Title
A Proposal for a Study Investigating Possible Somatic Mutations in Veterans Exposed to Atomic Radiation

Author
McClure, Katherine J

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A Proposal for a Study Investigating Possible Somatic Mutations in Veterans Exposed to Atomic Radiation

By

Katherine Jane McClure

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THESIS

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Approved: Charles A. Walker 5/13/85
Chairman Date

Allan H. Smith

H. Harrison Scaller

HUMAN SUBJECTS PROTOCOL FOR A PILOT STUDY INVESTIGATING POSSIBLE SOMATIC MUTATIONS IN VETERANS EXPOSED TO ATOMIC RADIATION

A Master's Thesis Project

Sponsored by Health and Medical Sciences

PRINCIPAL INVESTIGATORS:

Jane McClure, MSII
UCSF-UCB Joint Medical Program

Susan Lambert, M.D.
Radiation Research Institute

Date: _____
Jane McClure, MSII

Date: _____
Harrison Sadler, M.D.

BACKGROUND AND PURPOSE: Standard chromosomal aberration tests have been a well-established, effective way of determining chromosomal structural abnormalities resulting from exposure to ionizing radiation. Recently, G. Hirsch (1978) developed an assay to examine the genetic effects of radiation exposure by determining the number of base substitions (due to repair error) in the DNA coding for hemoglobin. This assay quantifies the number of isoleucines (not normally found in the subunit) which have been incorporated into the hemoglobin protein.

The purpose of our study is two-fold. Use of these two tests in tandem will make it possible to: (1) determine whether an increased incidence of chromosomal aberrations is correlated with exposure to ionizing radiation; and (2) compare the results of the standard and the experimental assays in order to determine a) whether they support one another and b) whether the new Isoleucine Substitution Test is significantly more sensitive than standard chromosomal damage assays.

SUBJECTS AND CONTROLS: Subjects- 25 enlisted men randomly selected from a roster of all those veterans (n 250) who participated in Operation Dominic in 1962 (on file with the National Association of Radiation Survivors, an atomic veterans advocacy group) will be contacted. The addresses and phone numbers of these 25 atomic veterans will be located and each prospective subject will be called. The study will be explained to them over the phone and an informational letter will be sent to prospective subjects who express interest. Next, potential subjects will be asked to answer a standard set of questions regarding known medical or occupational exposure to radiation or other toxic substances.

Controls- 25 men matched for age and smoking history with no known exposure to medical or occupational radiation will be randomly selected from another group. Potential controls will also be contacted by phone, sent an informational letter, and screened on the basis of the same health and occupational exposure questionnaire.

SUBJECT INVOLVEMENT: Initially, each subject and control will be asked to answer a standard set of questions on a radiation and toxic exposure questionnaire. A consent form must then be signed before a single venipuncture is performed. Two 10cc vacutainers of blood will be drawn once at the single experimental session. At blood drawing, the two 10cc samples will be taken by a trained phlebotomist (Jane McClure, MSII, a PI on this study), under the supervision of a physician (Dr. Susan Lambert, M.D., licensed in the state of California and a co-PI in this study). To insure impartiality during analysis, the blood samples will be coded. One tube from each subject will be sent to the laboratory of Dr. Sanford Sherman (Children's Hospital, Oakland) for chromosomal analysis and one tube to Dr. Gerald Hirsch's laboratory (D-Tech Inc., Texas) for hemoglobin studies.

BENEFITS: This study may provide a better understanding of the relationship between exposure to nuclear radiation and possible resultant chromosomal damage. It will test whether the Hirsch hemoglobin assay is as reliable and perhaps more sensitive than standard chromosomal studies in determining mutations associated with radiation exposure.

RISKS: There is the minor discomfort of venipuncture in the forearm. Occasionally a transient black and blue mark or a minor infection may result from the venipuncture. Dr. Lambert, one of the PI's and a licensed physician in the state of California, will be able to provide treatment if complications should arise.

CONFIDENTIALITY: This is a single blind study. After samples are collected, they will be coded by an investigator (Jane McClure) before being sent to the respective laboratories for assay. The names of the participants will be known to only two of the investigators (Susan Lambert and Jane McClure) and both their identities and records will be kept strictly confidential. In the event that they study is published, no names will be revealed and all records will also remain confidential.

SUBJECTS BILL OF RIGHTS: All subjects and controls will receive a copy of the signed consent form plus the "Medical Research Subjects Bill of Rights" in their native language.

HUMAN STUDIES CONSENT FORM

TITLE: A Pilot Study Investigating Possible Somatic Mutations in Veterans Exposed to Atomic Radiation.

PURPOSE: This study will compare chromosomal damage in 25 veterans exposed to ionizing radiation with 25 age-matched males who ahve not been exposed. Blood will be drawn and each sample will be assayed by two independent methods: (1) The recently developed Hirsch Isoleucine Base Substitution Test and (2) A standard chromosomal aberration test.

PROCEDURE: You will first be asked to answer a health screening questionnaire. If on the basis of your ansewrs you qualify for the study, you will be requested to give a one time donation of blood. A single venapuncture collecting two 10cc samples will be taken by Jane McClure, who is licensed to perform the procedure, under the supervision of Dr. Susan Lambert, a licensed physician in the state of California and a co-PI in this study. We wish to collect the samples and send them to two separate laboratories which will experimentally assay these bloods for evidence of genetic damage. To insure impartiality during analysis, the blood samples will be coded. From each subject, one tube will be sent to the laboratory of Dr. Sanford Sherman for chromosomal analysis and one tube to Dr. Gerald Hirsch's laboratory for hemoglobin studies.

RISKS: There is the minor discomfort of venipuncture in the forearm. Occasionally a transient black and blue mark or a minor infection may result from the blood drawing. If this should occur, Dr. Lambert's care will be available.

BENEFITS: This study may provide a better understanding of the relationship between exposure to nuclear radiation and possible resultant chromosomal damage. It will test whether the Hirsch hemoglobin assay is as reliable and perhaps more sensitive than standard chromosomal studies in determining mutations associated with radiation exposure.

INFORMATION: Any questions I may have concerning my participation in this study will be answered by Jane McClure, MSII, at 566-7173 or Susan Lambert, M.D. at the Radiation Research Institute, 848-8056.

CONFIDENTIALITY: If the results of this study are published, my name or identity will not be revealed and my records will remain confidential.

WITHDRAWAL: I have the right to withdraw from this study at any time.

CONSENT: I agree to participate in this study voluntarily.

RIGHTS OF HUMAN SUBJECTS: I have received a copy of both this consent form and the "Medical Research Subjects Bill of Rights."

I have read the above, understand it, and hereby do ___ or do not ___ consent to the proposed research procedures.

Date: _____
Volunteer

Date: _____
Investigator

Dear ,

We would like to invite you to participate in a pilot study. We will be looking for possible abnormalities in the genetic material of veterans exposed to radiation in above ground atomic tests. We will compare these veterans to men of similar ages who have not been exposed to any known radiation or other toxic substances. We hope to contact and involve 25 atomic veterans and 25 unexposed men or "controls." We hope to begin the study in September, 1984. Participation will involve answering a brief health questionnaire and a one time donation of a 20cc sample (four teaspoons) of blood.

We hope this study may provide a better understanding of the relationship between exposure to nuclear radiation and possible resultant chromosomal damage. Two assays will be used: 1) a standard chomosomal aberration test; and 2) a new and possibly more sensitive test which looks at hemoglobin.

If you would like to volunteer for this important study, or if you have any questions or wish any more information, please contact Jane McClure at 548-5488 or Susan Lambert, M.D. at the Radiation Research Institute, 848-8056. As a volunteer, please understand that you have the right to withdraw from the study at any time.

We will be able to pay a small travel subsidy if you need such help.

Sincerely,

QUESTIONNAIRE

NAME:

AGE: DATE OF BIRTH:

MARITAL STATUS:

TYPE OF EMPLOYMENT:

SMOKING HISTORY:

RADIATION EXPOSURE:

OCCUPATIONAL EXPOSURES:

CHRISTMAS ISLAND INFORMATION:

ACKNOWLEDGEMENTS

Many people have made significant contribution to this thesis. I would like to extend my special appreciation to Dorothy Legaretta and Susan Lambert for their help in developing the study, Dr. Allan Smith for demanding a rigorous study design, Dr. Charles Dekker for his help in clarifying this thesis and Dr. Harrison Sadler for the encouragement he has given me as well as his excellent editing skills.

This Master's thesis is dedicated to the many veterans who, in the service of their country, were exposed to radiation while witnessing atomic bomb testing.

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INTRODUCTION AND RATIONALE

In recent years there has been increasing concern regarding accidental and occupational exposure to ionizing radiation and its affect on human health. Popular opinions vary from believing that radiation is composed of harmless particles that bounce off our body to a realization of its ability to insidiously attack and alter the structure of all cells. Scientifically there is great debate as to what pathologies are produced by radiation. This question is further confused by the fact that there are different types of radiation (i.e., α , β , and λ particles) each of which have different Relative Biologic Effects (RBE)[1]. Studies of the survivors of the 1945 Hiroshima-Nagasaki atomic blasts have already shown a definitive increase in the numbers of leukemas (1), breast carcinomas (2), and thyroid carcinomas (3) as compared to normal populations. Radiation has also been implicated as an etiologic agent of genetic and chromosomal abnormalities, in utero teratogenesis and many other types of cancers in addition to those mentioned above. Some studies (4,5) have proposed and found evidence indicating an accelerated aging process in the survivors of Hiroshima and Nagasaki.

Each one of us is exposed to approximately 200 millirems[2] of radiation a year due to environmental isotopes or background radiation. Our yearly dose of radiation due to dental and medical X-rays is about 700 millirems; and those receiving diagnostic medical work-ups such as a GI series are exposed to an

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1. RBE (or Quality Factor) = an assigned value which shows the relative biological effectiveness of any type of radiation compared to that of X rays or gamma rays.

2. a rem = the amount of any type of radiation which will have the same biological effects on man as 1 roentgen of X-ray or gamma radiation

additional dose of radiation of approximately 720 millirems per workup. Radiologists, nuclear power plant workers, and many other individuals knowingly or unknowingly are exposed to radiation on a daily basis. Both the scientific community and the general population are now asking "What effect are these environmental and occupational exposures going to have on our health?"

There is a great deal of controversy in the area of radiation and health. Some scientists feel that an individual's susceptability to cancer as a result of radiation exposure may depend upon his immune status or genetic make up. Adding to the confusion regarding the effects of radiation exposure on man is the tremendous amount of variability in how a radiation dose is estimated, i.e., partial versus full body dose, dosemetry badges vs dose reconstruction and the choice of formulas used in the calculations. All informed scientists agree that radiation is harmful to health, yet questions remain, such as: 1) is there a "safe" dose, 2) what kinds of pathologies are produced by different types of radiation, 3) what is the pathogenesis of radiation induced illness and, 4) can we prevent or reverse the effects of radiation exposure? Much more work needs to be done before we can even attempt to answer these questions.

This pilot study focuses on the long term effects of radiation received by a group of Air Force veterans who were exposed to above ground atomic blasts while participating in Operation Dominic in 1962. Its specific aim is to evaluate the usefulness of two biological assays in determining somatic changes after exposure to radiation. 1) A Cytogenetic Assay and 2) A Hemoglobin Isoleucine Substitution Test.

The germinal question stimulating my own interest in this study is — Can chromosomal changes such as rings, dicentrics, translocations and deletions, etc. be detected in circulating lymphocytes and is there an increase in protein synthesis errors (as measured by an amino acid isoleucine substitution test) in

the hemoglobin chain of these Air Force veterans, who were exposed to α, β, and λ radiation while witnessing fourteen atomic tests and one hydrogen bomb explosion 23 years ago?

Background

Epidemiologic studies have been by far the best way to correlate the effects of radiation on human health. Yet, because of the long latency period between exposure to ionizing radiation and resultant diseases, epidemiologic studies need to be continued often, for greater than 20 years. For this reason, other model assays need to be evaluated for their ability to determine the mutagenicity and/or carcinogenicity of ionizing radiation in humans.

Animal studies have shown a link between exposure to radiation and induction of carcinomas- yet these studies are expensive and the resulting data can be applied to humans only after an extrapolation of dubious validity. The Ames test (6), using mutant bacteria and their reversion to wild type phenotypes after exposure to radiation is even further removed than animal data in its correlation with a human systems response to radiation. There is therefore, a need to develop and refine other human assays that can provide an indication of probable radiation exposure. Assays of this type might then be used for their predictive value in determining future adverse health effects following radiation exposure.

Cytogenetic Assay:

Cytogenetics is a discipline of science which is devoted to the study of cellular chromosomes. The use of chromosomal assays to determine radiation exposure and its effects on human cells has great potential as a research tool. Cytogenetic studies of X-ray induced aberrations were conducted as long ago as 1939, when Karl Sax noted an increase in the number of both chromatid and chromosome breaks in irradiated developing microspores of Tradescantia (7). Now the potential of cytogenetics for examining the integrity of human chromosomes is well accepted. Chromosome aberrations observed in peripheral blood lymphocytes of subjects receiving radiation therapy, were first reported

by Tough et al. (8) in 1960. In the mid-sixties, the use of chromosomal analysis revealed aberrations in the survivors of Hiroshima and Nagasaki. Bloom in 1967 (9) and Sasaki in 1968 (10) also demonstrated in this population, a direct dosimetric relationship between radiation exposure and the frequency of chromosomal aberrations. By the late 60's, chromosomal analysis was being used to look at lymphocytes of Marshallese Islanders (11) and Japanese fisherman (12) exposed to nuclear fallout in the atomic testing of 1954.

Although cytogenetic chromosomal analysis appears to be a good indicator of exposure to mutagenic or clastogenic substances -- there is a great deal of controversy as to how long various types of aberrations can be detected in different lymphocyte populations (i.e., long lived B cells vs T cells etc.). Many studies have been reported which have found a significant increase in stable chromosomal aberrations (able to persist and reproduce) such as deletions and symmetric translocations in T lymphocytes of the Hiroshima-Nagasaki survivors as long as 20 years after exposure to the blast. On the other hand, Sheldon Wolff at the University of California San Francisco has demonstrated a significant increase in sister chromatid exchange (an unstable chromosomal aberration) for a definitive period of only 6-12 months following exposure to toxic chemicals (13). Intuitively it would seem unlikely that unstable chromosome changes could be detected long after radiation exposure, but Buckton et al. in 1962 demonstrated an increase in the number of both stable and unstable chromosome aberrations in patients exposed to X-ray therapy for ankylosingspondylitis as long as 26 years after treatment (14). The probability of long term effects on chromosomes is also supported by research done by Norman et al. in 1966 (15) on women who received radiation therapy for cervical carcinoma. His study showed an increase in the number of both stable and unstable aberrations as long as 13 years after radiation exposure.

Isoleucine Substitution Test:

The biochemical assay this study proposes to use was developed by Dr. Gerald Hirsch in 1978 at Oakridge National Laboratories. He developed the assay, originally, to examine somatic effects of the aging process. The α and β chains of the protein (globin) of hemoglobin normally do not contain the amino acid isoleucine. Therefore, by determining the number of isoleucine base substitutions incorporated into the hemoglobin α and β chains, errors in protein synthesis can be quantified. Using this assay Hirsch found that synthetic errors increase with age. He also found that there was a significant increase in the isoleucine substitution content in the hemoglobin of Marshallese Islanders exposed in 1954 to above ground nuclear testing. (16)

It was noted in the data collected from this research that not all Marshallese Islanders exposed to radiation had abnormal isoleucine substitution values. Although this result could reflect individual variation in susceptability or exposure, another hypothesis was raised when it was noted that certain individuals showed abnormally high isoleucine values in one sample and normal values in another. This variable expression of isoleucine in the hemoglobin of single subjects suggested that red blood cells may be expressed in a clonal fashion.

It may be that each individual is born with a full compliment of stem cells and that a small percentage of these cells are expressed at one time. These stem cells then become exhausted and replaced by other stem cells which clonally proliferate. If any clones being expressed originate from radiation damaged stem cells there would be a possibility of an abnormally high isoleucine substitution value. If the clone originated from normal stem cells one would expect to find a negligible isoleucine content. Thus, blood samples drawn from the same individual at different times could very well reveal different isoleucine

values. Hirsch in fact observed one Marshallese Islander to have abnormal hemoglobin in 1974 with a return to a normal isoleucine content in 1976. (17)

There has been research to support the theory of clonal expression of stem cells. In 1977 Warner (18) found that in ten out of 47 allopenic mice studied, the composition of red blood cells as compared to white blood cells changed by more than 20% at different assay times. In another study by Fialkow et al. in 1977 (18) it was noted that 8 women with CML who were heterzygous for dual G6PD (A & B) phenotype posessed only a single G6PD pheontype in their CML granuloytes. This single G6PD phenotype was also seen in WBC's and RBC's and platelets implying clonal expansion of a common stem cell.

If the theory of stem cell clonal proliferation of blood cells is correct, the finding of stable and unstable chromosomal aberrations many years after exposure to radiation can more easily be explained. It would also account for the fluctuating levels, over time, of isoleucine substitution seen in the Marshallese Islanders heavily exposed to nuclear radiation.

The phenomenon of cloning may justify a continuing examination of the effects of both high and low dose radiation exposure, to insure that clonal expression of damaged stem cells has not ocurred many years after intitial exposure.

Study Population:

Scientific attention has been directed towards the survivors of Hiroshima and Nagasaki, the radium dial painters (20) of WWII, and nuclear dockyard workers (21), etc. yet the "Atomic Veterans" or the veterans exposed to nuclear bomb testing between 1945 and 1962 are just now receiving scientific and political attention. Our study population is a group of veterans who were exposed, while in the active service, to radiation resulting from the last above ground atomic

testing which occurred in 1962. Twenty to forty years after radiation exposure some of these men are now finding themselves with various and surprising uncatagorized syndromes including a devastating neuromuscular disorder (22). Atomic veterans groups have sprung up as advocates for these men. Such groups are working to bring these veterans' special medical and disability needs to the attention of the government. The Nuclear Regulatory Commission in a controversial report released on July 16, 1983 (23) suggested that the veterans' claims were "unwarranted" and that an epidemiologic study would probably be a waste of time. The National Association of Radiation Survivors (NARS) has been compiling information on their members which they hope will reflect an increase in the number of cancers within this special population and perhaps prove that epidemiologic studies of these veterans are indeed warranted.

An epidemiologic survey of British "atomic veterans" has recently been initiated in Britain. In this study the government is attempting to locate and follow up on the 8,000 English service men exposed to nuclear testing in the South Pacific between 1952 and 1958. Three hundred and thirty of these men have already been looked at so far and an abnormally high incidence of leukemia and other reticuloendothelial system neoplasms (i.e., lymphomas, polycythemia vera, etc.) have been reported. (24)

Both the cytogenetic and the biochemical assay described in this proposal, have been used to measure changes in other populations exposed to radiation, but using these assays to screen atomic veterans has not been attempted before. The technologies for performing the two assays are available and laboratories have agreed to participate in the pilot. The biochemical analysis of hemoglobin will be done (on a "in kind") contribution by the developer of the isoleucine substitution test and the cytogenetics will be performed at a reduced cost by Avery Sandberg's cytogenetics laboratory in Phoenix, Arizona. This will be a

single blind study and the persons conducting the assays will receive coded samples whose origin will be revealed only after all the assays have been completed.

The answers to the questions posed by this pilot may provide very useful and relevant information to the field of radiation sciences. If a greater than normal number of somatic changes are found in the lymphocytes and hemoglobin of the subject population being studied: 1) the results may be correlated with epidemiologic data in an attempt to determine the pathogenesis and resultant pathologies produced by radiation, 2) the government must be convinced to release information on service-related radiation exposure and, 3) a full-size study should be initiated which would include a larger sample size and an increase in the number of samples taken over time.

METHODS

Subject Selection:

A total of 25 veteran air force men will be recruited from a roster of veterans (n=250) who participated in Operation Dominic in 1962 (this roster is on file with the National Association of Radiation Surviors, an atomic veterans advocacy group). This operation was chosen for many reasons: 1) U.S. Air Force and U.S. Army administrative officials will not release names and addresses of veterans that participated in nuclear weapons testing and the NARS roster was the most readily available to us; 2) Operation Dominic was a "dirty" operation consisting of 14 atomic tests and one hydrogen bomb. This means that the enlisted men were probably exposed to a considerable amount of radiation, which increases the likelihood that laboratory analysis will reveal cellular changes; 3) the majority of men in this operation lived in the Bay Area at the time of the operation and hopefully a substantial number continue to reside in the greater Bay Area and; 4) a nuclear physicist has agreed to do an independent dose reconstruction for the participants of the operation which may contribute to analysis of the results.

In selecting subjects, enlisted men on the roster who were not living in the Bay Area at the time will be eliminated and the remaining men will be numbered consecutively. Using a table of random numbers, 25 men will be selected. Their names and addresses will be looked up on microfische phonebooks. If the same names and addresses cannot be located, a thorough search of the surrounding communities will be conducted and similar names and initials etc. will be noted. All numbers obtained will be called and every attempt to locate each person selected will be made. As necessary, a second or third list of 25 randomly selected men will be compiled and researched until at least 25 men have been located and agree to be in the study. It actually may be necessary to locate

and recruit 30 subjects realizing that there may be some attrition between telephone consent and actual blood drawing. A standardized subject recruiting format will be used over the phone to avoid selection bias in who agrees to participate. Informational letters (See Appendix B) will then be sent to subjects who have agreed to participate in the study.

Control Selection:

Twenty five "nearest neighbor controls" will be selected and recruited to participate in the study. After a subject has agreed to participate we will ask him for the name and address of the neighbors adjacent to him on the right. If this household does not include a male of approximately the same age (plus or minus 5 years) or if this man refuses to participate, the house on the left will be tried. If neither of these households provide a control, the house directly across the street will be contacted, followed by the house on its right and then the house on its left, etc. Potential controls may be eliminated if they are blood relatives of the subject, do not fall in the age range, have had significant radiation exposure, or if the subject has a strong objection to the choice of control. A similar telephone recruiting format will be used for the controls as was used with the subjects. An informational letter will be sent to controls who agree to participate.

Procedure:

The extent of participation by each subject and his control will be a single, joint 45 minute session. They will be asked to answer a standard set of epidemiologic questions as well as questions regarding their health and radiation exposure. A consent form must then be signed before the venipuncture is performed. (See Appendix C and D for forms used.) Two 10cc heparenized vacutainers of blood will be drawn from a single venipuncture on each participant

by a physician licensed in the State of California. Both blood tubes will be carefully coded, checked and recorded on a master sheet. One 10cc tube will be kept at room temperature and sent to a cytogenetics laboratory in Arizona and the other 10cc tube will be put on dry ice and shipped to Austin, Texas for hemoglobin analysis.

Laboratory Methods:

1. Cytogenetic analysis

Peripheral blood specimens will be cultured, harvested and prepared according to the modified technique of Moorhead's method (25) as outlined below.

Separation of leukocytes. Add Bacto-Phytohemagglutinin (Difco Laboratories, Detroit 1), 0.2ml/10ml blood. Mix and allow to stand 30-60 minutes in an ice bath. Centrifuge at 300-350 rpm (approximately 25 G) for 5-10 minutes at 5 degrees Centigrade to sediment most erythrocytes. Remove supernatant plasma containing leukocytes in suspension. Count leukocytes.

Culture of leukocytes. Plant cells in 60 x 22 mm screw-top bottle(s) in medium consisting of (a) 30-40 per cent frresh autologous or homologous plasma; (b) 60-70 per cent TC-199 (Difco); (c) penicillin and streptomycin. Plant 8-10 ml culture(s) with 1.0-1.2 x 10^6 cells/ml. Adjust pH to 7.0-7.2 initially and maintain in this range by daily adjustments with 0.1 N HCl or CO_2 gas. Incubate undisturbed at 37 degrees Centigrade with room air as gas phase and screw-top tightened.

Chromosome cytology. After 3 days (65-70 hours) add colchicine to the culture(s) in a final concentration of 0.5-1.0 x 10^{-6} M. After 6 hours exposure to colchicine (9-10 hours yields over-contraction of the chromosomes) sacrifice culture(s) as follows. Loosen cells from bottom of bottle by agitation with pipette and centrifuge at 800 rpm for 5 minutes,

using siliconized Pasteur piopette and 12-ml conical centrifuge tube, graduated. Remove most of supernatant medium and gently resuspend cells in 5-6 ml of warm Hands' or Earle's balanced salt solution (BSS) at pH 7.0. Centrifuge as before. Remove supernatant, leaving some known volume in tube (such as 0.5ml). Carefully resuspend cells in this small volume. With continual mixing, slowly add warm distilled water, adding 3 times original volume (such as 1.5 ml added to 0.5ml). Let stand in incubator at 37 degrees Centrigrade for 3-5 minutes. Centrifuge at 600 rpm for 5 minutes. Total time in hypotonic BSS is 8-10 minutes. Make fresh fixative (1:3 of glacial acetic acid and methyl alcohol, absolute). Remove all superenatant and add 3-4 ml of fixative without distrupting the "button" of cells. Let cells stand in fixative for at least 30 minutes. Break up button and cell clumps as completely as possible by careful and repeated pipetting. Centrifuge at 600 rpm for 5 minutes and replace supernatant fixative with more of the freshly made fixative. Add about one-half ml fixative to obtain a fairly dense cell suspension. Dip a pre-cleaned slide into chilled distilled water, shake off excess, and then pipette 2-3 drops of the cell suspension onto the wet slide. Immediately tilt slide and draw off all excess fluid by holding edge of slide on blotter. drying should be completed in 30-60 seconds by fanning or by gently warming slide over a spirit flame. The quality of the slide can be checked immediately under phase contrast optics. Inadequate spreading may often be corrected by an additional change of fixative, even after long storage in the refrigerator. A routine staining dish passage of the slides from freshly filtered stain (1 per cent natural orcein, Gurr Ltd., in 60% acetic acid) through a dehydration schedule and peremanent mounting in Diaphane or Permound complete the preparation.

2. Isoleucine Substitution Test

This assay will be performed according to Hirsch's protocol discribed below (16):

Erythrocytes are separated from plasma by centrifugation for 10 min at 600 g, washed 3 times in 10 vol of saline and resuspended in an equal volume of phosphate-buffered saline. The packed red cells are lysed by adding 4 vol of cold distilled water. Red cell stroma is removed by centrifugation for 15 min at 20,000 g. Hemoglobin in the supernatant is converted to the carbon monoxide form by bubbling with CO in a hood; one drop of octanol is added to prevent foaming. The hemoglobin solutions must be kept cold during all subsequent processing (cold room at 4 degrees Centigrade).

Hemoglobin is separated form nonhemoglobin proteins first by molecular seiving over Sephadex G-200 (column size, 1.9 x 120 cm; buffer, 50 mm sodium phosphate, pH 5.8, saturated with CO; flow rate, 6 ml/h. Fractions containing the majority of the carbon monoxyhemoglobin (60-90 ml) are pooled and placed on a column (3.7 x 100 cm) of carboxymethyl cellulose (Whatman CM 23), which are washed according to manufacturer's instructions, and equilibrated with 50 mM sodium phosphate buffer, pH 5.8 (9 parts NaH_2PO_4 and 1 part Na_2HPO_4). Carbon monoxyhemoglobin is eluted using a nonlinear gradient of 1.7 l of 50 mM sodium phosphate buffer, pH5.8, in a 2-liter reagent bottle, and 1.0 l of 50 mM Na_3PO_4, in a 1-liter reagent bottle (both solutions are bubbled briefly with CO); the flow rate is 1.5 ml/min. Carbon monoxyhemoglobin eluting between pH6.7 and 6.95 is pooled and concentrated by pressure dialysis to a volume of less than 20 ml to remove much of the salt and then diluted to 40 ml with distilled water;

then the pH is adjusted to 6.0 by adding 0.5 M H_3PO_4, and the carbon monoxyhemoglobin is converted in methemoglobin by making the solution 1 mm in $K_3Fe(CN)_6$. The second chromatography is performed on one of two columns (2.5 x 100 cm) using a gradient indentical (one gradient supply for two columns) to the previous one but without CO. Methemoglobin eluting between pH 7.05 and 7.35 is concentrated, diluted, and pH adjusted as before. Methemoglobin is converted to cyanmethemoglobin by making the solution 1 mM in KCN and the cyanmethemoglobin is chromatographed on one of four columns (1.8 x 100 cm) of carboxymethyl cellulose using a gradient identical to the previous one but now also containing 10^{-4} M KCN. Cyanmethemoglobin eluting between pH 6.7 and 7.0 is concentrated by pressure dialysis and used to prepare globin, which in a few cases in further separated into α and β-chains.

Globin or chains are hydrolyzed in 6 N HCL for 21 h at 110 degrees Centrigrade. 2% of each hydrolysate is used to determine by amino acid analysis the quantity of globin or chain in each sample. For reference markers, tracer amounts of l-leucine-^{14}C and l-isoleucine-4,5-^{3}H are added to the remainder of the hydrolysate before it is chromatographed on a preparative ion exchange column (1.9 x 60 cm) of 8% crosslinked sulfonated styrene divinylbenzene copolymer (Beckman Type 150A) to separate and recover the isoleucine in the hydrolysate. The resin is equilibrated with 0.2 m sodium citrate buffer, pH 3.25 and the amino acids are eluted with 0.2 m sodium citrate buffer, pH 4.25; the flow rate is 68 ml/h. 3-min fractions of the eluate are collected and the radiotracers are used to locate fractions containing isoleucine but excluding leucine. Isoleucine-^{3}H radioactivity counts are also used

to calculate the percentage of isoleucine eluting from the preparative column which is pooled for the quantitative analysis of isoleucine in the protein hydrolysate; ie., the small quantity of isoleucine that does not completely resolve from leucine could be included in computing the quantity of isoleucine in the original sample. The pooled eluate must not contain much leucine becasue it would interfere with an accurate determination for the small quantity of isoleucine there. After adjusting the pH to 2.2 by adding 1 N HCL, the quantity of isoleucine in the pooled eluate is determined using a Beckman Model 120C amino acid analyzer. The frequency at which isoleucine substitutes for other amino acids in human hemoglobin is calculated by dividing the nanomoles of isoleucine by the nanomoles of all other amino acids in the sample.

Measurements:

1. Cytogenetic analysis will measure the number of chromosomal structural abnormalities visible in 500 stained lymphocytes per subject or control under a microscope. Technicians will be looking for stable aberrations such as deletions and translocations and unstable changes such as rings and dicentrics. The number of abnormalities seen per person will be calculated as a percentage of the total cells looked at. This percentage will be compared to the normal population which generally shows a breakage or chromosomal aberration rate of 1-3% and to the number of aberrations in the control population.

2. The Isoleucine Substitution Test will measure the amount of isoleucine found in highly purified hemoglobin. According to the DNA sequence for this protein no isoleucine should be present. In reality there is a background mutation rate of 10^{-6} due to transcriptional and translational errors. We will be looking for

a significant rise in the rate of spontaneous mutations, as measured by amino acid substitutions, which we feel is most likely due to radiation induced errors in protein synthesis and in defective repair mechanisms.

ETHICS

Although the pilot involves human subjects there is virtually no risk involved. There will only be the minor discomfort of venipuncture in the forearm and possibly a transient black and blue mark. The identities and records of all subjects will be known to the Principal Investigators alone and will remain strictly confidential. Each participant will be identified by a coded number. In the event of publication, no names will be used. The results of these assays will be available to the participants along with an interpretation of their significance.

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HUMAN SUBJECTS PROTOCOL FOR A PILOT STUDY INVESTIGATING POSSIBLE SOMATIC MUTATIONS IN VETERANS EXPOSED TO ATOMIC RADIATION

A Master's Thesis Project

Sponsored by Health and Medical Sciences

PRINCIPAL INVESTIGATORS:

Jane McClure, MSII
UCSF-UCB Joint Medical Program

Susan Lambert, M.D.
Radiation Research Institute

Date: _____
Jane McClure, MSII

Date: _____
Harrison Sadler, M.D.

BACKGROUND AND PURPOSE: Standard chromosomal aberration tests have been a well-established, effective way of determining chromosomal structural abnormalities resulting from exposure to ionizing radiation. Recently, G. Hirsch (1978) developed an assay to examine the genetic effects of radiation exposure by determining the number of base substitions (due to repair error) in the DNA coding for hemoglobin. This assay quantifies the number of isoleucines (not normally found in the subunit) which have been incorporated into the hemoglobin protein.

The purpose of our study is two-fold. Use of these two tests in tandem will make it possible to: (1) determine whether an increased incidence of chromosomal aberrations is correlated with exposure to ionizing radiation; and (2) compare the results of the standard and the experimental assays in order to determine a) whether they support one another and b) whether the new Isoleucine Substitution Test is significantly more sensitive than standard chromosomal damage assays.

SUBJECTS AND CONTROLS: Subjects- 25 enlisted men randomly selected from a roster of all those veterans (n 250) who participated in Operation Dominic in 1962 (on file with the National Association of Radiation Survivors, an atomic veterans advocacy group) will be contacted. The addresses and phone numbers of these 25 atomic veterans will be located and each prospective subject will be called. The study will be explained to them over the phone and an informational letter will be sent to prospective subjects who express interest. Next, potential subjects will be asked to answer a standard set of questions regarding known medical or occupational exposure to radiation or other toxic substances.

Controls- 25 men matched for age and smoking history with no known exposure to medical or occupational radiation will be randomly selected from another group. Potential controls will also be contacted by phone, sent an informational letter, and screened on the basis of the same health and occupational exposure questionnaire.

SUBJECT INVOLVEMENT: Initially, each subject and control will be asked to answer a standard set of questions on a radiation and toxic exposure questionnaire. A consent form must then be signed before a single venipuncture is performed. Two 10cc vacutainers of blood will be drawn once at the single experimental session. At blood drawing, the two 10cc samples will be taken by a trained phlebotomist (Jane McClure, MSII, a PI on this study), under the supervision of a physician (Dr. Susan Lambert, M.D., licensed in the state of California and a co-PI in this study). To insure impartiality during analysis, the blood samples will be coded. One tube from each subject will be sent to the laboratory of Dr. Sanford Sherman (Children's Hospital, Oakland) for chromosomal analysis and one tube to Dr. Gerald Hirsch's laboratory (D-Tech Inc., Texas) for hemoglobin studies.

BENEFITS: This study may provide a better understanding of the relationship between exposure to nuclear radiation and possible resultant chromosomal damage. It will test whether the Hirsch hemoglobin assay is as reliable and perhaps more sensitive than standard chromosomal studies in determining mutations associated with radiation exposure.

RISKS: There is the minor discomfort of venipuncture in the forearm. Occasionally a transient black and blue mark or a minor infection may result from the venipuncture. Dr. Lambert, one of the PI's and a licensed physician in the state of California, will be able to provide treatment if complications should arise.

CONFIDENTIALITY: This is a single blind study. After samples are collected, they will be coded by an investigator (Jane McClure) before being sent to the respective laboratories for assay. The names of the participants will be known to only two of the investigators (Susan Lambert and Jane McClure) and both their identities and records will be kept strictly confidential. In the event that they study is published, no names will be revealed and all records will also remain confidential.

SUBJECTS BILL OF RIGHTS: All subjects and controls will receive a copy of the signed consent form plus the "Medical Research Subjects Bill of Rights" in their native language.

HUMAN STUDIES CONSENT FORM

TITLE: A Pilot Study Investigating Possible Somatic Mutations in Veterans Exposed to Atomic Radiation.

PURPOSE: This study will compare chromosomal damage in 25 veterans exposed to ionizing radiation with 25 age-matched males who ahve not been exposed. Blood will be drawn and each sample will be assayed by two independent methods: (1) The recently developed Hirsch Isoleucine Base Substitution Test and (2) A standard chromosomal aberration test.

PROCEDURE: You will first be asked to answer a health screening questionnaire. If on the basis of your ansewrs you qualify for the study, you will be requested to give a one time donation of blood. A single venapuncture collecting two 10cc samples will be taken by Jane McClure, who is licensed to perform the procedure, under the supervision of Dr. Susan Lambert, a licensed physician in the state of California and a co-PI in this study. We wish to collect the samples and send them to two separate laboratories which will experimentally assay these bloods for evidence of genetic damage. To insure impartiality during analysis, the blood samples will be coded. From each subject, one tube will be sent to the laboratory of Dr. Sanford Sherman for chromosomal analysis and one tube to Dr. Gerald Hirsch's laboratory for hemoglobin studies.

RISKS: There is the minor discomfort of venipuncture in the forearm. Occasionally a transient black and blue mark or a minor infection may result from the blood drawing. If this should occur, Dr. Lambert's care will be available.

BENEFITS: This study may provide a better understanding of the relationship between exposure to nuclear radiation and possible resultant chromosomal damage. It will test whether the Hirsch hemoglobin assay is as reliable and perhaps more sensitive than standard chromosomal studies in determining mutations associated with radiation exposure.

INFORMATION: Any questions I may have concerning my participation in this study will be answered by Jane McClure, MSII, at 566-7173 or Susan Lambert, M.D. at the Radiation Research Institute, 848-8056.

CONFIDENTIALITY: If the results of this study are published, my name or identity will not be revealed and my records will remain confidential.

WITHDRAWAL: I have the right to withdraw from this study at any time.

CONSENT: I agree to participate in this study voluntarily.

RIGHTS OF HUMAN SUBJECTS: I have received a copy of both this consent form and the "Medical Research Subjects Bill of Rights."

I have read the above, understand it, and hereby do ___ or do not ___ consent to the proposed research procedures.

Date: _____
Volunteer

Date: _____
Investigator

Dear ,

We would like to invite you to participate in a pilot study. We will be looking for possible abnormalities in the genetic material of veterans exposed to radiation in above ground atomic tests. We will compare these veterans to men of similar ages who have not been exposed to any known radiation or other toxic substances. We hope to contact and involve 25 atomic veterans and 25 unexposed men or "controls." We hope to begin the study in September, 1984. Participation will involve answering a brief health questionnaire and a one time donation of a 20cc sample (four teaspoons) of blood.

We hope this study may provide a better understanding of the relationship between exposure to nuclear radiation and possible resultant chromosomal damage. Two assays will be used: 1) a standard chomosomal aberration test; and 2) a new and possibly more sensitive test which looks at hemoglobin.

If you would like to volunteer for this important study, or if you have any questions or wish any more information, please contact Jane McClure at 548-5488 or Susan Lambert, M.D. at the Radiation Research Institute, 848-8056. As a volunteer, please understand that you have the right to withdraw from the study at any time.

We will be able to pay a small travel subsidy if you need such help.

Sincerely,

QUESTIONNAIRE

NAME:

AGE: DATE OF BIRTH:

MARITAL STATUS:

TYPE OF EMPLOYMENT:

SMOKING HISTORY:

RADIATION EXPOSURE:

OCCUPATIONAL EXPOSURES:

CHRISTMAS ISLAND INFORMATION: