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Some Studies of Immunological Aspects of Aging  
and Fallout Radiation Exposure in a Marshall Island Population!\*

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In 1954 the people of Rongelap in the Marshall Islands were accidentally exposed to radioactive fallout during the atomic bomb testing program. Fortunately the exposure proved to be sublethal. Numerous reports have documented both the early effects of radiation, on blood cells and skin (Cronkite, Bond, Conard, Shulman, Farr, Cohn, Cohn, Dunham, & Browning, 1955), and the late effects, particularly in regard to growth retardation of children and the development of thyroid nodules and malignancies (Conard, Sutow, Colcok, Dobyns, & Paglia, 1969). Among the many studies on this population, the investigation of the possibility that radiation causes premature aging had been of continuing interest, particularly because such findings have been reported in irradiated animals (Furth, Upton, Christenberry, Benedict, & Moshman, 1954). Some 200 unexposed Marshallese people served as an excellent comparison population for these studies since they are closely related and live in the same environment. For aging studies measurements were made during physical examinations on characteristics considered to be criteria of aging (Conard, 1960; Conard, Lowrey, Eicher, Thompson, & Scott, 1966). Among these were skin elasticity and looseness and hair grayness; accommodation, visual acuity, and arcus senilis of the eyes; hearing loss; nerve and neuromuscular function, vibratory sense, and hand strength; response to light extinction test and rapid movement test, systolic blood pressure; and levels of blood cholesterol and body potassium ( $^{40}\text{K}$ ). Most of these criteria showed varying degrees of correlation with age and afforded a means of arriving at a "biological age" score for each individual. However, none of the tests showed any significant indication of premature aging in the exposed group that might be associated with radiation exposure.

During the past several years we have extended these studies to include an examination of some aspects of the immune status in the exposed and unexposed Marshallese populations which might be indicative of aging and/or radiation exposure. The present studies include measuring transformation and replication of circulating lymphocytes from phytohemagglutinin (PHA) stimulation in culture, quantification of the various serum proteins by electrophoresis, immunodiffusion studies for immunoglobulin levels, and routine enumeration of peripheral blood elements. In contrast to results of previous studies some of the present tests showed differences in the exposed population compared with the unexposed group that might be interpreted as radiation effects. Therefore in this report the results in the unexposed population will be treated separately to determine the correlation of these criteria with aging in a normal Marshallese population. The results in the exposed group will then be compared with the unexposed group to evaluate possible radiation effects.

#### MATERIALS AND METHODS

In Table 1 the numbers of subjects on whom the various tests were done are listed according to age decades.

Lymphocyte cultures. - Blood cultures were set up as follows. The buffy coat was separated from 5 ml of heparinized blood by sedimentation and centrifugation. The culture medium consisted of Eagle's minimum essential medium supplemented with 1% glutamine, 15% fetal calf serum, penicillin (100 units/ml), and streptomycin (0.1 mg/ml). Five-ml cultures were seeded with  $10^6$  leukocytes/ml, PHA M Difco (0.32 mg/ml culture) was added and the cultures were incubated at 37 C. At exactly 72 hr the cells were harvested, and the number of transformed lymphocytes (blast-like cells) was determined as follows. The cells were prepared for counting by the method of Stewart and Ingram (1968). A 1-ml aliquot of each culture was treated with a proteolytic enzyme (pronase) to remove

cellular debris and a cytoplasmic stripping agent (cetrimid) to release intact nuclei. The nuclei were counted and sized with a Coulter electronic counter (Model A). Previous experiments (Conard, 1969) had shown that the transformed cells had nuclei which were larger than 47 cubic microns. The percent transformation was obtained by comparing the number of larger cells with the total number of cells present. With the above culture technique, the leukocytes removed from the buffy coat are predominantly lymphocytes, but with varying fractions of other leukocytes, principally neutrophils. Although the total number of cells was constant at the beginning of culture for each individual, the number of lymphocytes varied because of slight differences in differential counts. However, by 72 hr, when the final counts were done, practically all neutrophils had disappeared from the cultures so that the percentage transformation of lymphocytes was not significantly affected by this variable.

Serum proteins. - Serum was collected from non-heparinized aliquots of blood from each individual. Total serum proteins (g/100 ml) were determined with a refractometer (American Optical-TS). Separation of serum proteins into albumen and alpha-1, alpha-2, beta, and gamma globulin fractions was done by microelectrophoresis with strips of cellulose acetate (Phoroslides) and a Millipore cell (Millipore Corp.). Barbitol buffer (pH 8.6, ionic strength 0.075) was used with a run separation of 17 min at 100 volts. The protein bands were stained with Ponceau-S dye and then quantified by using a Beckman/Spinco Analytrol with a microzone scanning attachment.

Serum immunoglobulins. Immunodiffusion procedures for the determination of immunoglobulins IgA, IgD, IgG, IgM, and kappa and lambda light chains were carried out by Dr. John L. Fahey and Dr. Roy Woods of the National Cancer Institute Immunoglobulin Center (Springfield, Virginia). The technique

used for quantifying the serum immunoglobulins in antibody-agar plates has been previously described (Fahey & McKelvey, 1965).

Peripheral blood elements. - The enumeration of peripheral blood elements was part of the routine medical examinations of the Marshallese. Leukocyte counts (Richar & Breakell, 1959) were carried out electronically (Coulter A counter). Platelet counts (Bull, Schneiderman & Brecher, 1965) were done by phase microscopy. Differential counts of leukocytes (200 cells) were performed on Wright stained smears. Hematocrits were determined by the micro-capillary method (Strumia, Sample & Hart, 1954), and sedimentation rates by the method of Wintrobe (1

Statistical analysis of data. - An analysis of variance was used to determine differences among groups for age, sex and radiation exposure. These data were programmed and analyzed on a high speed digital computer. Since sex differences were not apparent the sexes were combined and each criterion analyzed for age correlation (r value). The level of significance (p) of differences in the group exposed to fallout compared with the unexposed group (radiation effects) was expressed.

## RESULTS

The results are summarized in Table 2, and the values of the various criteria are plotted as a function of age in figures 1 through 4. Most of the changes noted generally reached a maximum effect in the 40-50 year age group with little further change in the older groups. Therefore most of the age-dependent correlation was due to differences between the younger (15-50 years) and older age groups (beyond 50 years of age).

Response of lymphocytes to phytohemagglutinin stimulation. The transformation of lymphocytes into blast forms as a result of PHA stimulation in peripheral blood cultures showed a decreasing response with increasing age (Fig. 1) which

was well correlated with age ( $r=0.89$ ). Lymphocyte cultures in the exposed group showed no significant differences from the unexposed group in response to PHA stimulation ( $P>0.68$ ).

Peripheral blood elements. - The changes in various blood elements as a function of age are presented in Tables 1 and 2. In the unexposed population the decreases in lymphocyte levels showed the greatest correlation with age ( $r=0.91$ ) and appeared to reach a maximum in the 50-60 year age group. Slight depressions in platelet counts, white blood counts, and hematocrit were noted but were less strongly correlated with age. An increase in sedimentation, however, was fairly well correlated with age. In the exposed population the mean levels of neutrophils and platelets were significantly depressed ( $P<.04$ ) below levels of the unexposed population particularly in the older age groups. (See Table 2, Fig. 2). The other blood findings were not notably different.

Serum protein patterns. - The results for serum protein studies as determined by electrophoretic analysis are shown in Fig. 3 and Table 2. A slight increase was noted with increasing age in the unexposed Marshallese, but it was not statistically significant. The gamma globulins increased significantly in older people. Alpha and beta globulin levels tended to show some increase though the correlation with age was not significant. Albumen levels tended to decrease slightly in older people. Pronounced differences were noted in the exposed population. The albumen levels were significantly higher and the globulin levels significantly lower than in the unexposed group. Alpha 1, Alpha 2 and gamma globulins showed the most pronounced depression in the exposed group (Table 2). Serum proteins, particularly gamma globulins

showed greatest deficits in the older exposed age groups.

The results of the immunodiffusion studies are shown in Table 2 and in Fig. 4. Parallel to the increase in serum gamma globulin levels, the immunoglobulins showed increasing values in the older age groups of the unexposed people. The increase in IgG moiety was most pronounced and showed significant correlation with age ( $r=0.78$ ). The increase in the other immunoglobulins did not show a high correlation with age. The increase in the K light chains was highly correlated with age ( $r=0.96$ ). Higher K/L ratios were noted in the older people though there was no significant correlation with age ( $r=0.46$ ). In the exposed population all of the immunoglobulins were depressed below levels of the unexposed group, the most pronounced depression was in the IgG and IgA moieties and in the L light chains.

#### DISCUSSION

Though the role of immune mechanisms in the aging process has never been clearly defined, it is generally agreed that such mechanisms are impaired in senescence. Ram(1967) in an excellent review of the subject pointed out that ".....it is well established that the capacity for immune responses increases during neonatal and juvenile life to a maximum in the young and adult animal, it remains constant for a time and then gradually decreases as the animal ages." The results of the present studies in the Marshallese people seem generally to support this thesis.

Before discussing the results of these studies, a few pertinent remarks should be made concerning the Marshallese people under study. This population is in many ways not comparable with a population in the United States in view of certain environmental and racial differences. As is generally true

throughout Micronesia, even though improvements in living standards have occurred, the people are subject to frequent infections such as gastroenteritis, upper respiratory infections, skin lesions and intestinal parasites. Therefore it is not surprising that the gamma globulin levels in the Marshallese have been found to be elevated. The A/G ratio is reduced or reversed in most of the people. The serum protein levels are generally elevated and may in part be related to dehydration associated with tropical environment where dependence for water is on rainfall which is scant at certain seasons. We have noted that the specific gravity of the urine is frequently high, and reduced red cell and blood volume have been found in this population. In spite of these factors the present studies should be valid for comparison of the exposed and unexposed Marshallese populations, since both groups are generally exposed to the same environmental factors, and studies of genetically inherited characteristics show them to be a relative homogeneous population.

These studies are believed to test the immunological status of the individuals to some degree though interpretation of some of the results is not readily apparent. The discussion which follows will first consider the results of the various tests in the unexposed population in terms of correlation with aging. The differences in the results in the exposed population will then be discussed in regard to radiation-induced effects.

In regard to aging effects in the unexposed comparison population it is quite obvious from the various graphs that even in the tests showing changes more closely correlated with aging, that changes are maximum by middle years (usually in the 40-50 age group) with little or no change, or in some cases even slight reduction after middle age. The reason

for this is not apparent.

The response of lymphocytes to PHA stimulation in peripheral blood cultures was tested because of the active role of the lymphocyte in maintaining immunological integrity. PHA is generally believed to evoke a non-specific blastogenic response in such cultures, since many more lymphocytes are transformed and proliferate with this agent than with specific antigens (such as tuberculin, tetanus, thyroid, pertussis, etc.). It is controversial whether or not PHA in this situation induces antibody formation. The exact mechanism of action of the mitogen is unknown. Conard and Demoise (1970), using autoradiographic and subcellular fractionation techniques, showed that a tritiated PHA was localized largely in the cytoplasm of transformed lymphocytes, with the greatest concentration in the mitochondrial fraction, which suggested that such organelles may be involved in initiation of lymphocyte transformation.

The Marshallese in this study showed a decreasing transformation of lymphocytes with PHA stimulation which was well correlated with increasing age. These results indicated that the percentage of lymphocytes that can respond to PHA was reduced as the Marshallese grew older. Such a finding appears to indicate a general decrease in immunological capacity of the lymphocytes, assuming that the response of these cells to specific antigens is similarly impaired.

Peripheral blood counts showed that a decrease in lymphocytes was well correlated with aging and compatible with cellular depletion and reduction in immunological capacity generally noted in the aged (Ram, 1967). It would appear, therefore, from these results that with aging there is on an absolute basis a greater loss of PHA-committed lymphocytes than of non-committed types. The slight decrease not

in the hematocrit and platelet levels may also be part of the phenomenon of age-related cellular depletion. Such reduction, however, was not noted in the case of neutrophils. The increasing sedimentation rate noted with aging in the Marshallese could be related to a cumulative effect of chronic infections and debility in the older age groups.

Electrophoretic studies of the serum showed serum protein levels (both albumen and globulin) well above the usually accepted norms for Caucasians. This finding may be related in part to dehydration and lowered blood volumes mentioned earlier. The increasing gamma globulin levels in the aging Marshallese is consistent with many reports in the literature (Karel, Wilder & Beber, 1956; Rafsky, Brill, Stern, & Corey, 1952; Goldbloom, 1955; Das & Bhattacharya, 1961). It is possible that the slightly lower albumen levels noted in the older Marshallese may be related to lowered food intake.

As would be expected from the serum electrophoretic data, the immunodiffusion studies showed increasing immunoglobulin levels with increasing age (Fig. 4). The most pronounced and most age-correlated change was in the IgG group. Since the K light chains are twice as prevalent as the L light chains in the IgG immunoglobulins (Ritzman & Levin, 1967), it is not surprising that there was a significant increase in the K light chains paralleling the increase in the IgG group. The K/L ratios in the Marshallese are similar to Caucasians (Fahey, 1969) and show a slight but insignificant increase in older people.

The increased immunoglobulins in the older Marshallese people is probably related to an accumulation of immunological reactions to infections. Parfentjev (1954) suggested that the increase in gamma globulins he noted in aging dogs and chickens was related to continuous contact with infectious

organisms resulting in hyperimmunization. Such a situation may be present in the Marshallese.

The increased gamma globulin levels would seem to be incompatible with decreased immunological reactions in the aged. It has been suggested that the increased globulin levels may be partly related to the development of autoantibodies with age. Blumenthal and Berns (1964) state that "while antibodies to exogenous antigens decrease with age there may be an age-related increase in gamma globulins, presumably containing antibodies to endogenous substances." On the other hand the changes may be of a compensatory nature. Perhaps in older people there is a greater conservation of immunoglobulins by some mechanisms which would decrease catabolism or excretion. Or there might be increased activity of those cells still capable of producing antibodies. If it had been possible to study age-related integrity of plasma cells and other lymphatic and reticuloendothelial tissues in these people, further light might have been shed on the problem. Finally, no attempt was made in the studies to separate "normal" older people from those with various morbid changes (if that is possible). Therefore some of the age-correlated changes may be associated with such morbid processes. But are not these processes part of aging? It will be interesting in future studies of this population, to see if longitudinal changes on an individual basis agree with the cross-sectioned results.

Let us now consider the differences noted in the exposed population as compared with the unexposed. Radiation-induced aging is a poorly understood phenomenon. It is generally considered to be a late effect of radiation, a manifestation of non-reparable injury since such aging effects are usually

not recognizable early. It seems likely that the relative depression of the peripheral blood elements noted in the exposed Marshallese is probably a continuing manifestation of incomplete recovery of the hematopoietic injury originally sustained. Therefore if such an effect, is a part of the aging process, it has been present since exposure since we have noted continuing depression of blood elements on annual examinations. The significant depression of the serum globulins and increase in serum albumens in the exposed population is a notable finding. The derpression in the gamma and alpha globulins and particularly in the IgA moiety and to a lesser extent in the IgG group and the L light chains would seem to indicate a reduction in relative immunological capacity or at least lowered antibody reserves in the exposed people. The tendency for the depression of these moieties to be relatively greater in the older age groups may imply a radiation-induced aging effect. Since these studies were not carried out earlier it is not possible to say when these changes developed. However, at 3 years post exposure the people were challenged with tetanus toxoid and primary and secondary responses were found not to be significantly different in the exposed compared with the unexposed population (Conard, Meyer, Rall, Lowrey, Bach, Cannon, Carter, Eicher, & Hechter, 1958). Also in spite of slight depression of blood elements and reduced serum globulin levels the exposed Marshallese people, based on our observations over a 15 year period since the accident, have not been observed to have any recognizable impairment of immunological capacity based on the incidence or susceptibility to illness or diseases. If the serum protein changes are a recent development then such deficiency may yet become apparent if the people are faced with a virulent antigenic challenge in the future.

The lack of any differences in transformation of lymphocytes in response to PHA stimulation in the exposed compared to the unexposed people is somewhat unexpected in view of the above findings. However, the lymphocyte levels in the exposed group have not been as severely depressed as some of the other blood elements indicating that there may be no significant depression of lymphocytes capable of responding to PHA stimulation

SUMMARY

Age related and/or radiation-induced age effects on immunological competence were tested in a Marshallese population of about 150 people, 50 of whom had been exposed to fallout radiation in 1954. In the unexposed group decreasing immunological competence with aging was indicated by :

(1) decreasing percentage transformation of peripheral blood lymphocytes by phytohemagglutinin stimulation indicating decreasing immunological competence of lymphocytes; (2) significant increase in gamma globulin reflected also in increases in IgG, IgA immunoglobulins and also increase of K light chains. These latter findings are believed to be associated with age-accumulated effects of repeated exposures to infectious agents and perhaps to increased autoimmune reactions.

In the irradiated population there were certain significant differences in the findings compared with the unexposed population which may indicate radiation effects. A relative depression of platelet and neutrophil levels of the peripheral blood was noted. Some slight degree of depression of peripheral blood elements has, however, been noted since exposure. Also noted was reduced gamma globulin levels (also IgG, IgA and K light chains on immunodiffusion analysis), more pronounced in older age groups which may indicate a radiation-induced aging effect. Evidence, however, for relative loss of immunological capacity in the exposed population has not been evident based on incidence or severity of diseases compared with the unexposed population

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Figure 1 Age-related change in lymphocyte transformation in peripheral blood cultures showing the mean percent transformation for each decade with standard deviation.

Figure 2 Age-related changes in the blood elements [total leukocytes (WBC), neutrophils, hematocrit, lymphocytes, platelets, and sedimentation rate] showing the mean level for each decade with standard deviation.

Figure 3 Age-related changes in serum proteins [total serum proteins, albumen, alpha-1 globulin, alpha-2 globulin, beta globulin, and gamma globulin] showing the mean level for each decade with standard deviation.

Figure 4 Age-related changes in immunoglobulin [G(IgG), A(IgA), D(IgD), M(IgM), lambda light chains, and kappa light chains] showing the mean level for each decade with standard deviation.

Table 1. Numbers of Marshallese Subjects Tested in Various Studies<sup>a</sup>

Age Group	Lymphocyte Transformation		Serum Proteins		Immuno-Globulins		Blood Elements <sup>b</sup>	
	<u>Unexposed</u>	<u>Exposed</u>	<u>Unexposed</u>	<u>Exposed</u>	<u>Unexposed</u>	<u>Exposed</u>	<u>Unexposed</u>	<u>Exposed</u>
13-20	11	11	12	11	6	4	29	15
21-30	11	9	11	10	9	2	16	7
31-40	25	10	26	10	9	2	20	7
41-50	19	4	19	4	11	2	11	8
51-60	15	6	17	6	11	3	5	5
61-70	12	3	12	3	12	2	11	3
71-80	9	1	8	1	7	1	6	1
Total Number	102	44	105	45	65	16	98	46

<sup>a</sup>Sexes were combined since the results on males and females showed no significant differences.

<sup>b</sup>The blood element studies were carried out in 1967, the others in 1968.

Table 2. Criteria Correlation with Age and Radiation Exposure

<u>Criterion</u>	<u>Unexposed Group</u>		<u>Exposed Group</u>	Significance (p value)
	<u>Change with age</u>	<u>Correlation with age(r value)</u>	<u>x percent dif. from unexposed</u>	
Lymphocyte Transformation	decrease	0.89	-1.1	.68
Serum Proteins				
Total Serum Proteins	increase	0.35	-1.5	.24
Albumen	decrease	0.45	+15.0	.01
Total Globulins	increase	0.58	-17.1	.01
Alpha-1	increase	0.37	-31.0	.01
Alpha-2	increase	0.43	-20.0	.01
Beta	increase	0.32	- 6.0	.03
Gamma	increase	0.75	-18.3	.01
Immunoglobulins				
A(IgA)	increase	0.49	-17.0	.05
D(IgD)	increase	0.20	- 3.0	.98
M(IgM)	increase	0.20	- 4.0	.74
G(IgG)	increase	0.78	- 8.0	.22
Kappa light chains	increase	0.96	- 3.0	.69
Lambda light chains	increase	0.24	-14.0	.15
K/L ratio	increase	0.41	+ 0.4	.74
Blood Findings				
Hematocrit	decrease	0.57	+ 2.9	.07
Sedimentation rate	increase	0.72	+11.4	.08
Total leukocytes	decrease	0.43	- 2.5	.59
Lymphocytes	decrease	0.91	- 0.1	.51
Neutrophils	increase	0.44	-13.8	.04
Platelets	decrease	0.65	- 8.4	.04

# IN VITRO TRANSFORMATION

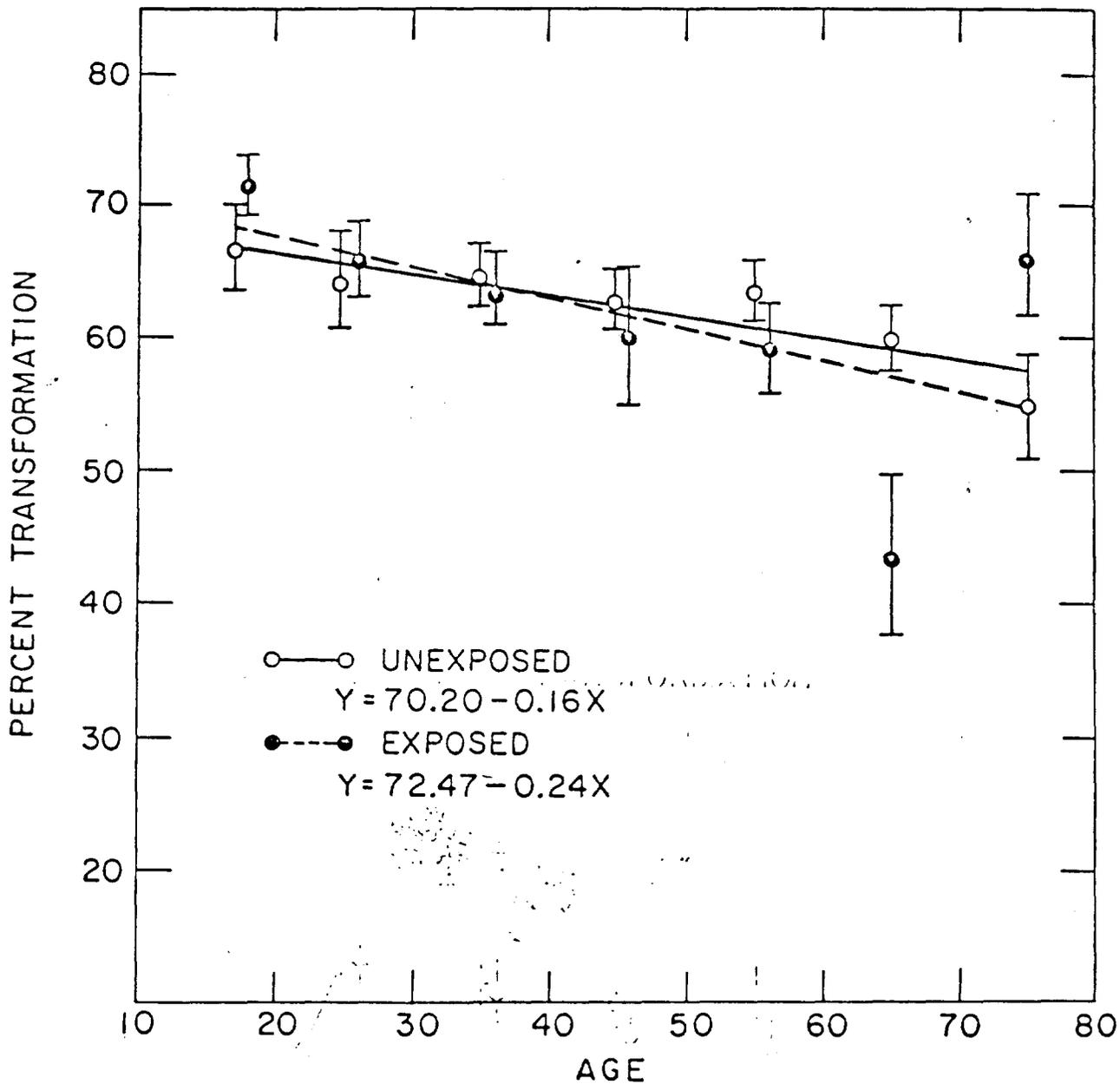


Figure 1 - Neg #1-579-70

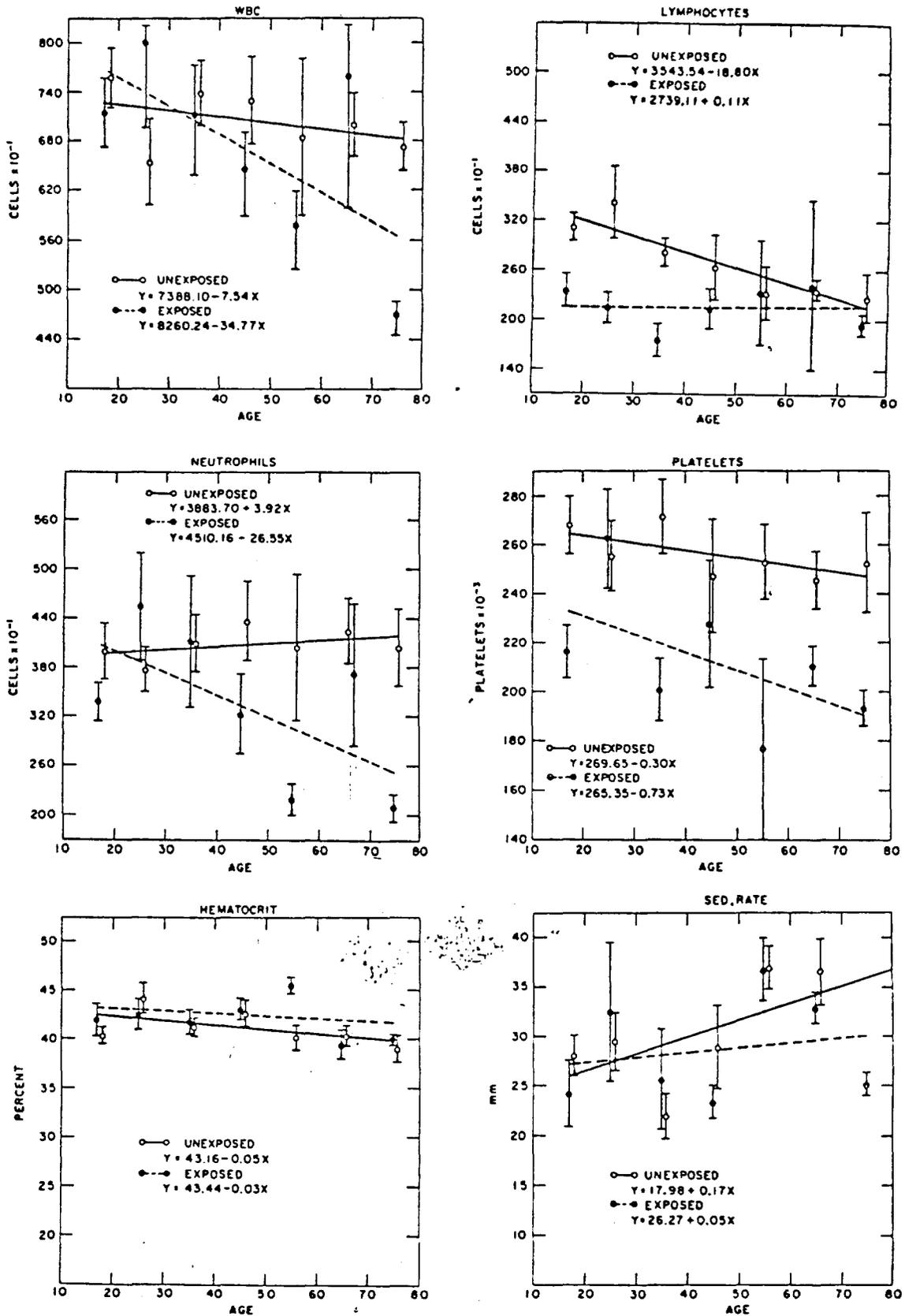


Fig. 2 Neg. #1-802-70

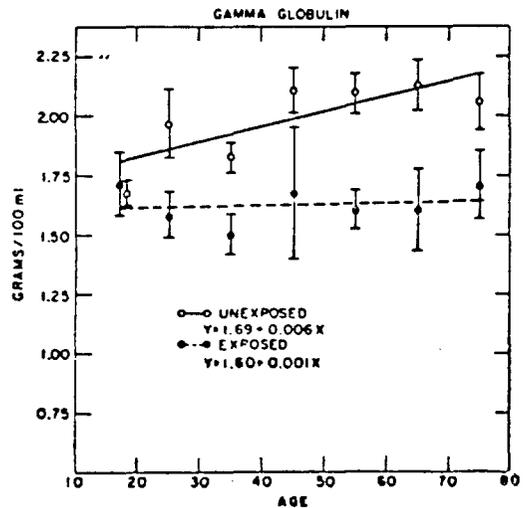
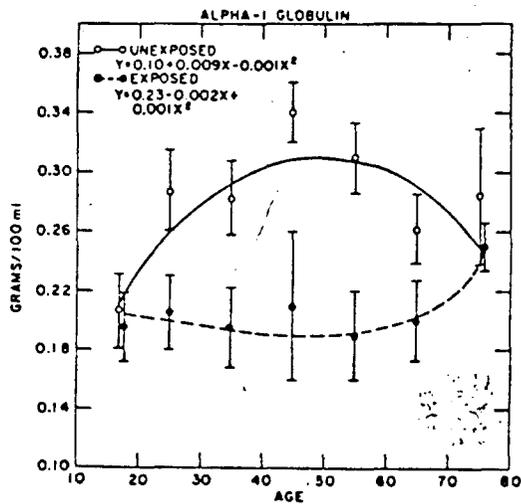
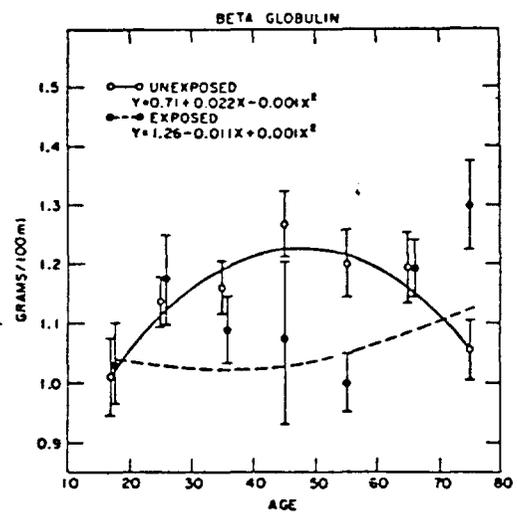
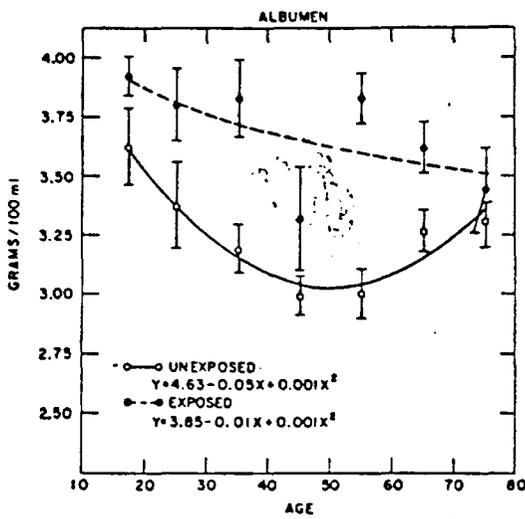
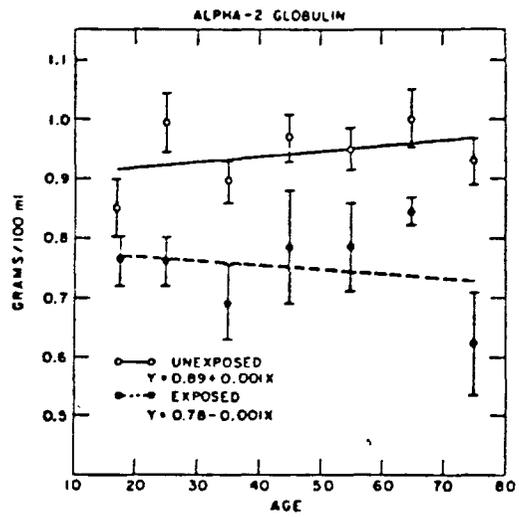
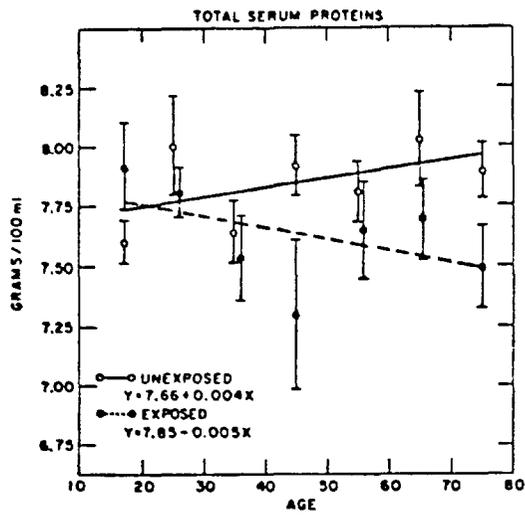


Fig. 3 - Neg. #1-801-70

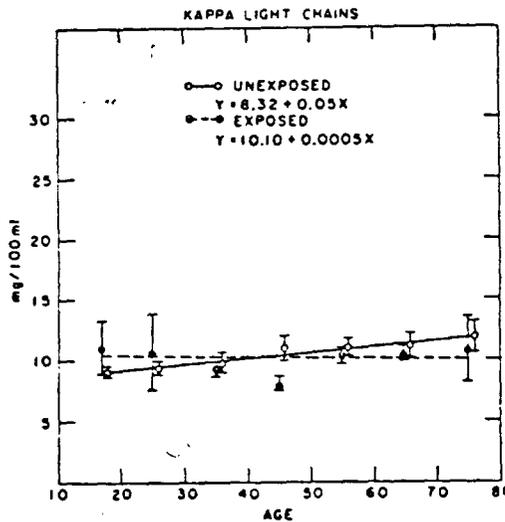
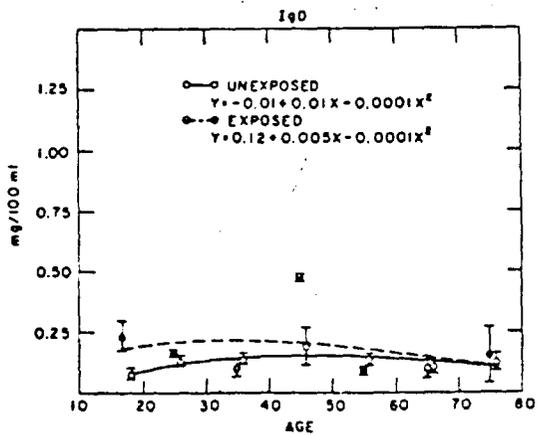
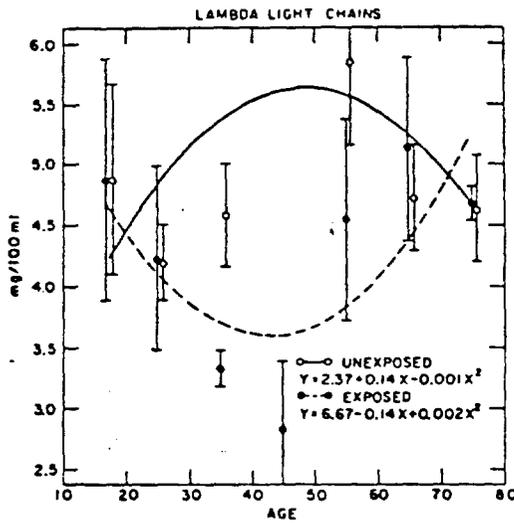
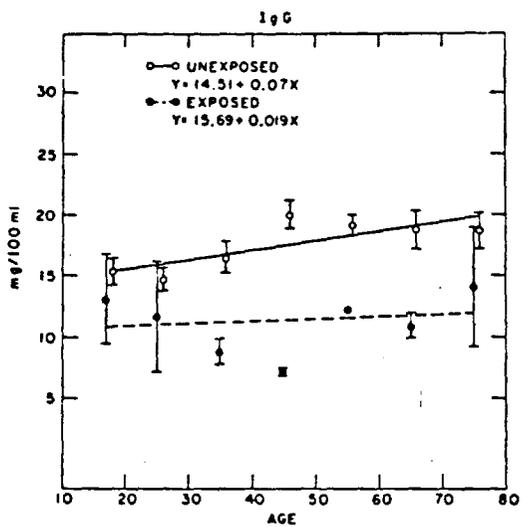
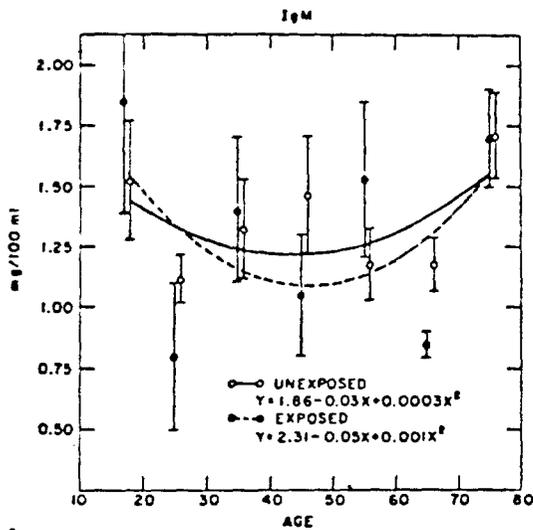
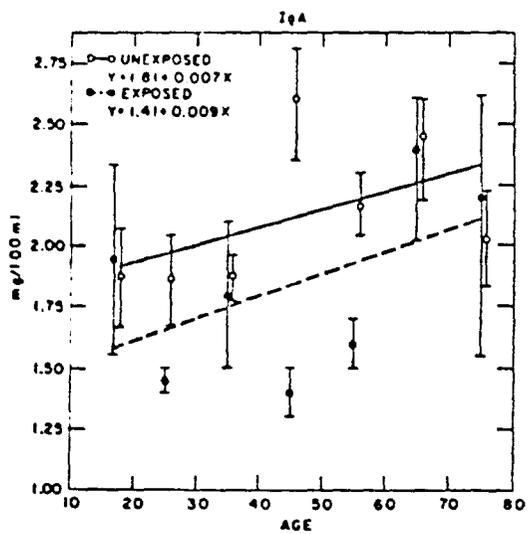


Fig. 4 - Neg. #1-800-70