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EFFECTS OF SPACE RADIATION ON HUMORAL AND CELLULAR IMMUNITY IN RHESUS MONKEYS

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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.

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13. ABSTRACT *(Maximum 200 words)*

The objective of this study was to complete profiles of immune competence in rhesus monkeys more than 25 years after single exposures to protons of different energies. Access to irradiated animals provided a unique opportunity to study late effects on the immune systems of nonhuman primates; late immunological defects could be relevant to astronauts and high-flying pilots. Working with the primate model allowed us to assess the possible late effects of ionizing radiations on parameters associated with B-cell and T-cell functions. Antibody-mediated immune (AMI) function was investigated by measuring immunoglobulin (Ig) levels, hemolytic complement activity, and autoantibodies. Cell-mediated immune (CMI) function was evaluated by measuring selected T- and B-cell activities as well as responses to mitogens and interleukin production. There were no significant differences between control and irradiated animals for most parameters measured in this survey, but some reduction in spontaneous proliferation was noted in irradiated primates. With regard to late risks for humans following exposure(s) to ionizing radiations, it is encouraging that few late immunobiological effects were exhibited by primate survivors of low and intermediate doses of protons.

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EFFECTS OF SPACE RADIATION ON HUMORAL AND CELLULAR IMMUNITY IN RHESUS MONKEYS

INTRODUCTION

Background

The rhesus monkeys (Macaca mulatta) in the Brooks Air Force Base Delayed Effects Colony (DEC) in San Antonio, Texas, have been maintained for over 25 years to gather information regarding late effects of exposure to ionizing radiations. The most dramatic effects found to date have been life shortening associated with organ degeneration, cancer, endometriosis, and opportunistic infections (Yochmowitz, et al., 1985).

Since it is well known that radiation affects the immune system, the availability of the DEC of rhesus monkeys provided a unique opportunity to study the possible immune effects of the radiation treatment. We were particularly interested in effects which would be potentially relevant to human diseases that may occur in astronauts and high-flying pilots who are exposed to radiation.

These studies actually began in 1986 but were interrupted several times because of lack of funding. The overall objectives of this study were as follows:

1. To assess the possible effects of radiation on antibody-mediated immune (AMI) function by measuring immunoglobulin (Ig) levels, hemolytic complement activity, and auto-antibodies. These parameters are associated with B-cell function.

2. To assess the possible effects of radiation on 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.

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

Preliminary Results

Early studies indicated that there were no significant effects of radiation on any of the B- or T-cell functions measured. However, since most of the monkeys were older than 25 years of age,

we initiated a study to determine if age had an effect on any of these immune parameters.

We were fortunate to be able to obtain serum samples from rhesus monkeys of various ages. We gratefully acknowledge the University of Wisconsin Regional Primate Research Center (WRPRC), Madison, WI, for its generous cooperation.

Even during the periods when we were not funded, we continued, at a caretaker's level, to collect and analyze sera obtained from the DEC monkeys. Our inventory of sera is shown in Table 1.

ASSAY OF B-CELL FUNCTION

Immunological Levels

Methodology. The serum samples were assayed by a single radial immunodiffusion technique using commercially available (Kallestad Laboratories [now Sanofi Diagnostics Pasteur], 1000 Lake Hazeltine Drive, Chaska, MN 55318-1084) 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 old world primate species; and because our aim was to compare the levels of Ig between the irradiated and control monkeys of different ages. 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.

Repeatability of Ig Assay

Before analyzing the results in detail, we set up a number of experiments to determine how repeatable the radial immune assay was. The results were as follows:

Retest I

Retest I was conducted to determine the reliability of Ig measurements that fell outside of the range of the standards included in the testing kit. Of the animals that had at least one Ig measurement out of range, 16 were randomly selected. The blood samples of these animals were used to obtain a new reading of IgA, IgG, and IgM, at both 18 and 72 h.

These new measurements were compared to the original measurements in two ways. First, all measurements were converted to

units, and correlations between the new and old measurements were computed. Second, those new measurements that had been made at the same dilution as the old measurements were compared to each other and correlations were computed. In all of these comparisons, except for IgM at 72 h on the unit scale, the correlations were very low and were not significantly different from zero. Some correlations were actually negative. Nine of the 16 tested animals had originally had IgM 72-h readings that were out of the range of the standards. Comparing the new measurements for these animals (on a unit scale) with the original measurements yielded a correlation of 0.851 ($p=0.004$).

By changing the dilutions for retest I, all of the retested values for IgA and IgG fell within the range of the standards. All of these values had originally been lower than the lowest standard, and the new values were all higher than the original values, strongly suggesting that values that fall below the lowest standard result in unit measurements that are too small.

Of the 16 values of IgM that originally fell out of the range of the standards, retesting at a new dilution brought only 4 of them into the range of the standards. Of those that had originally fallen below the lowest standard, all of the new readings were higher (on a unit scale) than the originals. Of those that had originally fallen above the lowest standard, half had new measurements that were lower than the originals, and half higher.

The general conclusion of this test was that the observations that fell outside of the range of the standards were subject to a substantial amount of error.

Retest II

Because retest I results did not match well with the original measurements, a second retest was performed to determine if original and retest measurements matched more closely for animals whose original measurements had not fallen out of the range. Twelve such animals were randomly chosen. Additionally, 4 animals that did have outside measurements were chosen. Each of the 6 readings (IgA, IgG, IgM, all at 18 and 72 h) was redone four times for each of these 16 animals. Two of the readings were made on plates from one lot, and two on plates from another lot. Each sample was diluted separately. This experiment was designed to determine how much variability could reasonably be expected in the measurements.

An analysis was performed on the diameter readings. No significant lot effect was found. The variability of the four measurements for a single animal was generally small. For IgA, IgG, and IgM at both 18 and 72 h, the estimated standard deviation of the measurements for an animal was about 0.15 (diameter unit).

The correlations of the four repetitions with each other were quite high (>0.9).

The results of the first of the four repetitions of retest II were compared with the original measurements for the animals whose original measurements had been within the range of the standards. These comparisons were made using International Unit measurements and, where possible, diameter measurements. The results are shown in Table 2.

Note that the IgA measurements correspond fairly closely to the original measurements in both units and diameters, while the match for IgG and IgM is less satisfactory. For the unit and diameter correlations that can be directly compared, the retested diameter measurements match the original diameter measurements just slightly better than the retested unit measurements match the retested unit measurements.

Overall conclusions from this retest are that for measurements within the range of the standards:

1. There is little change in diameter measurements from lot to lot.

2. The estimated standard deviation for repeated measurements on the same animal is about 0.15 (diam.).

3. Repeated measurements on the same animal match each other more closely than they match the original measurements. Only the match with the original IgA measurement is good.

4. There is a very slight tendency for diameters to match the original diameters better than for units to match original units.

Retest III

A third retest was performed on the animals that were used in retest I. The purpose of this retest was to determine whether another set of recently collected observations would correspond more closely to the original measurements or to the retest I measurements. The results of this test are summarized in Table 3.

Clearly, the measurements match the more recently calculated values more closely than the original values. The low correlation between the two retests on IgG at 18 h seems unusual.

The so-called "original data" were obtained on samples that were stored at -70°C for a relatively short time (perhaps less than 1 year). In contrast, the retest I and II used the same samples, but they had been stored at -70°C for more than 3 years. Thus, it is possible that prolonged storage accounts in part for the poor correlation between the original data and the retest I data. The same operator performed the original and the retests. It is possible that her technical skills improved between the time of the original tests and the retests. This conclusion seems reasonable in view of the high correlations between retests I and II. In any case, we have considerable confidence in the reliability of the results for most of the samples tested to date.

Effect of Radiation on Ig Levels

We believed that a rational way to look at the data was to group the irradiated monkeys into 4 groups according to radiation dose and whether or not the type of radiation would be expected to 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.

Using this irradiation classification, we have been analyzing the data using various statistical methods. The analyses are not completed, but our preliminary results show that the irradiated monkeys show no significant differences either among the different groups or between any single group and the unirradiated controls. These preliminary results 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.

(Note: Graphs depicting some of these results have been reported in USAFSAM-TR-90-39.)

Analysis of Effect of Treatment vs. Sex

We have not yet completed this analysis, but our preliminary data suggest no effect of treatment on sex, as might be expected from our preliminary analyses of the total population. The number and sex of the animals and the various treatments are shown in Table 5.

Analysis of Effect of Treatment vs. Age

The Air Force and WRPRC data were pooled, thus adding a large number of animals to the aged group. These data are being analyzed now. Our preliminary results indicate:

1. There was no difference among the different treatments and age.

2. There was a significant increase in the Ig levels with age as summarized in Table 6.

Although there was no significant sex difference, apparently the males changed more rapidly with age than the females.

Effect of Treatment on Levels of Hemolytic Complement

Methodology. The serum samples were assayed using a commercial radial immune assay. Serum 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.

Results. We have tested over 600 samples from the DEC animals representing 5 different bleedings over a period of 3 years (1984 to 1987). We have also tested all of the 477 samples from the WRPRC. The data have not yet been statistically analyzed, but preliminary analyses indicate no significant differences in complement activity between irradiated and control DEC monkeys. There apparently is no significant difference between the complement activity of the DEC monkeys and the monkeys from the 2 WRPRC, except for the 1984 and 1985 samples. The low complement activities in the DEC 1984-85 samples are likely the result of degradation during handling and storage.

Incidence of Autoantibodies

Methodology. Serum samples were assayed using an indirect fluorescent antibody test kit (Kallestad Laboratories, now Sanofi Diagnostics, Chaska, MN). 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. The results of our preliminary analysis of the autoantibody frequency in the DEC vs. treatment are shown in Table 7.

It is safe to conclude from these data that there is no significant difference in the frequency of autoantibodies in any of the treatment groups or in the control. It is interesting to note that the average frequency over all treatments including the control is 21.2%. It will be most interesting to see what the frequency is in the age-matched group of monkeys from WRPRC. Preliminary data indicate that the incidence of autoantibodies is higher in the DEC monkeys than among monkeys of other colonies.

By far, the majority (90%) of strong reactions were for antireticulin antibodies that are associated with celiac disease in humans (Eterman & Feltkamp, 1978). About 10% were antinuclear antibodies that are associated with renal disease, especially systemic lupus erythematosus (Schwartz, 1986).

ASSAY OF T-CELL FUNCTION

Exposure to radiation is known to cause a rapid decrease in the number of circulating B and T lymphocytes in humans (Yamakido, et al., 1982; Gaston, et al., 1988; Sieber, et al., 1985; Manori, et al., 1985; Trentham, et al., 1981; Fuks, et al., 1976 and Campbell, et al., 1973). 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 number and function of human B and T cells may recover within as little as 3 months after exposure (Gaston, et al., 1973 and Fuks, et al., 1976). The time necessary for full recovery depends upon the amount of radiation received, the areas of the body exposed, and the age of the subject at the time of exposure.

Our preliminary results on the DEC monkeys indicate that there is no observable relationship between radiation dosage and immune function, as measured by proliferative responses to Con A, PHA, and pokeweed mitogens.

MATERIALS AND METHODS

Monkeys

A total of 106 monkeys were studied, including control monkeys

which received no irradiation and monkeys that received varying amounts of radiation (Table 9).

Isolation of Peripheral Blood Mononuclear Cells

Blood was obtained by venipuncture in tubes containing heparin. The blood was transported to the University of Texas Health Science Center at San Antonio. Peripheral mononuclear cells were obtained using Ficoll-Hypaque.

Cell Culturing

After adding 5 ml enriched RPMI media, the cells were adjusted to a concentration of 200,000 cells per ml and 260 µl of enriched media with cells was placed into each well of 96-well microtiter plates. The plates were incubated at 5% CO_2 for 48 h. To the wells was then added 50 µl of either enriched media (control group) or the appropriate mitogen (Con A, PHA or pokeweed, usually 10 µg). After incubating for at least another 48 h, 50 µl of tritiated thymidine (1 µCi/ml) was added 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. Counts per minute (CPM) were obtained in a scintillation counter. The results were analyzed using an IBM personal computer and database/spreadsheet software.

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, 5 ml of HEPES buffer, and 5 ml of non-essential amino acids. Next was added 5 ml of L-glutamine, 5 ml of pyruvic acid, and 3.5 ml of penicillin/streptomycin. Finally, 5 x 10^{5}M 2-mercaptomethanol was added.

RESULTS

We examined both the actual CPM value for each monkey and the monkey's stimulation index (SI). The SI for each mitogen is that mitogen's CPM value divided by the monkey's control (no mitogen) CPM value. SIs allow compensation for responses that displayed excessive proliferation without mitogen stimulation. The monkeys were categorized into groups according to radiation dosages. (See Table 4).

Proliferative Responses in the Absence of a Mitogen

The average control CPM values in the absence of a mitogen are shown for each monkey in Figure 1. As more monkeys were studied, the proliferative abilities of peripheral blood mononuclear cells

no longer tended to decrease as radiation dosage increased, as had been previously reported.

Proliferative Responses of Cells Stimulated by Con A

The SI of cells stimulated by Con A are shown in Figure 2. As can be seen, there was no observable relationship between dosage and the proliferative responses to this mitogen.

Proliferative Response of Cells Stimulated by PHA

The SI of cells stimulated by PHA (10 μg) which largely stimulates T-cells, are shown in Figure 3. As can be seen, there is no significant effect of irradiation dose on the SI.

Proliferative Response of Cells Stimulated by Pokeweed Mitogen

The stimulation indexes of cells stimulated by the B-lymphocyte pokeweed mitogen 2.5 μg (PWM) are shown in Figure 4. As can be seen, there is no observable relationship between dosage and proliferative responses to this mitogen.

DISCUSSION

Based on our preliminary data, exposure to radiation has not affected the proliferative responses of T cells to PHA or Con A in the DEC monkeys. Some drop in spontaneous proliferation was noted, which could be due to several factors. Radiation exposure may have decreased the number of cells available for proliferative responsiveness. However, studies of humans have shown that lymphocyte counts of irradiated subjects eventually recover to levels equal to or higher than those of nonirradiated control subjects (Trentham, et al., 1981 and Sado, et al., 1978). Alternatively, radiation-induced genetic defects might result in inhibition of proliferative responses. Other experiments have shown that the proliferative responses of mitogen-activated lymphocytes are significantly lower in experimental groups consisting of subjects with chromosomal aberrations, than in control groups (Yamakido, et al., 1982 and Buckton, et al., 1967). It would be interesting to examine the DEC monkeys for chromosomal abnormalities.

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Table 1. Inventory of Sera from DEC Rhesus Monkeys

Year of Sampling	Approximate Number of Samples*	
	DEC	WRPRC
1986	441	103
1987	274	0
1988	255	374
1989	240	0
1990	95	0
1991	87	0
TOTALS	1392	477

* In 1986 there were 3 bleedings annually; thereafter, there were only 2 bleedings. The WRPRC samples were obtained over the entire year period.

Table 2. Analysis of Repeatability of Immunoglobulin Assay Results

Item	Reading*	Units			Diameter		
Ig	Hr.	corr.	p	n	corr.	p	n
IgA	18	0.784	0.001	14			
		0.965	0.002	6	0.976	0.001	6
IgA	72	0.749	0.002	14			
		0.928	0.008	6	0.932	0.007	6
IgG	18	0.384	0.175	14	0.521	0.057	14
IgG	72	0.381	0.179	14	0.402	0.155	14
IgM	18	no conversion information			0.517	0.126	10
IgM	72	no conversion information			0.539	0.108	10

* Note that results on the same line pertain to the same animals.

Table 3. Retest III Results

a. Correlation of Results of Ig Level Assays with Original Assay Results

Item	Reading	Units			Diameter		
Ig	Hr.	corr.	p	n	corr.	p	n
IgA	18	0.222	0.516	11	(dilutions differ)		
IgA	72	0.333	0.318	11	(dilutions differ)		
IgG	18	0.103	0.826	7	0.257	0.579	7
IgG	72	0.348	0.444	7	0.403	0.370	7
IgM	18	**			0.907	0.057	7
IgM	72	0.826	0.006	9	(dilutions differ)		

b. Correlation with Retest I data

Item	Reading	Units			Diameter		
Ig	Hr.	corr.	p	n	corr.	p	n
IgA	18	0.848	0.000	11	0.808	0.008	9
IgA	72	0.906	0.000	11	0.844	0.004	9
IgG	18	0.294	0.522	7	0.699	0.122	6
IgG	72	0.767	0.044	7	0.782	0.066	6
IgM	18	**			(dilutions differ)		
IgM	72	0.968	0.000	9	(dilutions differ)		

** Several outlying values distort these statistics.

Table 4. Grouping of Irradiated Monkeys by Dose and Probability of Effects on Blood Forming Organs (BFO)

Group	Dose (rads)	Penetration to BFO (MeV)
1.	High dose (>2.0 Gray)	No: (32 or 55)
2.	Low dose (<2.0 Gray)	No: (32 or 55)
3.	High dose (>2.0 Gray)	Yes: (138,400 or 2,300)
4.	Low dose (<2.0 Gray)	Yes: (138,400 or 2,300)

Table 5. Analysis of Data Comparing Irradiation Effect and Sex

GROUP / Frequency / Percent / Row Pct / Col Pct	SEX ♀	♂	Totals
BFO/High Dose	4 0.80 23.53 1.29	13 2.60 76.47 6.84	17 3.40
BFO/Low Dose	0.15 3.00 31.25 4.84	33 6.60 68.75 17.37	48 9.60
Controls	9 1.80 28.13 2.90	23 4.60 71.88 12.11	32 0.40
No BFO/High Dose	8 1.60 34.78 2.58	15 3.00 65.22 7.89	23 4.60
No BFO/Low Dose	1 0.20 12.50 0.32	7 1.40 87.50 3.68	8 1.60
WRPRC	273 54.60 73.39 88.06	99 19.80 26.61 52.11	372 74.40
Totals	310 62.00	190 38.00	500 100.00

Table 6. Effect of Age on Ig Levels

Ig	Increase/year Diameter	Units
A	0.053	21.5
G	0.026	25.5
M	0.020	3.07

Table 7. Results Comparing Irradiation Treatment vs. Presence of Autoantibodies

TREATMENT	AUTOANTIBODIES		
Frequency Percent Row Pct Col Pct	NO	YES	TOTAL
CONTROL	0.26 19.70 78.79 25.00	7 5.30 21.21 25.00	33 25.00
NO BFO/High Dose	0.18 13.64 75.00 17.31	6 4.55 25.00 21.43	24 18.18
NO BFO/Low Dose	5 3.79 62.50 4.81	3 2.27 37.50 10.71	8 6.06
BFO/High Dose	15 11.36 83.33 14.42	3 2.27 16.67 10.71	18 13.64
BFO/Low Dose	40 30.30 81.63 38.46	9 6.82 18.37 32.14	49 37.12
TOTAL	104 78.79	28 21.21	132 100.00

Note: Weak autoantibody has been recoded as yes

Table 8. Statistical Analyses of Data on Irradiation vs. Incidence of Autoantibodies

Statistic (N=132)	Degrees of Freedom	X^2 Value	Prob.
Chi-Square	4	1.936	0.748
Likelihood Ratio Chi-Square	4	1.782	0.776
Mantel-Haenszel Chi-Square	1	0.332	0.565
PHI	-	0.121	-
Contingency Coefficient	-	0.120	-
Cramer's V	-	0.121	-

Table 9. Inventory of 106 Monkeys Assayed for T-cell Function

Number of Monkeys	Dose (Gray)
32	controls
3	0.25
4	0.50-0.56
12	1.0-1.13
18	2.0-2.8
10	3.0-3.95
10	4.0-6.0
8	9.0-10.0
9	10.0-15.0

FIGURES

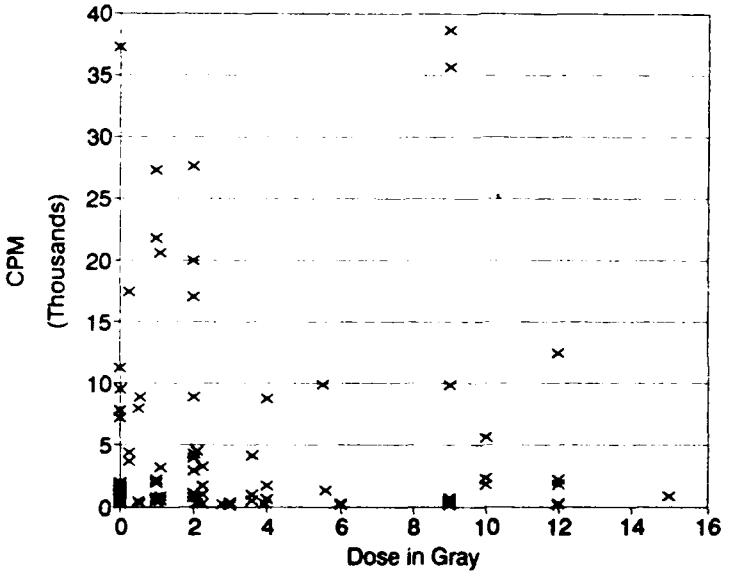

Figure 1. The spontaneous proliferative abilities of PBMC from irradiated monkeys, as measured by thymidine uptake.

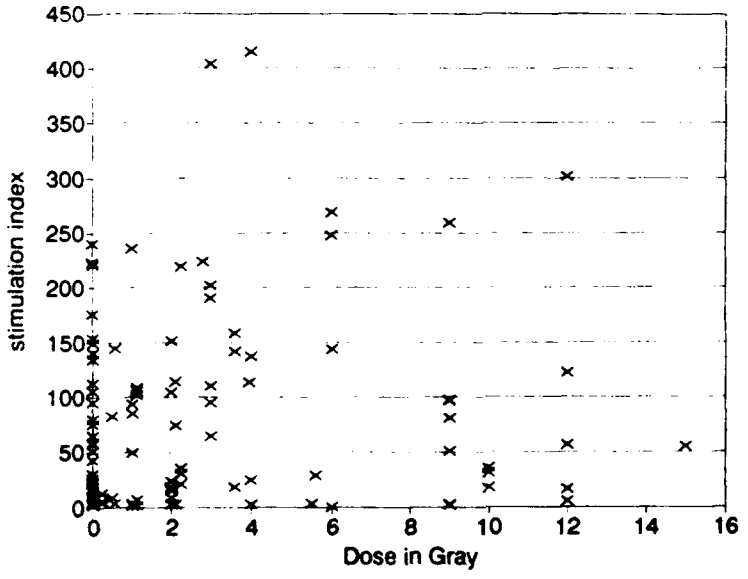

Figure 2. The proliferation of PBMC from irradiated monkeys in response to 10 μg/ml Con A, as measured by SI.

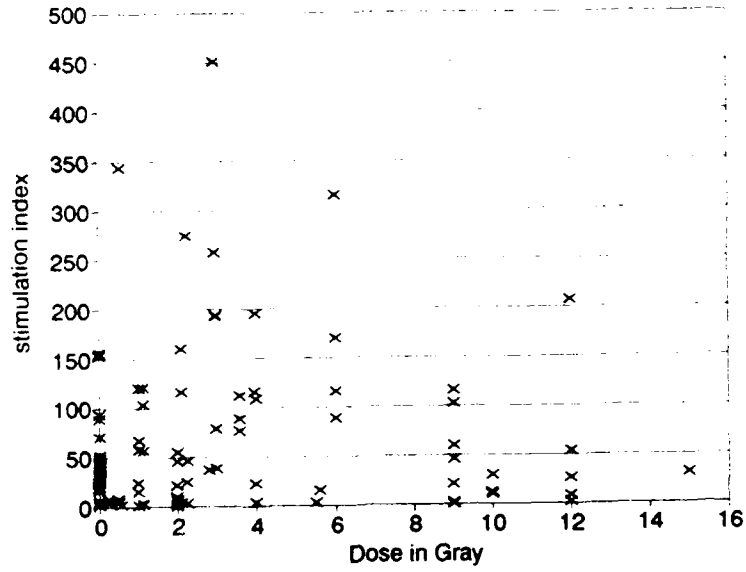

Figure 3. The proliferation of PBMC in response to 10 μg/ml PHA, as measured by SI.

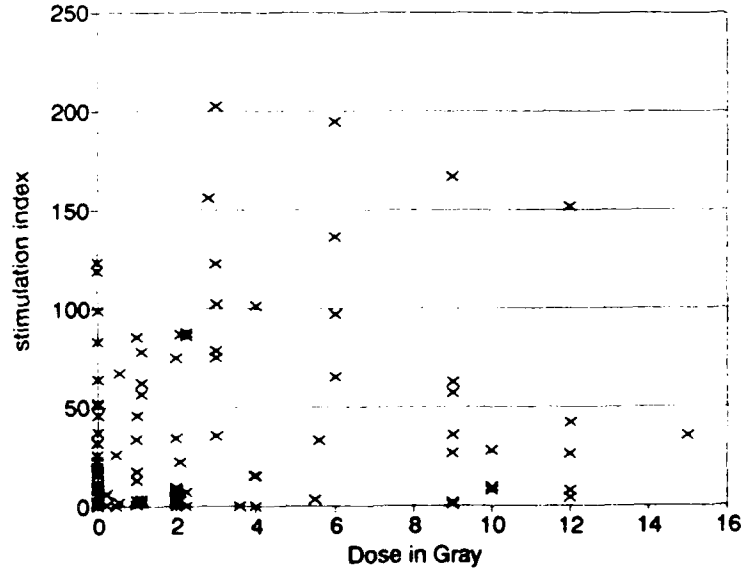

Figure 4. The proliferation of PBMC from irradiated monkeys in response to 2.5 μg/ml PHA, as measured by SI.

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