

USAFSAM-TR-90-39

AD-A242 590



van
②

LATE IMMUNOBIOLOGICAL EFFECTS OF SPACE RADIATION

William H. Stone, Ph.D.
Michael L. Miller, M.D.

Trinity University
715 Stadium Drive
San Antonio, TX 78212

December 1990

Final Report for Period February 1989 - December 1989

Approved for public release; distribution is unlimited.

Prepared for
USAF SCHOOL OF AEROSPACE MEDICINE
Human Systems Division (AFSC)
Brooks Air Force Base, TX 78235-5301



91-15629



NOTICES


This final report was submitted by Trinity University Department of Biology, 715 Stadium Drive, San Antonio, Texas, under contract F33615-87-D-0626/0005, job order 7757-04-51, with the USAF School of Aerospace Medicine, Human Systems Division, AFSC, Brooks Air Force Base, Texas. Dr. Ann B. Cox (USAFSAM/RZB) was the Laboratory Project Scientist-in-Charge.


When Government drawings, specifications, or other data are used for any purpose other than in connection with a definitely Government-related procurement, the United States Government incurs no responsibility or any obligation whatsoever. The fact that the Government may have formulated or in any way supplied the said drawings, specifications, or other data, is not to be regarded by implication, or otherwise in any manner construed, as licensing the holder or any other person or corporation; or as conveying any rights or permission to manufacture, use, or sell any patented invention that may in any way be related thereto.

The animals involved in this study were procured, maintained, and used in accordance with the Animal Welfare Act and the "Guide for the Care and Use of Laboratory Animals" prepared by the Institute of Laboratory Animal Resources - National Research Council.

The Office of Public Affairs has reviewed this report, and it is releasable to the National Technical Information Service, where it will be available to the general public, including foreign nationals.

This report has been reviewed and is approved for publication.


ANN B. COX, Ph.D.
Project Scientist


STANLEY L. HARTGRAVES, Lt Col, USAF, BSC
Supervisor


GEORGE E. SCHWENDER, Colonel, USAF, MC, CFS
Commander

REPORT DOCUMENTATION PAGE

Form Approved
OMB No 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to: Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503.

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE December 1990	3. REPORT TYPE AND DATES COVERED Final February 1989 - December 1989	
4. TITLE AND SUBTITLE Late Immunobiological Effects of Space Radiation		5. FUNDING NUMBERS C - F33615-87-D-0626 PE - 62202F PR - 7757 TA - 04 WU - 51	
6. AUTHOR(S) William H. Stone Michael L. Miller*			
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Trinity University 715 Stadium Drive San Antonio, TX 78212		8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) USAF School of Aerospace Medicine/RZB Human Systems Division (AFSC) Brooks Air Force Base, TX 78235-5301		10. SPONSORING / MONITORING AGENCY REPORT NUMBER USAFSAM-TR-90-39	
11. SUPPLEMENTARY NOTES *Dr. Miller is with the University of Texas Health Science Center at San Antonio. USAFSAM Technical Monitor: Dr. Ann B. Cox, (512) 536-3416 Animal Protocol No. RZB-82-01			
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for public release; distribution is unlimited.		12b. DISTRIBUTION CODE	
13. ABSTRACT (Maximum 200 words) The objective of this investigation was to obtain profiles of immune competence in primates more than 25 years following exposures to protons. The availability of irradiated animals provided a unique opportunity to study immune defects which could be relevant to astronauts and high-flying pilots. The Delayed Radiation Effects Colony at Brooks Air Force Base provided blood samples. Antibody-mediated immune function (associated with B-cell function) was assessed by measuring immunoglobulin levels, hemolytic complement activity and autoantibodies; cell-mediated immune function (associated with T-cell function) was evaluated by measuring selected T- and B-cell activity plus response to mitogens and interleukin production. There were no significant differences between control and irradiated animals for most parameters measured in this preliminary survey, but lymphocyte proliferation tended to decrease as radiation dosage increased. Survivors of low and intermediate doses of proton irradiation apparently show few late immunobiological effects, which is encouraging. Additional monkeys will be measured in future confirmatory studies.			
14. SUBJECT TERMS Late effects Proton irradiation Immunobiology Aging		15. NUMBER OF PAGES 32	
		16. PRICE CODE	
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT UL

TABLE OF CONTENTS

	<u>Page</u>
INTRODUCTION.	1
Specific Objectives.	1
Background	1
Methodology	3
Results	3
Effects of Freezing and Thawing on Ig Levels	12
Methodology.	12
Results	12
Level of Functional Hemolytic Complement	13
Methodology.	13
Results.	13
Incidence of Autoantibodies.	14
Methodology.	14
Results.	14
Studies of Cellular Immune Functions	16
Background.	16
MATERIALS AND METHODS	16
Isolation of Peripheral Blood Mononuclear Cells.	16
Cell Culturing	16
Procedure for Making Enriched RPMI Media	17
RESULTS	17
Proliferative Response in the Absence of Mitogens.	17
Proliferative Response of Cells Stimulated by Con A.	17
Proliferative Response of Cells Stimulated by PHA.	17
Proliferative Response of Cells Stimulated by Poke- weed Mitogen.	18
DISCUSSION.	24
CONCLUSIONS	24
ACKNOWLEDGMENTS	25
REFERENCES.	25

APPROVED FOR
 RELEASE BY
 AUTHORITY
 DATE
 11/11/2011
 BY
 A-1

List of Figures

Fig.
No.

1a.	IgG Levels for Controls (BFO=C Dose=C)	4
1b.	IgG Levels for BFO=Y Dose=H	5
1c.	IgG Levels for BFO=N Dose=H	5
1d.	IgG Levels for BFO=Y Dose=L	6
1e.	IgG Levels for BFO=N Dose=L	6
2a.	IgA Levels for Controls (BFO=C Dose=C)	7
2b.	IgA Levels for BFO=Y Dose=H	7
2c.	IgA Levels for BFO=N Dose=H	8
2d.	IgA Levels for BFO=Y Dose=L	8
2e.	IgA Levels for BFO=N Dose=L	9
3a.	IgM Levels for Controls (BFO=C Dose=C)	9
3b.	IgM Levels for BFO=Y Dose=H	10
3c.	IgM Levels for BFO=N Dose=H	10
3d.	IgM Levels for BFO=Y Dose=L	11
3e.	IgM Levels for BFO=N Dose=L	11
4.	Average control response	18
5.	Average proliferative response to Con A	19
6.	Stimulation indices for responses to Con A	19
7.	Stimulation indices for responses to PHA	20
8.	Average proliferative response to pokeweed	20
9.	Stimulation indices for responses to pokeweed	21

List of Tables

Table
No.

1.	Inventory and Tests of Serum Samples from the CRC	2
2.	Effect of Freezing and Thawing on Ig Levels in Serum of Rhesus Monkeys Stored at -70°C.	12
3.	Complement Activity (As Measured by Diameters of Hemolytic Plaques) and CH ₁₀₀ Hemolytic Units of Rhesus Monkeys	14
4.	Frequency of Autoantibodies in Serum of Rhesus Monkeys.	15

LATE IMMUNOBIOLOGICAL EFFECTS OF SPACE RADIATION

INTRODUCTION

Specific Objectives

The overall objective here was to obtain a profile of the immune competence in rhesus monkeys more than 25 years after exposure to simulated space radiation. To this end, the studies were divided into 2 subtasks:

- (1) To assess the antibody-mediated immune (AMI) function by measuring immunoglobulin (Ig) levels, hemolytic complement activity, and autoantibodies. These parameters are associated with B-cell function.
- (2) To assess the cell-mediated immune (CMI) function by measuring selected T- and B-cell activity as well as response to mitogens and interleukin production. These parameters are associated with T-cell function.

Subtask 1 was performed at Trinity University, Department of Biology, under the direction of primary investigator, Professor William H. Stone. Subtask 2 was performed at the University of Texas Health Science Center under the direction of Dr. Michael L. Miller.

Background

The rhesus colony at Brooks Air Force Base Chronic Radiation Colony (CRC) has been maintained for about 25 years to gain information regarding late effects of exposure to ionizing radiations. To date, the most dramatic effects have been life-shortening associated with organ degeneration, cancer, endometriosis, and opportunistic infections (Yochmowitz, et al., 1985).

The availability of the CRC of rhesus monkeys provides a unique opportunity to study immune defects which would be of great potential relevance to human diseases that may occur in astronauts and high-flying pilots.

Previously, some pilot studies were completed which suggested that these syndromes could have a common etiology related to aberrant immunologic function. Three immunologic parameters of the serum were studied: (1) Ig levels, (2) total complement activity, and (3) the frequency of autoantibodies. Some differences were

observed in all 3 of these parameters. Most notable was the exceptionally high frequency of autoimmune antibodies as compared to an age-matched unirradiated control group of rhesus monkeys. These preliminary studies suggested that we should include a series of function assays of immune competence. The monkeys have already passed more than their median life span; therefore, it was important to initiate these studies as soon as possible while the animals were still alive.

In Table 1, we present an inventory of the serum samples from the CRC that are available for this study. In all, we have over 2,500 samples in our freezers, of which over 1,500 are from the CRC monkeys obtained over a span of 5 years, representing 13 bleeding dates. The other samples are from nonirradiated monkey colonies other than the CRC. Whereas the task called for the testing of only 60 samples from the CRC, we have tested more than 800 samples to obtain a complete picture of the variation in these parameters over time. We have over 1,000 serum samples from the Wisconsin Regional Primate Research Center (WRPRC). These samples will provide age-matched unirradiated controls for the CRC animals, as well as animals of different ages. This setup will permit us to evaluate the effects of aging in the CRC colony, since most of the monkeys in the CRC colony are over 25 years old.

TABLE 1. INVENTORY AND TESTS OF SERUM SAMPLES FROM THE CRC

Sample Bleeding Date Year/Mo.	Approximate Number of Samples	Tested for 3 AMI parameters	Data Entered in Computer (Y=yes, N=no)
85/03	80	Y	Y
85/08	71	N	N
85/11	80	N	N
86/03	142	Y	Y
86/08	150	N	N
86/11	149	N	N
87/03	139	Y	Y
87/08	135	Y	Y
88/01	130	Y	Y
88/08	125	N	N
89/01	122	Y	N
89/08	118	N	N
90/03	104	Y	N

Methodology. The serum samples were assayed by a single radial immunodiffusion technique using commercially available (Kallestad Laboratories, Inc., Austin, TX) immunoplates that are routinely used for quantitative assays of human Ig levels. It seemed justified to use the human system to test the monkeys, because the major Ig classes are about 90% homologous among the various primate species; and because our aim was to compare the levels of Ig among the monkeys themselves. All of the tests were set up and read at 18 and 72 h by the same operator, taking careful note of the lot number and the standard reference curves for each test kit. The samples were suitably diluted to obtain clear-cut reactions (i.e., diameters of precipitation rings). A computer program was written that converted precipitation ring diameters to international units based on the standard curve for each lot of Ig plates. IgG, IgA and IgM levels were assayed.

Results. A preliminary analysis of the data from bleed sample 87/08 indicated that there was no significant ($p > 0.05$) difference between the irradiated and control monkeys in mean levels of the 3 classes of Ig. On the other hand, there were as many as 10 monkeys in both treated and control groups with Ig levels 2 or more standard deviations away from the mean values suggesting some pathological condition. However, upon checking the health records of these 10 monkeys, we could find no clear-cut correlation between the aberrant Ig values and their health status.

At this point in our studies, we decided to group the irradiated monkeys into 4 groups according to radiation dose and whether or not the type of radiation would or would not reach the blood-forming organs (bone marrow, spleen, liver, etc.). This decision seemed justified on 2 grounds: (1) the immunologic parameters that we were studying are elaborated by the blood-forming organs (BFO), and (2) there were too few monkeys in each of the several dose and energy radiation classes with which to carry out reliable statistical analyses.

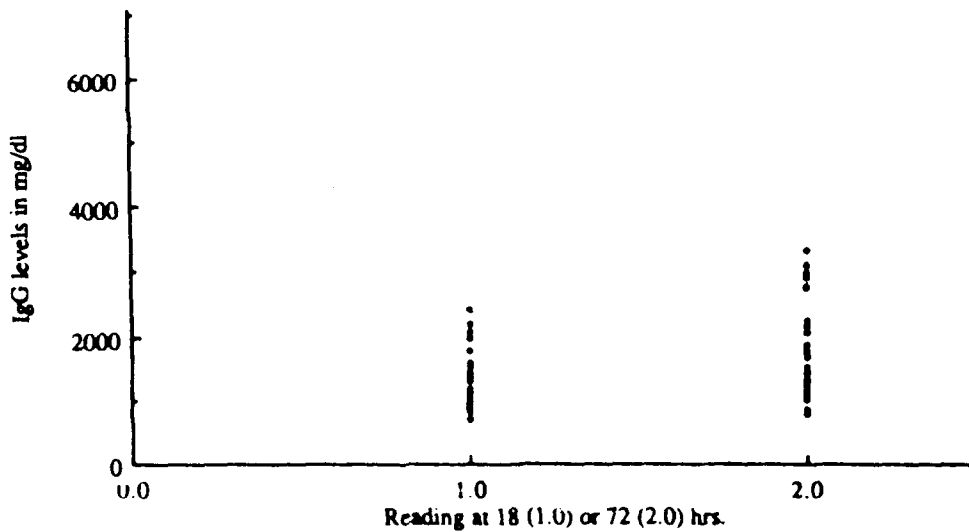
Thus, the 4 groups were:

<u>Dose (rads)</u>	<u>Penetration to BFO</u>
1. High dose (More than 200 rads)	: No BFO (32 or 55 MeV)
2. Low dose (Less than 200 rads)	: No BFO (32 or 55 MeV)
3. High dose (More than 200 rads)	: Yes BFO (138, 400 or 2,300 MeV)
4. Low dose (Less than 200 rads)	: Yes BFO (138, 400 or 2,300 MeV)

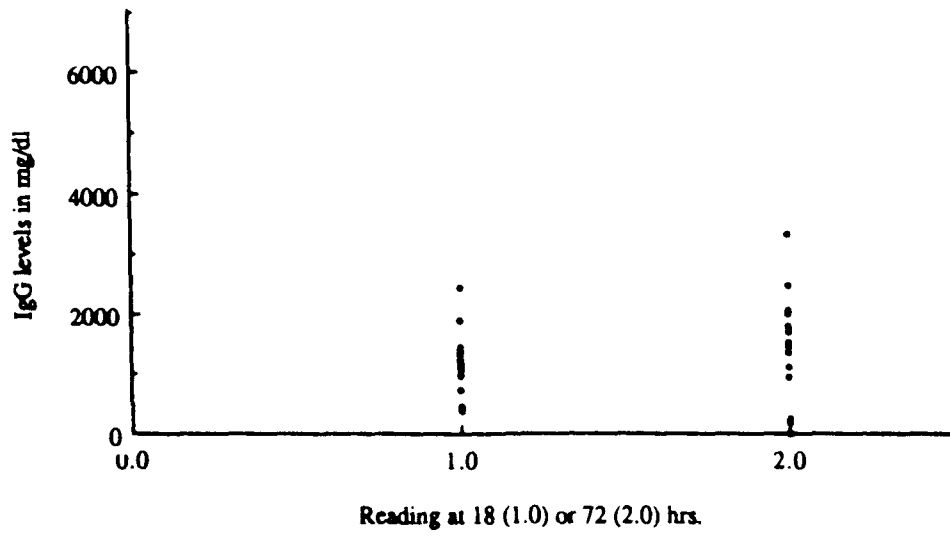
These analyses have just begun. Figures 1, 2, and 3 (a, b, c, d, and e) are dot plots of the data on the Ig levels (G, A, and M) among the samples obtained in August 1987 (sample bleeding date 87/08). Each Ig class is plotted for both the 18- and 72-h readings in the 4 different treatment classes.

A comparison of the results of the controls vs. the irradiated monkeys shows no significant differences. The preliminary results (not yet statistically analyzed) would seem to confirm our earlier analyses suggesting that there is no detectable effect of irradiation on the Ig (G, A, and M) levels in the survivor monkeys. Similar analyses of data for the other bleeding dates are underway.

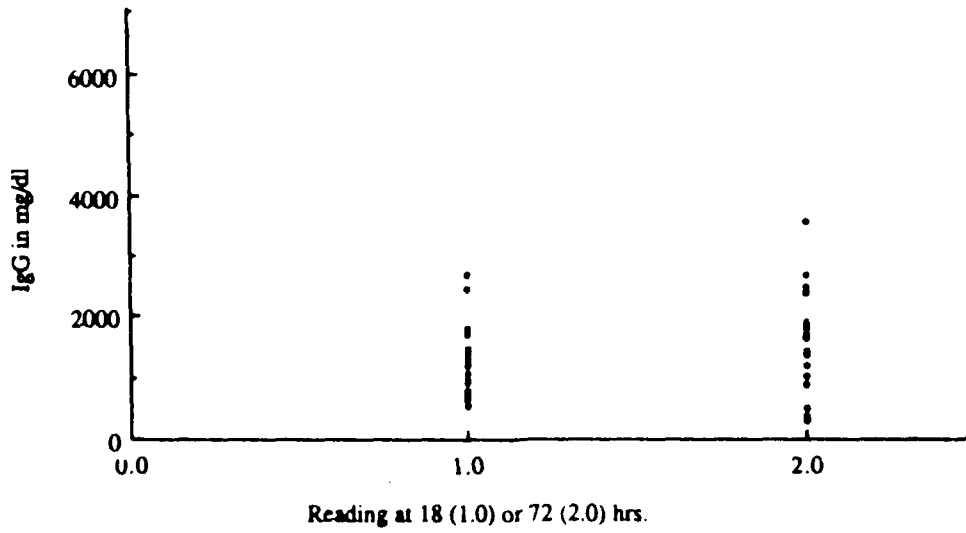
FIGURES 1, 2 & 3 (a, b, c, d and e): Dot plots of the IgG, A, and M levels in each of the 4 treatment categories and the levels in the untreated controls, expressed in mg/dl at the 18- and 72-h readings.



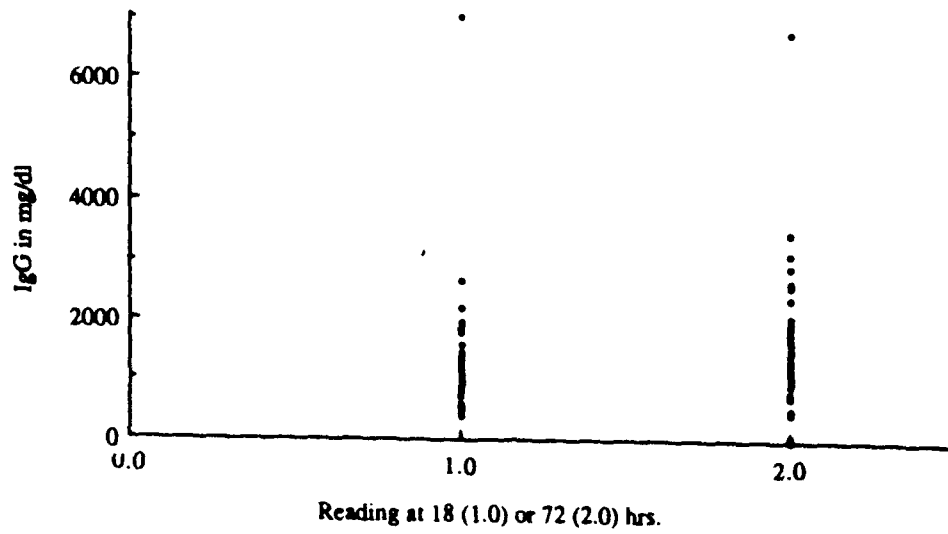
1a. IgG levels for controls (BFO=C DOSE=C).



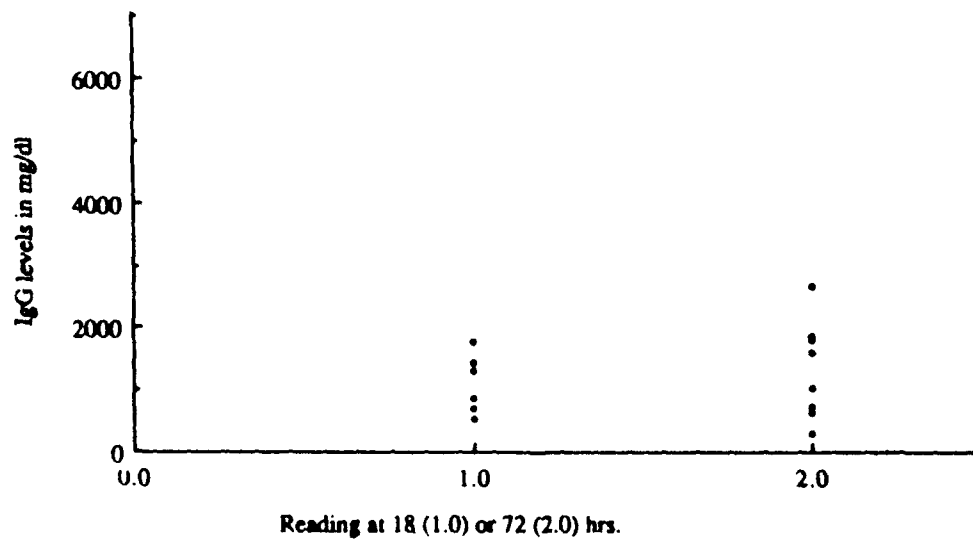
1b. IgG levels for BFO=Y Dose=H.



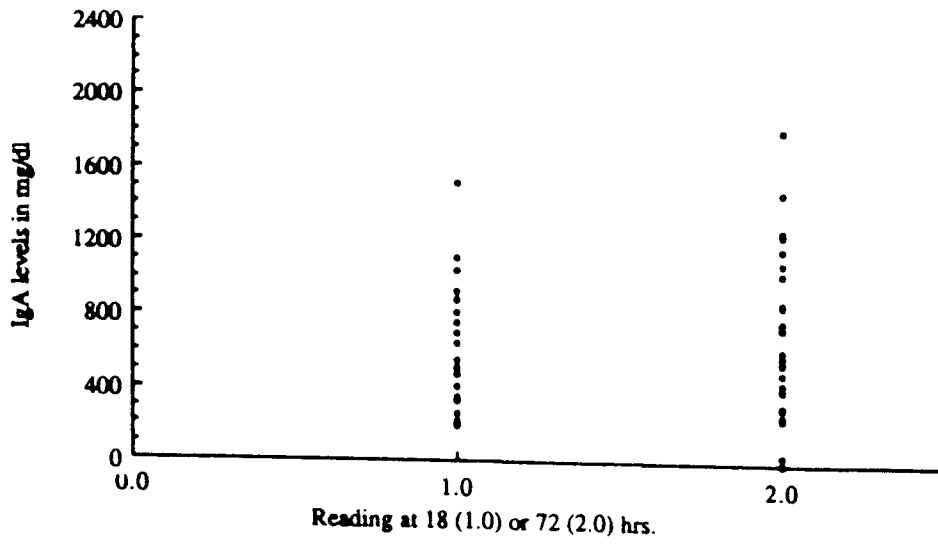
1c. IgG levels for BFO=N Dose=H.



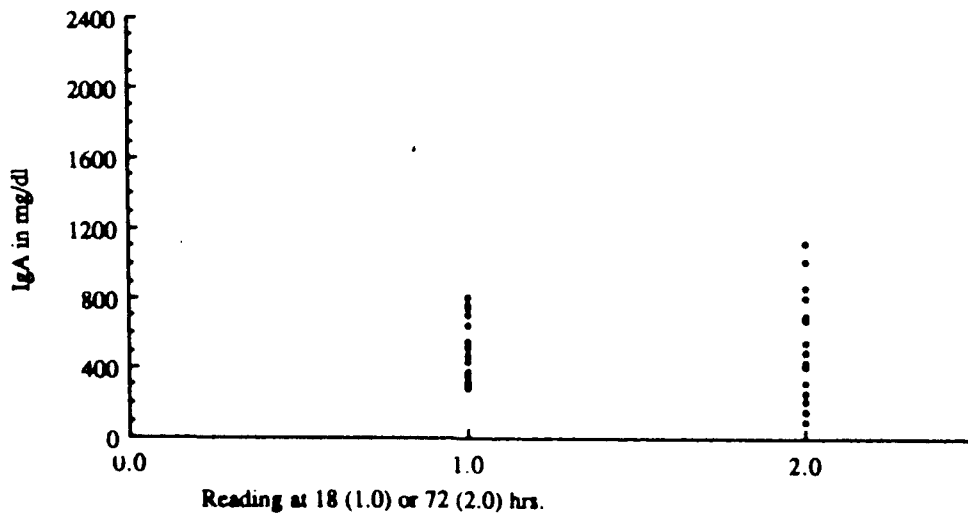
1d. IgG levels for BFO=Y DOSE=L.



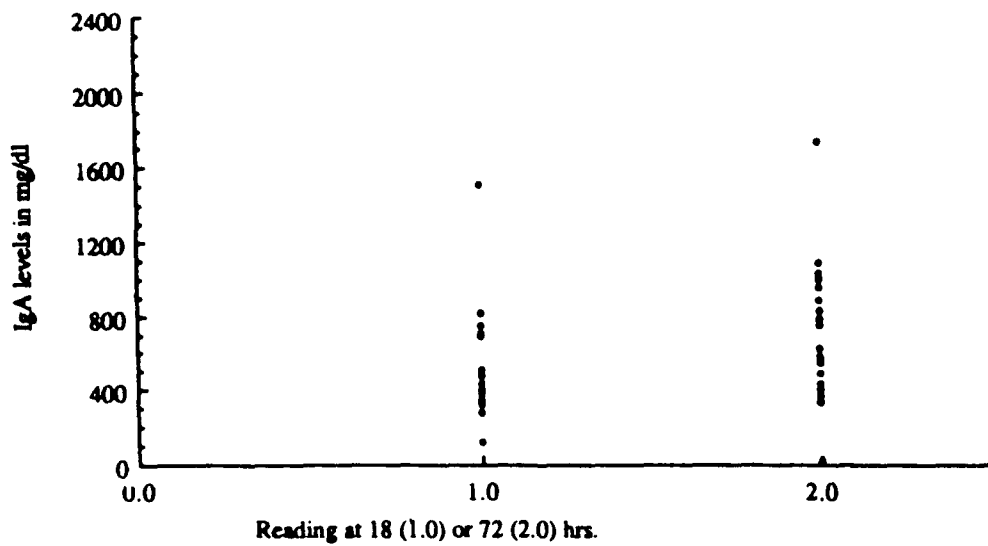
1e. IgG levels for BFO=N DOSE=L.



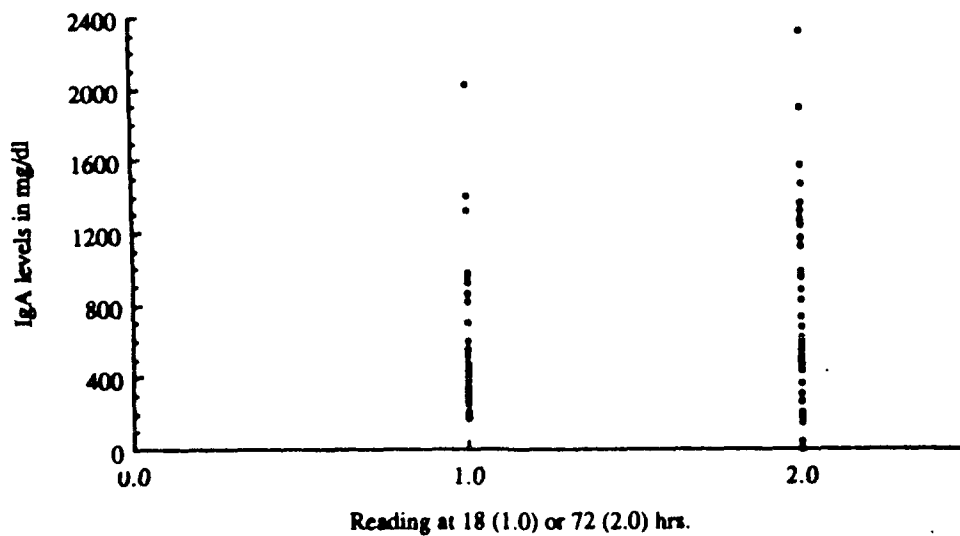
2a. IgA levels for controls (BFO=C DOSE=C).



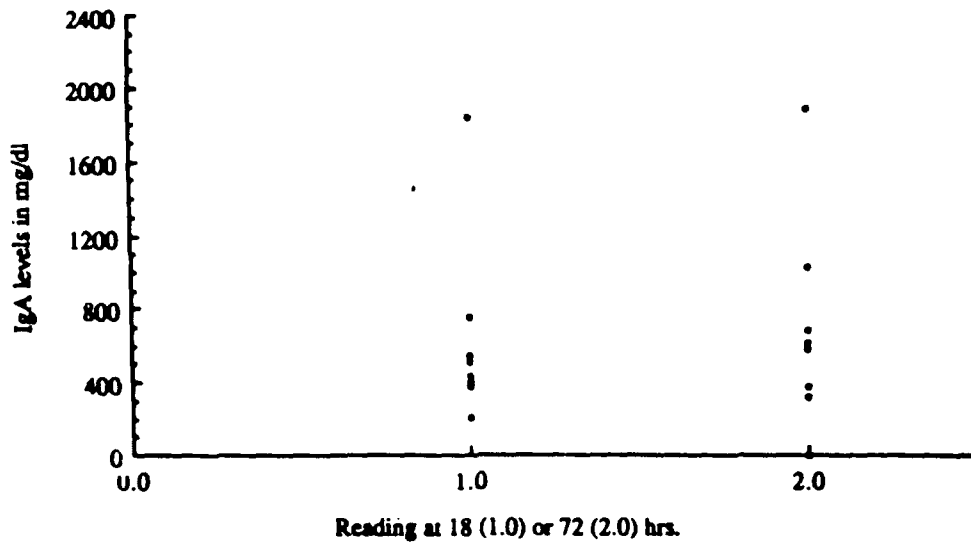
2b. IgA levels for BFO=Y DOSE=H.



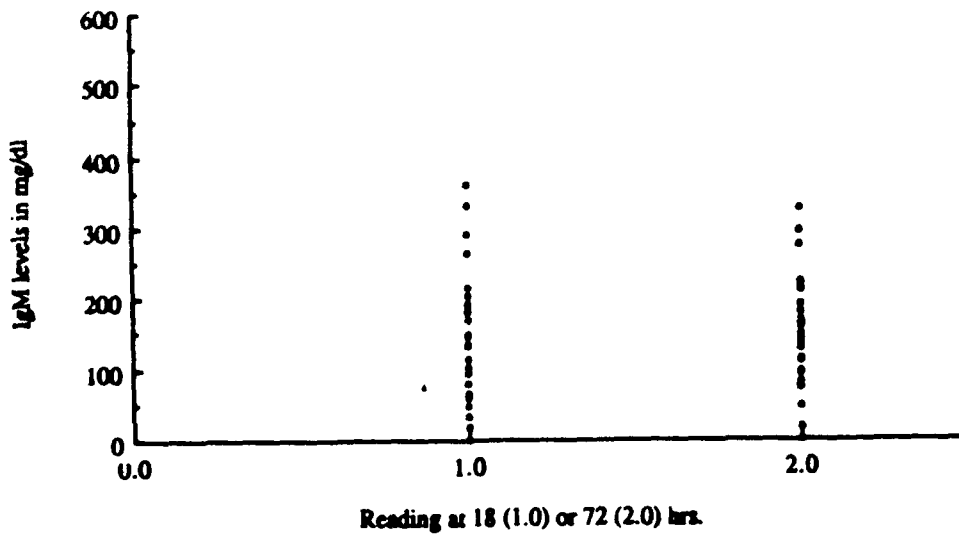
2c. IgA levels for BFO=N DOSE=H.



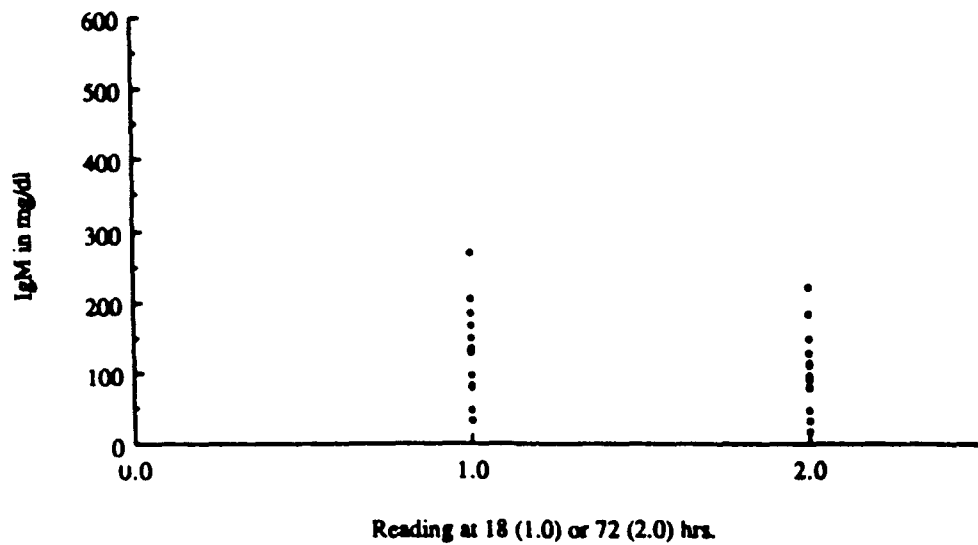
2d. IgA levels for BFO=Y DOSE=L.



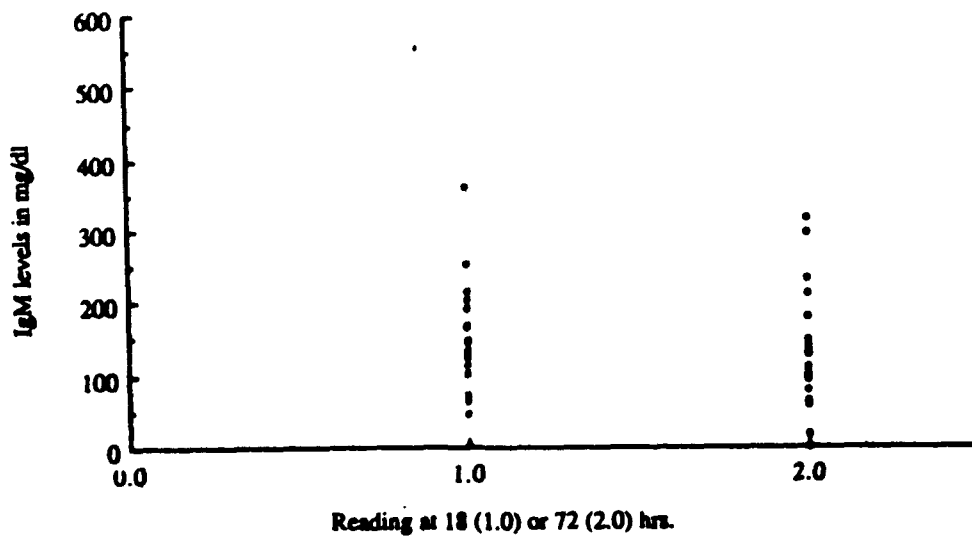
2e. IgA levels for BFO=N DOSE=L.



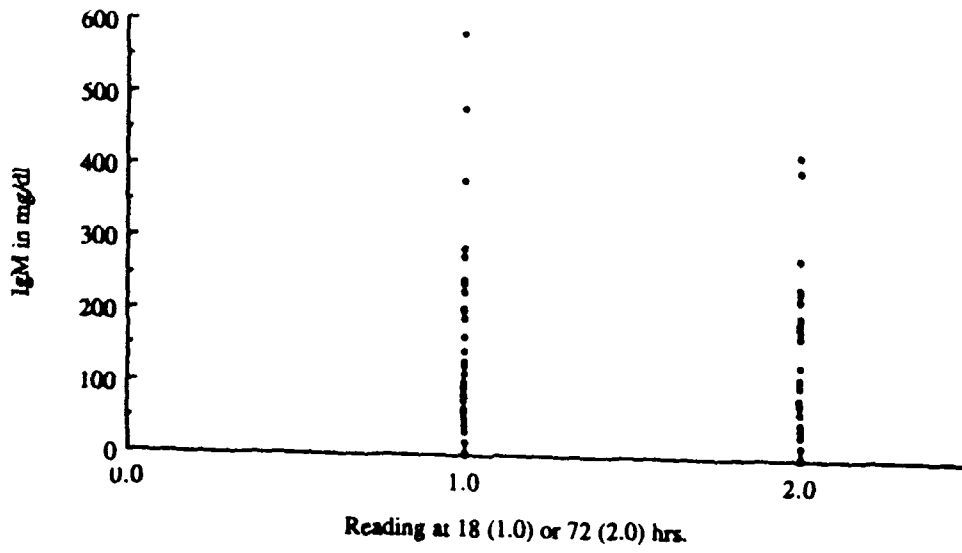
3a. IgM levels for controls (BFO=C DOSE=C).



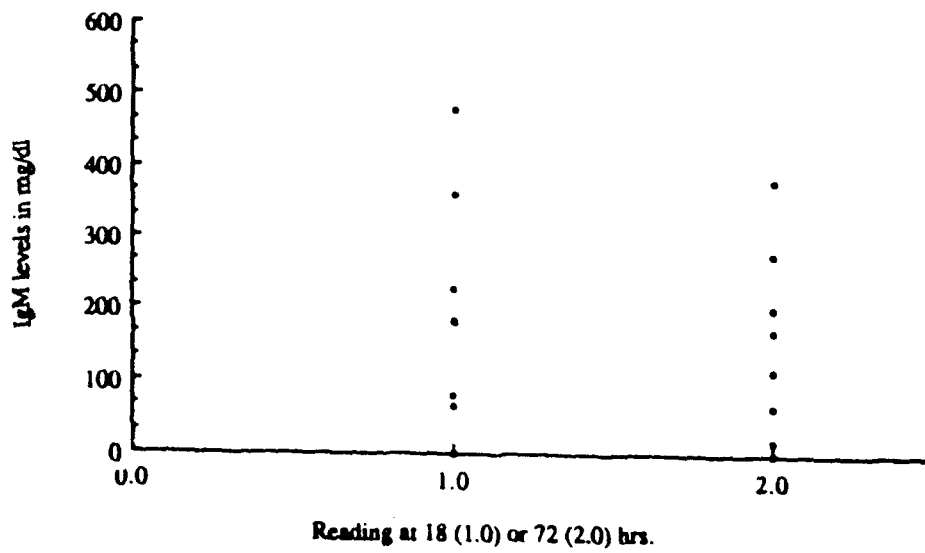
3b. IgM levels for BFO=Y DOSE=H.



3c. IgM levels for BFO=N DOSE=H.



3d. IgM levels for BFO=Y DOSE=L.



3e. IgM levels for BFO=N DOSE=L.

Preliminary analyses also indicate a significant sex difference for all 3 Ig classes. Female monkeys tend to exhibit lower levels of IgG and IgA, but higher levels of IgM. The biologic significance of this finding is unexplained, but a similar sex difference has been observed in humans (Grundbacher and Shreffler, 1970). We believe that our data represent the first time this difference has been reported in rhesus monkeys. This sex difference occurred within both the irradiated and control CRC monkeys (data not shown because analysis is not complete).

Effects of Freezing and Thawing on Ig Levels

Methodology. Because the serum samples are stored frozen (at -70°C) for varying periods of time and frozen and thawed on more than one occasion, we felt that it was important to test the effects of freezing and thawing on the Ig levels. Serums from 1 male monkey and 1 female monkey were frozen and thawed at weekly intervals for 6 weeks. A total of 500 µl of each serum was kept frozen at -70°C. Each week the sample was thawed and 50 µl removed. The remaining serum was refrozen. This procedure was repeated for 6 weeks. Each aliquot was tested in 8 wells of the radial immunodiffusion plate for all 3 Ig (G, A and M).

Results. The results are summarized in Table 2. There are no significant differences among the various thawed samples in any of the Ig's. Thus, we think it is safe to conclude that freezing and thawing as many as 6 times has no effect on the Ig levels.

Table 2. EFFECTS OF FREEZING AND THAWING ON Ig LEVELS IN SERUM OF RHESUS MONKEYS STORED AT -70°C

Weeks frozen	<u>Mean Diameters of Precipitation Rings at 72 h</u>					
	IgG		IgA		IgM	
	J51	J70	J51	J70	J51	J70
0	5.65	7.47	5.60	7.14	5.04	6.24
1	5.70	7.62	5.60	7.20	5.09	6.20
2	5.08	NT	NT	NT	5.20	7.30
3	5.68	7.41	6.05	7.08	5.44	6.15
4	5.76	7.65	5.90	7.15	5.15	6.28
5	5.82	7.72	5.99	7.16	5.17	6.23
6	5.71	7.65	5.80	7.18	5.11	6.44

NT = not tested

Level of Functional Hemolytic Complement

Methodology. The serum samples were assayed by a commercial radial immune assay similar in principle to the test used for measuring Ig levels. Serum to be tested is placed in wells of precise dimensions and diffuses through the agarose gel containing standardized sheep erythrocytes sensitized with hemolysin. If all complement proteins are present in sufficient quantity, the sheep red blood cells are lysed to form a clear zone ring. A reference curve is constructed on semi-logarithmic graph paper; ring diameters cleared by reference sera are plotted on a linear scale and their corresponding concentrations on the logarithmic scale. The points are connected, and unknown concentrations are determined by locating the cleared zone ring diameter of the sample on the reference curve. All tests were set up and read after 6 h by the same operator.

During the previous contract period, we determined that for reliable complement assays the clotted blood must be cooled immediately after bleeding the animal, and that the serum must be harvested within a few hours after bleeding. Also, the sera must be stored at -70°C . An experiment to determine the effect of freezing and thawing on the hemolytic complement level was completed during the previous project period. The experiment indicated that there was little or no effect of freezing-thawing on the complement level. During this project period, we tested the hemolytic complement level of sera from 3 additional bleeds.

Results. As shown in Table 3, we have tested 611 samples from the CRC animals representing 5 different bleedings over a period of 3 years (1984 to 1987). We have also tested 133 samples from the Wisconsin Regional Primate Research Center (WRPRC) in Madison, WI, and 114 samples from the National Institute of Aging (NIA) in Bethesda, MD. These other samples served as controls to the CRC, both age matched and age mismatched. The data have not yet been statistically analyzed, but preliminary analyses indicate no significant differences in complement activity between irradiated and control CRC monkeys. There apparently is no significant difference between the complement activity of the CRC monkeys and the monkeys from the 2 other colonies, except for the 1984 and 1985 samples. The low complement activities in the CRC 1984-85 samples are likely the result of degradation during handling and storage.

It is worth noting that in humans, complement activity less than 70 CH_{50} units is suggestive evidence of some pathology. Whereas most of the average values we obtained were not less than 70 units, some individual samples fell well below this level. Additional tests and analyses on the other samples are continuing.

Table 3. COMPLEMENT ACTIVITY (AS MEASURED BY DIAMETERS OF HEMOLYTIC PLAQUES) AND CH₁₀₀ HEMOLYTIC UNITS OF RHESUS MONKEYS

Sample Source	Bleeding Date	Number Samples Tested	Approx. Animal Age (Yrs.)	Avg. Diam. (mm)	CH Units	Diam. Range (mm)
CRC	85/03	167	23	3.4	<26 ^a	2.6-5.3
CRC	85/11	22	22	4.1	<26 ^a	2.6-5.4
CRC	86/03	147	24	5.4	72	4.4-6.4
CRC	87/03	140	25	5.4	72	4.7-6.2
CRC	87/08	155	25	5.3	80	4.3-6.2
CRC	88/01	128	26	5.1	86	4.4-6.2
WRPRC ^b	85/06	133	20	4.0	<26	2.6-5.5
WRPRC ^b	86/07	100	0≥20	5.2	71	4.2-6.0
NIA ^c	87/03	114	<5≥20	5.6	85	4.6-6.3

^a Diameters with complement values of <26 CH₁₀₀ units are considered critically low.

^b Wisconsin Regional Primate Research Center

^c National Institute of Aging, representing 30 individual monkeys bled at different times during the 87/3,8 period.

Incidence of Autoantibodies

Methodology. Serum samples were assayed using an indirect fluorescent antibody test kit (Kallestad Laboratories, Inc., Austin, TX). Autoantibodies in a test sample bind to homologous antigens in the substrate. Excess (unbound) serum is then removed from the substrate by washing. Fluorescein isothiocyanate (FITC) antiserum is added to the substrate and attaches to the bound autoantibody. After a second washing step to remove excess FITC, the substrate is coverslipped and viewed with a fluorescent microscope for specific fluorescent patterns which indicate the presence of autoantibodies in the test sample.

Results. Table 4 shows the results of our preliminary analysis of the autoantibody frequency in the CRC as compared to age-matched groups of monkeys at the WRPRC and NIA.

Table 4. FREQUENCY OF AUTOANTIBODIES IN SERUM OF RHESUS MONKEYS

Sample Source	Bleeding Date	Number Samples Tested	Fluorescent Patterns*		
			Positive (%)	Weak Positive (%)	Negative (%)
CRC	85/03	143	25 (17)	27 (19)	91 (64)
CRC	86/03	149	21 (14)	11 (7)	117 (79)
CRC	87/03	141	24 (17)	20 (14)	97 (69)
CRC	87/08	135	22 (16)	6 (5)	107 (79)
WRPRC	85/07	157	18 (11)	44 (28)	95 (61)
NIA	87/03	30	1 (3)	11 (37)	18 (60)
TOTALS		755	111 (14.7)	119 (15.8)	525 (69.5)

* By far the majority (74%) of the strongly positive reactions were for anti-reticulin antibodies. The next most frequent type (12%) was anti-nuclear antibodies. The remaining (14%) was distributed among anti-smooth muscle, anti-parietal cell, and undetermined types.

We have completed tests on 568 serum samples from 4 different bleedings of the CRC monkeys. We also tested 187 samples representing the 2 other monkey colonies. In total, we have assayed 755 serum samples for the presence of autoantibodies. We found that 111 of the 755 samples (i.e., 14.7%) exhibited strong autoantibodies, while an additional 15.8% exhibited weak antibodies. Taken together, about 30% of all of the sera had autoantibodies. In the CRC monkeys, we observed an average of about 27% with autoantibodies (strong and weak). The frequency of strong reactors averaged 16% among the CRC monkeys, 11% among the WRPRC monkeys and 3% among the NIA monkeys. The values have not been tested statistically, but apparently the CRC monkeys exhibit the highest frequency of autoantibodies. Our analyses are not complete, but in the 87/08 sample bleed date, there is no apparent significant difference between the frequency of autoantibodies in the irradiated monkeys (16.2%) and the control monkeys (18.2%). A sex difference could not be ascertained because most of the animals are males. By far the majority (90%) of strong reactions were for anti-reticulin antibodies that are associated with celiac disease in humans (Eterman & Feltkamp, 1978). About 10% were anti-nuclear antibodies that are associated with renal disease, especially systemic lupus erythematosus (Schwartz, 1986).

We have not yet completed our analyses of whether or not there is a correlation between the presence of autoantibodies and the source or energy of the radiation. However, preliminary analyses indicate that there are no significant differences in the frequency of autoantibodies among the various treatment classes.

Studies of Cellular Immune Functions

Background. Exposure to radiation is known to cause a rapid decrease in the number of circulating B and T lymphocytes in humans (Sado, et al., 1978; Yamakido, et al., 1982; Sieber, et al., 1985; Fuks, et al., 1976; and Stratton, et al., 1975). The ability of these cells to proliferate in response to mitogens also decreases following radiation exposure. These conditions are often short-lived, and both the numbers and function of human B and T cells may recover within as little as 3 months after exposure. The time necessary for full recovery depends upon the amount of radiation exposure, the areas of the body exposed, and the age of the subject at the time of exposure. However, little is known about long-term effects of radiation on the immune system.

MATERIALS AND METHODS

Isolation of Peripheral Blood Mononuclear Cells

Sterile blood samples were obtained from the monkeys by venipuncture, placed in tubes containing heparin anticoagulant and transported from Brooks AFB to the University of Texas Health Science Center at San Antonio (UTHSC-SA). Blood was placed on an equal volume of Ficoll-Hypaque and spun at 1,800 rpm (400 G) for 45 min. The buffy coat (white cell) layer was removed and Hank's balanced salt solution (HBSS) was added until the total volume was 50 ml. The cells were then centrifuged for 7 min at 1,800 rpm (400 G). HBSS was removed and fresh HBSS added to 50 ml. The tubes were centrifuged as before. HBSS was removed and 5 ml of enriched (FCS) RPMI media was added.

Cell Culturing

After adding 5 ml enriched RPMI media, the cells were adjusted to 200,000 cells per ml; 2,600 μ l of enriched media with cells was then placed into each well of 96-well microtiter plates. The plates were incubated for 48 h under 5% CO₂ at 37°C. Then, 50 μ l of either enriched media (control group) or the appropriate mitogen (Con A, PHA or pokeweed) was added. The cells were incubated for another 48 h. Then, 50 μ l of tritiated thymidine (2 μ Ci/ml) was added to each well 6 h before placing the plates into a cell harvester. Filter papers containing harvested cells were placed into tubes with 2 ml of scintillation fluid to allow uniform dispersion of radioactivity. Tubes containing the filter papers

were placed into a scintillation counter for measurement of counts per minute (CPM). As this effort was a direct measurement of the amount of thymidine which the cells had incorporated, it reflects cellular proliferation. The results obtained were analyzed by computer.

Procedure for Making Enriched RPMI Media

Enriched RPMI media was made by mixing 500 ml of RPMI media with 50 ml of fetal calf serum (FCS), 5 ml of HEPES buffer, and 5 ml of nonessential amino acids. Next was added 5 ml of L-glutamine, 5 ml of pyruvic acid, and 3.5 ml of penicillin/streptomycin. Finally, 5×10^{-5} M 2-mercaptoethanol was added.

RESULTS

We tested 93 monkeys during the experiment. Data analysis examined both the actual CPM value and stimulation index for each monkey. The stimulation index for each mitogen is the CPM value divided by the control (no mitogen) CPM value. Stimulation indices allow compensation for responses that display excessive proliferation without mitogen stimulation. The monkeys were grouped according to radiation dosages.

Proliferative Response in the Absence of Mitogens

The average control CPM values in the absence of a mitogen are shown for each radiation dosage group in Figure 4. The proliferative abilities of peripheral blood mononuclear cells decreased as radiation dosage increased. The numbers above each bar on the histogram represent the number of monkeys in that dosage group.

Proliferative Response of Cells Stimulated by Con A

The stimulation indices of cells stimulated by the T-lymphocyte mitogen Con A ($10 \mu\text{g}$) are shown for each group vs. radiation dosage in Figures 4-6. As can be seen, there is no observable relationship between dosage and the proliferative responses to mitogen.

Proliferative Response of Cells Stimulated by PHA

The stimulation indices of cells stimulated by PHA ($10 \mu\text{g}$, which largely stimulates T cells) are shown in Figure 7 for each group vs. radiation dosage. As can be seen, there is no observable relationship between dosage and the proliferative responses to this mitogen.

Proliferative Response of Cells Stimulated by Pokeweed Mitogen

The average CPM and stimulation indices of cells stimulated by the B-lymphocyte mitogen pokeweed (2.5 μ g) are shown for each group graphed against radiation dosage in Figures 8 and 9. As can be seen, there is no relationship between dosage and proliferative responses to this mitogen. The numbers above each point represent the number of monkeys in that radiation dosage group.

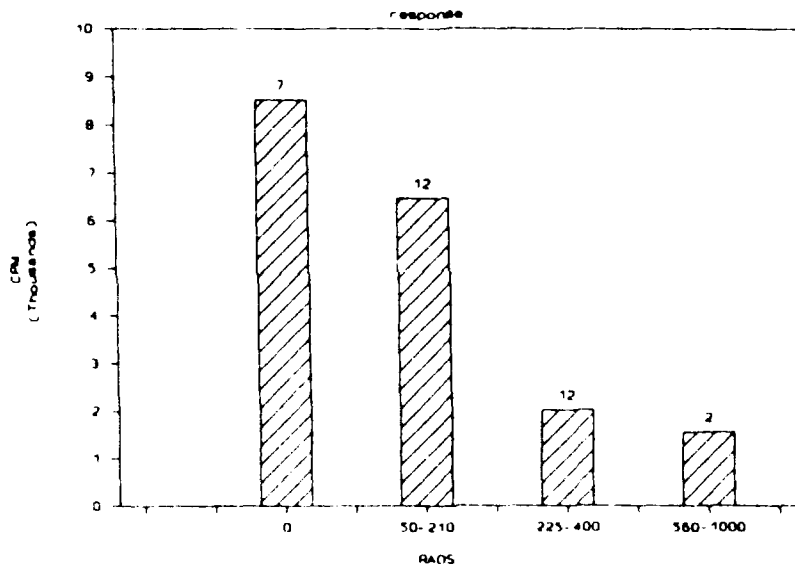


Figure 4. The spontaneous proliferative abilities of peripheral blood mononuclear cells (PBMC) from irradiated monkeys, as measured by thymidine uptake, is inversely related to radiation dosage. Cells are cultured for 48 h as described in Materials and Methods. The numbers shown above each point on the graph represent the number of monkeys in that radiation dosage group.

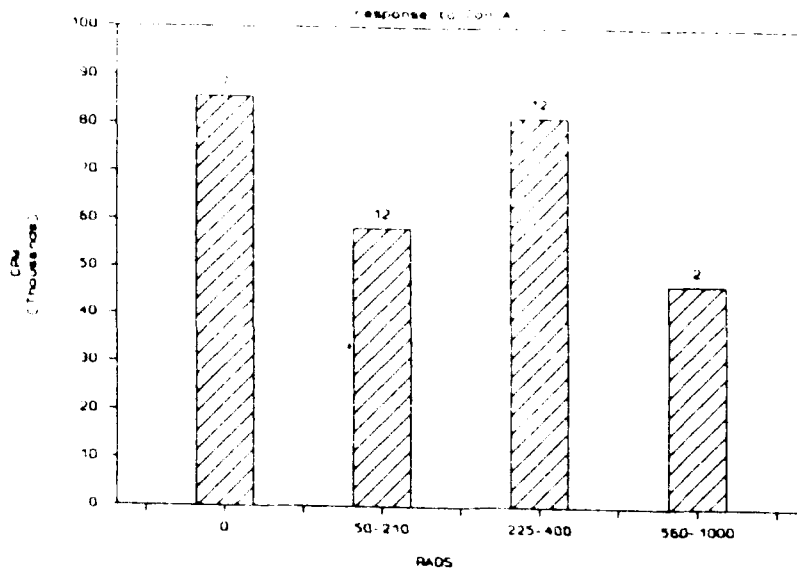


Figure 5. The proliferation of PBMC from irradiated monkeys in response to 10 $\mu\text{g/ml}$ Con A,, as measured by thymidine uptake, is not related to radiation dosage. Cells are cultured for about 48 h as described in Materials and Methods.

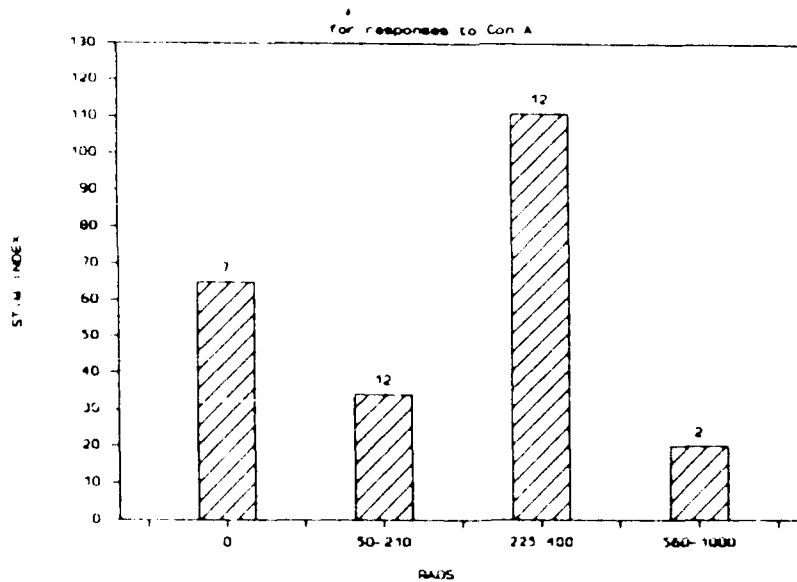


Figure 6. The proliferation of PBMC from irradiated monkeys in response to 10 $\mu\text{g/ml}$ Con A, as measured by stimulation indices (see Materials and Methods), is not related to radiation dosage. Cells are cultured for 48 h as described in Materials and Methods. The numbers shown above each point on the graph represent the number of monkeys in that radiation dosage group.

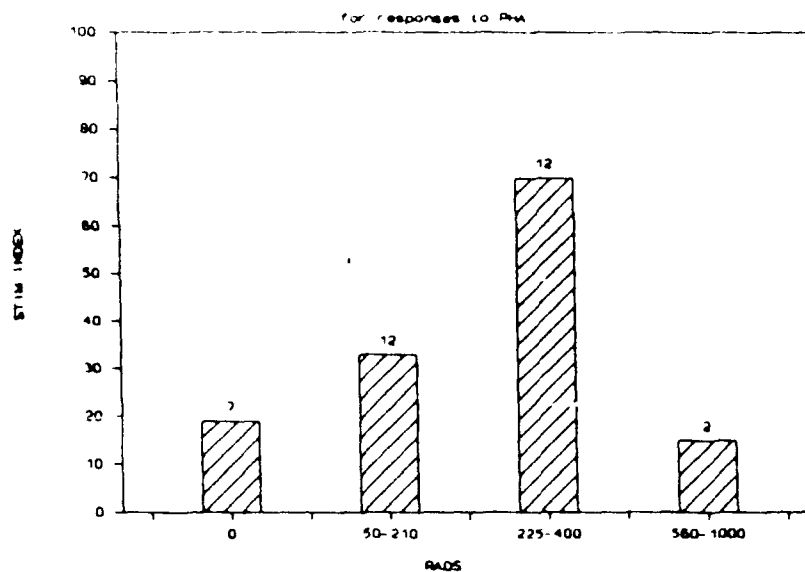


Figure 7. The proliferation of PBMC from irradiated monkeys in response to 10 $\mu\text{g/ml}$ PHA, as measured by stimulation indices (see Materials and Methods), is not related to radiation dosage. Cells are cultured for 48 h as described in Materials and Methods. The numbers shown above each point on the graph represent the number of monkeys in that radiation dosage group.

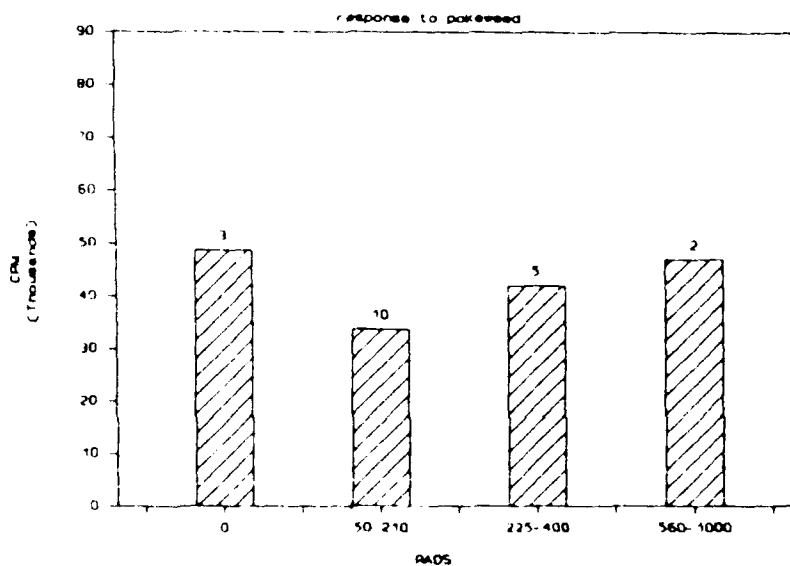


Figure 8. The proliferation of PBMC from irradiated monkeys in response to 2.5 $\mu\text{g/ml}$ pokeweed mitogen, as measured by thymidine uptake, is not related to radiation dosage. Cells are cultured for 48 h as described in Materials and Methods. The numbers shown above each point on the graph represent the number of monkeys in that radiation dosage group.

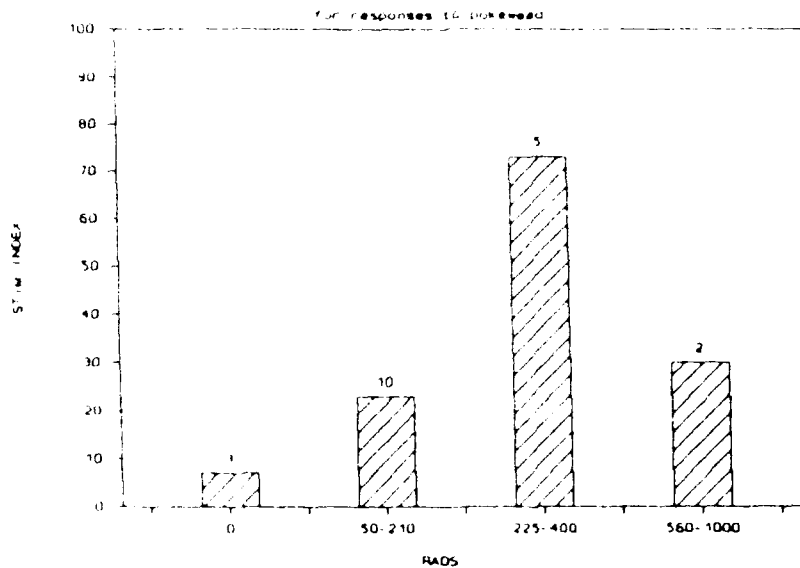


Figure 9. The proliferation of PBMC from irradiated monkeys in response to 2.5 $\mu\text{g/ml}$ pokeweed, as measured by stimulation indices (see Materials and Methods), is not related to radiation dosage. Cells are cultured for 48 h as described in Materials and Methods. The numbers shown above each point on the graph represent the number of monkeys in that radiation dosage group.

DISCUSSION

Based on our preliminary data, there does not appear to be an effect of radiation irrespective of source or dose on any of the B cell (AMI) immunologic parameters. However, rigorous statistical analyses have not been completed. There is suggestive evidence that exposure to radiation decreases the proliferative ability of unstimulated T and B cells in rhesus monkeys. However, this exposure does not appear to affect the immune cell function, measured by proliferative responses of B and T lymphocytes to Con A, PHA, and pokeweed mitogens. The drop in spontaneous proliferation could be due to several factors. Radiation exposure may have decreased the number of cells available for continuous proliferation. However, human studies have shown that lymphocyte counts of irradiated subjects eventually recover to levels equal to or higher than those of non-irradiated control subjects (Sado, et al, 1978). Alternatively, radiation-induced genetic defects might result in inhibition of proliferative abilities. Other experiments have shown that the proliferative abilities of mitogen-activated lymphocytes are significantly lower in experimental groups consisting of subjects with chromosomal aberrations than in control groups (Yamakido, et al., 1982). That lymphocytes with some types of chromosomal aberration may be present in vivo after irradiation also supports this hypothesis (Buckton, et al., 1967).

We have the expertise of Professor Diane Saphire in the Department of Mathematics, Trinity University, to help guide our statistical analyses. We have embarked on an active program of data entry and analysis. However, to date, very few statistical analyses have been done, because we have continued to collect and test serum samples. We expect to concentrate on data analysis during the remainder of the current contract period. It is also important to note that we are dealing with an aged, survival population of rhesus monkeys. There is some evidence that there are changes in Ig levels with age in humans (Grundbacher and Shreffler, 1970) and data are also available on Ig levels in rhesus (Eitzman, 1970). The occurrence of autoantibodies in nonhuman primates has also been studied (Stone and Nandy, 1979). Thus, it should be informative to compare the immunologic parameters in the CRC with those in other rhesus colonies consisting of both age-matched monkeys, as well as monkeys of various ages. These studies are currently underway.

CONCLUSIONS

Preliminary analyses indicate that there are no significant differences in the immunoglobulin (IgA, G and M) levels among the control (unirradiated) and treated (irradiated) monkeys in CRC. Some monkeys showed Ig levels more than 2 standard deviations different from the mean, which may be indicative of an immunologic perturbation.

There did not appear to be a significant difference in the hemolytic complement activity between the control and irradiated monkeys. However, some monkeys had complement activity below the level which in humans is indicative of pathology. These monkeys will be followed up.

The frequency of autoantibodies (mostly anti-reticulum) was not different between the controls and irradiated monkeys. The overall frequency of autoantibodies in the entire colony seems relatively high and must be compared with those of a different age-matched colony as well as with monkeys of different ages.

By isolating peripheral blood mononuclear cells from the CRC monkeys and measuring their proliferation by the incorporation of tritiated thymidine in response to mitogen, we found no observable relationship between radiation dosage and response Con A, PHA, and pokeweed mitogens. In contrast, lymphocyte proliferation correlated with radiation dosage in the absence of mitogen: cell proliferation tended to decrease as radiation dosage increased.

ACKNOWLEDGMENTS

We wish to acknowledge the assistance of Dr. Diane Saphire, who has provided the computer programming and statistical analyses.

Also, Ms. Susan Hackleman and Ms. Pamela Pennington provided invaluable technical help in testing the serum samples for the AMI parameters. Also, Ms. Hackleman has been responsible for data entry and the graphics. Mr. Scott Manis has provided technical assistance at Trinity University, and Ms. Jeya A. Chacko and Sandra Hernandez, who under Dr. Miller's direction have done the CMI functional studies (mainly the mitogen responses) at UTHSC-SA.

Finally, we thank Dr. Ann Cox, Dr. Jeff Wigle, and Dr. David Wood for their undiminished confidence and encouragement in these studies, and Ms. Yolanda Salmon for her efficient cooperation.

We gratefully acknowledge support from the USAF.

REFERENCES

1. Buckton, K. E., W. M. C. Brown, and P. G. Smith. Lymphocyte survival in men treated with x-rays for ankylosing spondylitis. *Nature* 214:470 (1967).
2. Eitzman, D. V. Immunoglobulin levels in the Macaca mulatta. *Folia Primat* 12:313 (1970).
3. Eterman, K. P. and T. E. W. Feltkamp. Antibodies to gluten and reticulin in gastrointestinal diseases. *Clin Exp Immunol* 31:92 (1978).
4. Fuks, Z., S. Strober, A. Bobrove, T. Sasazuki, A. McMichael, and H. S. Kaplan. Long term effects of radiation on T and B lymphocytes in peripheral blood of patients with Hodgkin's disease. *J Clinical Invest* 58:803 (1976).
5. Grundbacher, F. J. and D. C. Shreffler. Changes in human serum immunoglobulin levels with age and sex. *Z Immun-Forsch Bd* 141:20 (1970).
6. Sado, T., S. Kobayashi, H. Kamisaku, H. Kurokawa, Y. Kataoka. Immunological competence of aging mice exposed to x- or gamma rays during young adulthood. *Late Biological Effects of Ionizing Radiation: Proceedings of the Symposium on the Late Biological Effects of Ionizing Radiation held by the International Atomic Energy Agency in Vienna, International Atomic Energy Agency, Vienna, 13-17 March 1978.*
7. Schwartz, R. S. Anti-DNA antibodies and the problem of autoimmunity. *Cellular Immunol* 99:38 (1986).

8. Sieber, G., P. Zierach, F. Herrmann, V. J. Brust, and H. Ruhl. Impaired B lymphocyte reactivity in patients after radiotherapy. *Int J Radiation Oncology Biol Phys* 11:777 (1985).
9. Stone, W. H. and K. Nandy. Autoimmune antibodies and other characteristics. *In Aging in Nonhuman Primates*, Bowden, D. M. (ed.), pp 112-122. New York: Van Nostrand Reinhold Co., 1979.
10. Stratton, J. A., P. E. Byfield, J. E. Byfield, R. C. Small, J. Benfield, and Y. Pitch. A comparison of the acute effects of radiation therapy, including or excluding the thymus, on the lymphocyte subpopulations of cancer patients. *J Clin Invest* 56:88 (1975).
11. Yamakido, M., M. Akiyama, D. Dock, H. Hamilton, A. Awa, H. Kato, S. Iida, and K. Yoshimoto. T and B cells and PHA response of peripheral lymphocytes among atomic bomb survivors. *Radtn Effects Res. Fdtn, Technical Report 23-81. Radtn Res Fdtn* (1982).
12. Yochmowitz, M. G., D. H. Wood, and Y. L. Salmon. Seventeen-year mortality experience of proton radiation in Macaca mulatta. *Radiation Res* 102:14 (1985).