps and isochromatid gaps, all of which are chromatid abnormalities (Figure 4). These changes are usually artifacts caused by tissue culture.

Group C abnormalities are chromosomal in character and fall into three types:

1) C-1 represents acentric fragments, dicentrics and rings (Figure 4). These abnormalities are unstable because the involved chromosomes are unable to replicate themselves due to the lack of a centromere or due to multiple centromeres. The acentric fragment represents one hit, the x-ray penetrating both arms which causes them to break away from the chromosome. Dicentrics represent two hits on different chromosomes, allowing a rearrangement of chromatin material and the addition of a second centromere.

2) C-2 cells include abnormalities such as translocations, deletions and inversions. These are stable abnormalities capable of continuing through chromosomal replication.

3) C-3 cells are also stable cells and are due to multiple breaks, deletions and translocations. They are normal in modality but display an abnormal karyotype. For example, one may have a trisomy in the No. 15 pair associated with a missing chromosome in the No. 6-12 pairs.

From two control blood cultures 43 metaphase plates were karyotyped. Six blood cultures obtained from the post-irradiated samples grew out 87 suitable metaphase plates for karyotyping. The data obtained from these karyotypes are tabulated in Table XVIII.

These results indicate that no significant increase in chromatid or chromosomal abnormalities occurred in peripheral blood lymphocytes following irradiation to the testis. This was expected and rules out technical difficulties in our study.

Ten control testicular cultures were obtained which produced 201 suitable cells for karyotyping. From three post-irradiation biopsy specimens the culture material produced 90 cells suitable for comparison. These data are found in Table XVIX.

The only significant increase in chromosomal abnormality was of the C_1, C_2 and C_3 types ($p = <0.01$). The analyses are listed in Table XX.

These preliminary studies show that by using our techniques and test system (testis tissue) we can demonstrate increases in chromosome abnormalities similar to those reported by others. Further studies will be required before we can state that a dose response relationship exists. Bender's data on three men exposed to neutron and gamma irradiation indicates that the regression curve may be the most important part of the dose-response relationship. (36)

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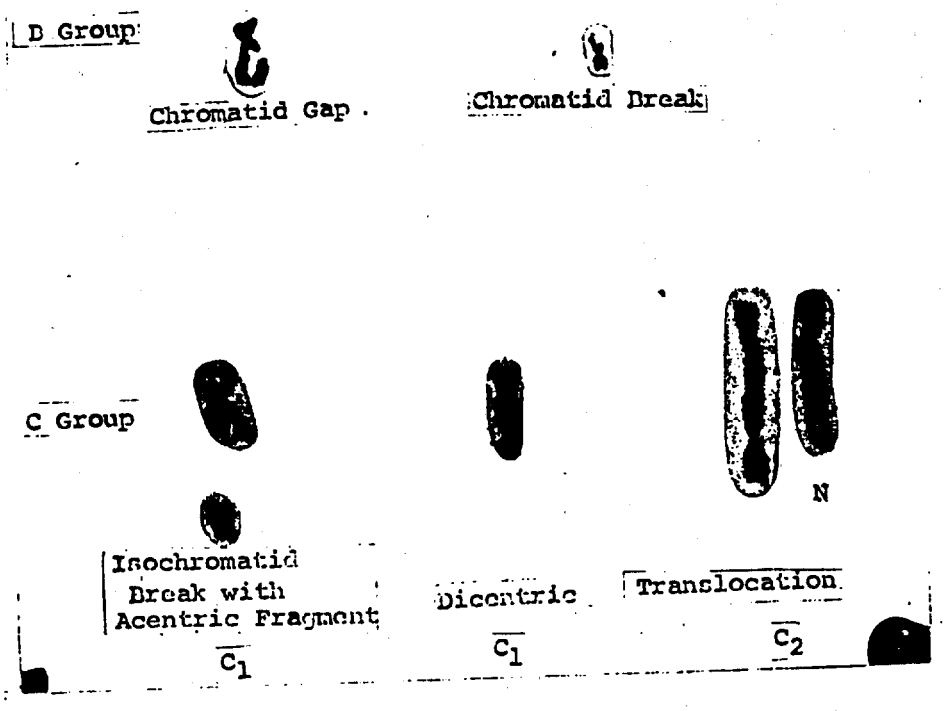
B Group
Chromatid Gap
Chromatid Break
C Group
N
Isochromatid Break with Acentric Fragment
C_1
Dicentric
C_1
Translocation
C_2

FIGURE 4

TABLE XVIII

Status	# of Cultures	Type A Cells		
		<46	46	>46
Control	2	3	35	2
Post-Irrad. (100r) 1/2 hr.-15 days	6	3	75	3

Status	Type B Cells	Type C Cells		
		C_1	C_2	C_3
Control	1	1	1	0
Post-Irrad. (100r) 1/2 hr.-15 days	1	3	1	1

TABLE XIX

Status	No. of testicular cultures	Total no. of cells karyotyped	Type A cells			Type B cells	Type C cells		
			<46	46	>46		C_1	C_2	C_3
Control	10	201	16	153	5	21	2	0	4
Post-irradiation (100r)	3	90	5	65	1	5	7	4	3

TABLE XX

	C_1 Cells (Unstable) / Modal A Cells	Significance
Control	$\frac{2}{153}$	p=<0.01
Post X-Ray (100r)	$\frac{7}{65}$	

	$C_2 + C_3$ Cells (Stable) / Modal A Cells	Significance
Control	$\frac{4}{153}$	p=<0.01
Post X-Ray (100r)	$\frac{7}{65}$	

The phenomenon of endoreduplication was also studied in a preliminary fashion. Documentation of our preliminary studies will be found as enclosure #2.

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REPOSITORY: DOE-RICHLAND
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PRISONER STUDY

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ASSIGNED NUMBER: RLHTS 94-0056

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