To be presented at the 12th Hanford Biology Symposium on "Radionuclide Carcinogenesis," May 11-13, 1972, Richland, Wash.

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RADIATION-INDUCED IMMUNOSUPPRESSION: DEMONSTRATION OF ITS ROLE IN RADIATION LEUKEMOGENESIS IN THE INTACT RF MOUSE[1]

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[1] Research sponsored jointly by the U.S. Atomic Energy Commission under contract with Union Carbide, the National Cancer Institute, and the National Aeronautics and Space Administration.

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ACKNOWLEDGMENTS

The authors are indebted to Dr. B. Batzing for performing the fluorescent antibody tests, and to Mrs. J.O. Procotor and M.L. Hayes and Miss A.E. Walker for their excellent assistance.

ABSTRACT

Through an analysis of the immunologic and virologic nature of glomerulosclerosis in the RF mouse, it has been established that this disease results, at least in part, from an immune response to vertically transmitted wild type Gross leukemia virus (MuLV) antigens. The complexes of antigen and antibody lodge in the glomeruli, giving rise to glomerulosclerosis. Analysis of the leukemia patterns in these glomerulosclerotic mice has demonstrated that the immune response which this disease reflects reduces the probability of leukemia by a factor of 2. Further, radiation doses in excess of 100 rads suppress the development of this immune response, and this suppression contributes to the increased leukemia risk in this dose range.

INTRODUCTION

The hypothesis that the immunosuppressive effects of rapidly delivered large total doses of ionizing radiation contribute to the increased cancer risk associated with these exposures has been entertained for a number of years. This proposal states that cells which have undergone malignant transformation are antigenically different from the host in which they reside, and radiation suppresses the ability of the host to recognize and destroy them. In this view, radiation not only induces malignant transformations, but also reduces the ability of the host to cope with them. The importance of this proposal stems from the corollaries which would accompany its unequivocal proof: 1. exposure regimens which are only weakly immunosuppressive, such as low total doses and chronic or fractionated administration [1] should be less carcinogenic, on a per rad basis, than large rapidly delivered doses, and 2. pre-irradiation immunization and post-irradiation specific therapy should be possible.

The existence of antigenic differences between the host and a malignant neoplasm which it carries ~~has~~ have been established repeatedly as has the ability of the host to respond to these foreign antigens. Further, the immunosuppressive effects and the carcinogenic effects of radiation exposure are well documented, but the importance of this radiation-induced immunosuppression in the carcinogenic process has not been unequivocally established. Demonstration of further increases in the cancer yield in irradiated mice given immunosuppressive drugs or biologicals [2] has little relevance to the intact irradiated animal, for a variety of reasons, including the possible co-carcinogenic effects

of the added immunosuppressive agent [3]. Ideally, this proposal should be tested in a system in which a cumulative record of the individual animals response to tumor antigens could be compared to its pathology record without requiring external intervention.

The possibility that such a system might exist was recently demonstrated in our laboratory [4]. RF mice which died with severe glomerulosclerosis showed only one-half the leukemia incidence which was observed in non-glomerulosclerotic mice which died at the same time. No such reduced incidence of non-lymphatic tumors could be demonstrated for these mice with severe glomerulosclerosis; in fact the expected and observed incidences coincided almost exactly [4]. These data in combination with the established etiology of glomerular lesions in other systems [5] and the viral etiology of murine leukemias in general [6] led us to propose that glomerulosclerosis itself and the associated reduced risk of leukemia results from an endogenous humoral immune response to a murine leukemia virus which the RF mouse carried [4]. By attacking the virus itself or the antigens induced on the cell surface following infection, this response would reduce the probability that the mouse would develop leukemia, while at the same time the antigen-antibody complexes would accumulate in the glomeruli and lead to histologically detectable glomerulosclerosis. If this proposal is correct, then similar analyses of glomerulosclerosis and leukemia in irradiated mice, would provide a useful means of assaying the role of radiation induced immunosuppression in radiation leukemogenesis.

The present report summarizes our direct testing of the immunologic and virologic aspects of glomerulosclerosis in the RF mouse, as well as the effects of graded doses of radiation on its importance in leukemia development.

MATERIALS AND METHODS

The mice used in the present study were RF females which were part of an experiment designed to assay the relative biological effectiveness (RBE) of 60 MeV protons relative to 300 kVp X-rays for the induction of late radiation effects [7]. Mice were exposed, at the age of 70 days, to graded doses of protons or X-rays and then allowed to live out the remainder of their lifespan in conventional animal quarters. Since the RBE for the protons approximated unity for all of the late effects studied, we have pooled the data for the two types of radiation within each dose level. Separate analysis of each radiation type would lead to the same conclusions but suffer from the variation which accompanied the reduced sample sizes.

In order to be included in the present study, a mouse record had to include microscopic diagnoses (either positive or negative) for at least the following organs: heart, lung, kidney, spleen, ovary, uterus, lymph nodes, and liver. These sections include all of the major sources of positive pathological lesions in this mouse. Most records included additional information on organs such as the skin, which were taken only if a possible lesion was observed on gross autopsy. The second requirement for inclusion in the present study was that the sections had to have been read by the principal pathologist (NKC). This latter point is especially critical in the grading of glomerulosclerosis, which varies widely among pathologists. The criteria used in the grading of glomerulosclerosis have been detailed elsewhere [8]. As in our original study, we classify all

mice with the negative to moderate grades of glomerulosclerosis as "non-glomerulosclerotic" and reserve the term "glomerulosclerotic" for those mice with the severe forms of the disease. Additional details regarding the classification of the diseases has been published in our original report [4].

We could not demonstrate any significant differences in the survival times of the selected samples included in the present study and the original populations from which they were drawn (maximum difference in survival time equalled 23 days at the 100 rad dose), and conclude therefore that the results obtained in our analyses are reflective of the original population.

The method used to calculate the expected number of individuals in one sub-population from the incidence in another sub-population has been given elsewhere [4]. Briefly, one determines the fraction of mice which died without disease A but with disease B ($A^-B^+/ [A^-B^- + A^-B^+]$) in each 100 day interval, multiplies these fractions by the respective number of mice dying with disease A in each interval, and then sums these expected cases of A^+B^+ over the entire lifespan, or

$$\text{Expected } (A^+B^+) = \sum_{i=1}^{i=\infty} \left[\left[\frac{A^-B^+}{A^-B^+ + A^-B^-} \right]_i \quad \left[A^+B^- + A^+B^+ \right]_i \right]$$

Further details of the analysis are given below.

RESULTS

Before the pathology records of irradiated mice could be used to analyze the role of immunosuppression in radiation leukemogenesis, it was first necessary to establish the fact that severe glomerulosclerosis resulted, at least in part, as a by-product of a humoral immune response to wild-type Gross murine leukemia virus (MuLV) antigens. Presumably the less severe forms of glomerulosclerosis result from the same process, but we have pooled them with the mice showing no glomerulosclerosis in order to increase the sample size in the base population and force us to obtain conservative estimates of any deviation from non-random patterns. Direct testing of the immunologic and virologic nature of glomerulosclerosis in the RF mouse has been completed and will be reported in detail elsewhere [9]. The results of these investigations are summarized briefly below.

Twenty to 50 day old RF mice were injected with ^{125}I-labelled human gamma globulin in order to determine the rate at which they would clear the antigen from the blood and localize it within the spleen. The 20 and 30 day old mice were unable to clear the antigen (Figure 1), but by the age of 50 days this ability had adequately developed. Autoradiographic studies of the spleens in this latter group demonstrated both follicular localization and germinal center development.

Since 50 day old animals can clear and localize soluble antigens, and viruses are processed in the same manner [10] one would expect that clearance of the MuLV antigen to occur at or soon after the age of 50 days. Figure 2 shows that shortly after the age of 50 days, the frequency of MuLV positive spleens and thymuses decreases. In addition, MuLV antigen does not appear in the kidney until after this competence develops, but before the first histologically detectable signs of glomerulo-

sclerosis appear. While these data are strictly correlational in nature, they are consistent with our proposed etiology for glomerulosclerosis.

For the assay of the antigen and antibody in the kidney, we studied 1 and 1.5 year old RFM males in order to obtain maximum development of glomerulosclerosis. All of the kidneys were positive for MuLV antigen, as determined by complement fixation tests. Fluorescein-labelled rabbit antisera directed against murine IgG was used to determine the presence of the murine immunoglobulin in the glomeruli. Figure 3 shows the basic glomerular lesion and the fluorescent antibody labelling patterns observed in the glomeruli of 1 and 1.5 year old mice. Although both ages are positive for IgG in the glomeruli, it is our impression that the glomeruli of the older mice was stained more intensely. We are currently attempting to develop quantitative methods for assaying the intensity of the fluroescent labelling.

In order to determine whether this IgG in the glomeruli was specific for the MuLV antigens, we eluted it from the kidney, incubated it with cells which did or did not express the MuLV antigens, and then stained these cells with the same fluroescent antibody preparation used above. Specific labelling was only observed in those cells which expressed the MuLV antigen, indicating that the eluate contained an IgG which was directed against the MuLV antigen. These studies are continuing in two directions: further qualitative and quantitative description of the immune response in the RF mouse, and extension of the basic observation to include other viral leukemia systems.

Based on these observations and others presently accumulating in our laboratory, we conclude that glomerulosclerosis does reflect, at least in part, an endogenous humoral immune response to the MuLV antigens, and therefore analysis of the effects of radiation on its development and its relation to leukemia incidence should provide an adequate test of the role of immunosuppression in determining the increased leukemia risk in irradiated RF mice.

If we were to use a simple method of estimating disease coincidence, such as the final incidence of leukemia in two sub-groups, a potential bias would exist since the risk of having both an early and late occuring disease would appear artificially low. The expected incidences calculated according to the methods given above are based on the simple premise that animals dying at the same time should all have the same leukemia risk, independent of whether they do or do not have some other disease. Even if only 10% of the glomerulosclerotic deaths occur during the time that leukemias are appearing, the method used will be valid since the population considered to be at risk is the 10% which died at the appropriate time. It should be noted that estimates of reduced risk of leukemia in glomerulosclerotic animals obtained in this manner are highly conservative since they do not include those glomerulosclerotic mice which survived past the leukemic period because they had mounted the immune response and developed glomerulosclerosis. We are currently studying this problem directly by using a series of transplantable leukemias in the RF mouse.

The first point to be determined in this analysis was the effect of radiation on the development of glomerulosclerosis. If radiation has no effect on the development of this disease, then the glomerulosclerosis in the shorter lived irradiated mice should be identical to that observed in the unirradiated mice which lived the same amount of time. We calculated the expected incidences of each of the glomerulosclerosis grades for each irradiated group from the patterns observed in the unirradiated group, i.e., if 20% of the unirradiated mice dying in the first interval had moderate glomerulosclerosis, we would predict that 20% of all the deaths occuring in the same interval in each of the irradiated groups should show moderate glomerulosclerosis. For ease in presenting the data, each grade of glomerulosclerosis was assigned a numerical rating (negative = 0, mild = 1, moderate = 2, and severe = 3), and average expected and observed values were calculated. Figure 4 gives the expected and observed glomerulosclerosis grades as a function of radiation dose. Expected and observed values deviate significantly at the 200 and 300 rad levels. In this comparison or in one in which we compare severe glomerulosclerosis to all other grades, it is clear that doses of 200 rads or more suppresses the development of glomerulosclerosis.

For the sake of this discussion we will assume that the number of malignant transformations induced is a linear function of radiation dose. If this is the case, then the immune response has to cope with a greater number of antigenically foreign cells as the radiation dose is increased. We would predict therefore that the leukemia incidence would increase in both glomerulosclerotic and non-glomerulosclerotic mice, but that the risk of leukemia within a radiation dose group should always be reduced by the same factor in the glomerulosclerotic mice or those which mounted the immune response. If the leukemia risk in the glomerulosclerotic mice re-

mained constant over the entire dose range, it would imply a biological efficiency of remarkable proportions.

Table 1 gives the expected and observed incidences of leukemia in glomerulosclerotic mice as a function of radiation dose. Each expected value is calculated from the leukemia patterns observed in the non-glomerulosclerotic mice within that dose group. As is apparent from the table, the relative risk of leukemia in mice with glomerulosclerosis is constant over the 0 to 200 rad dose range, but the risk increases at the 300 rad level. For at least the 0 to 200 rad range, however, the immune response, if mounted, is able to reduce the probability of developing leukemia by a factor of 2.

In combination these data indicate that the effectiveness of the immune response, as reflected by the appearance of glomerulosclerosis, is radiation dose independent, at least through doses of 200 rads, but that a radiation dose dependence exists for the fraction of animals which can mount such a response. The question then arises regarding the efficiency of this response for each of the three major types of leukemia observed in the RF mouse: lymphoma, myeloid leukemia, and reticulum cell sarcoma. Table 2 summarizes the expected and observed cases of each of these leukemic types in glomerulosclerotic mice which had or had not been irradiated. The expected values were estimated within each dose group and then summed, as were the observed values. In the 5 comparisons which can be made(there were no cases of myeloid leukemia in the unirradiated group) glomerulosclerosis is associated with a statistically significant reduced risk of leukemia.

DISCUSSION

Three points have been established by these studies: glomerulosclerosis in the RF mouse is, at least in part, the by-product of an endogenous immune response to MuLV antigens, the effectiveness of this immune response in reducing the probability of developing leukemia is largely dose independent, and doses of radiation in excess of 100 rads inhibit the function of this immune response. In combination, these data indicate that at least part of the leukemogenic effectiveness of rapidly delivered high doses is owed to the suppression of the mouses ability to cope with malignantly transformed cells or the viruses which cause them.

The demonstration of an endogenous immune response to MuLV antigens presented here, and in detail elsewhere [9] confirms the observation of a similar response in the AKR mouse strain [11] and seriously questions the proposal of "tolerance" to the MuLV antigens [12]. Although this immune response has been demonstrated in two mouse strains, RF and AKR [11], it is obvious that reduction of the leukemia risk is not a necessary consequence of the immune response, since almost all of the AKR mice die with lymphoma within the first year of life [11]. Resolution of this paradox will require quantitative and qualitative analyses of the viral burden and the immune response in these two mouse strains, as well as others.

From the data presented above, it appears that the immune response against the MuLV antigens, as reflected by the development of glomerulosclerosis, is as efficient in the presence of radiation as it is in its absence, i.e., whatever the probability of developing leukemia is in the non-glomerulosclerotic mice, glomerulosclerosis is associated with a two-fold reduction in the probability. The possible exception to this occured following the

in the probability. The 300 rad group is a possible exception to this generality in that the risk of leukemia in glomerulosclerotic mice is 78% of that expected. Without further information, we can only speculate as to the reason for this alteration, but it should be remembered that high doses of radiation can induce kidney alterations which are histologically indistinguishable from those caused by the deposition of MuLV antigen/antibody complexes [13]. If we are in fact observing a radiation induced glomerulosclerosis component in the 300 rad group, it would account for the "dilution" of the reduced relative risk.

The only point which can be raised in support of this interpretation at the present time is seen in the plot of expected versus observed glomerulosclerosis ratings (Figure 4). Based on the rate at which the observed curve drops below expected values, the 300 rad observed point appears too high. If we restrict our consideration to severe glomerulosclerosis versus all other grades, the observed incidence of severe glomerulosclerosis in the 300 rad group actually exceeds that of the 200 rad group.

As a last point, the data in Table 2 indicate that all three of the major forms of leukemia show a reduced incidence in mice with glomerulosclerosis. Additional data presently accumulating in our laboratory confirms this association for the lymphomas and reticulum cell sarcomas, but indicates that the reduction in the incidence of myeloid leukemia is only of marginal importance, as would be indicated by the statistical testing results given in the table.

In summary, we conclude that part of the radiation leukemogenic mechanism involves the suppression of the animals ability to cope with the malignantly transformed cells or the viruses which induce them.

Table 1. Expected and observed cases of leukemia in glomerulosclerotic RF mice as a function of the radiation dose received at 70 days of age.

	RADIATION DOSE [rads]				
No. of Mice	311	339	350	278	306
No. with Glomerulosclerosis	114	113	129	69	75
Expected Cases of Leukemia	91.9	88.1	103.7	59.5	57.6
Observed Cases of Leukemia	50	47	67	34	45
Relative Risk	0.54[a]	0.53[a]	0.65[a]	0.57[a]	0.78[b]

a - $P < .0005$

b - $P < .05$

Table 2. Expected and observed cases of lymphoma, myeloid leukemia, and reticulum cell sarcoma in unirradiated and irradiated RF mice which had glomerulosclerosis.

	UNIRRADIATED	IRRADIATED
Glomerulosclerotic Mice	114	386
LYMPHOMA		
Expected No. of Cases	14.8	54.3
Observed No. of Cases	4	26
Relative Risk	0.27[b]	0.48[c]
MYELOID LEUKEMIA		
Expected No. of Cases	-[a]	48.7
Observed No. of Cases	-	30
Relative Risk	-	0.62[d]
RETICULUM CELL SARCOMA		
Expected No. of Cases	77.1	205.8
Observed No. of Cases	46	137
Relative Risk	0.60[e]	0.66[e]

a - no cases of myeloid were observed in unirradiated mice
b - $P < .01$
c - $P < .001$
d - $P < .05$
e - $P < .0005$

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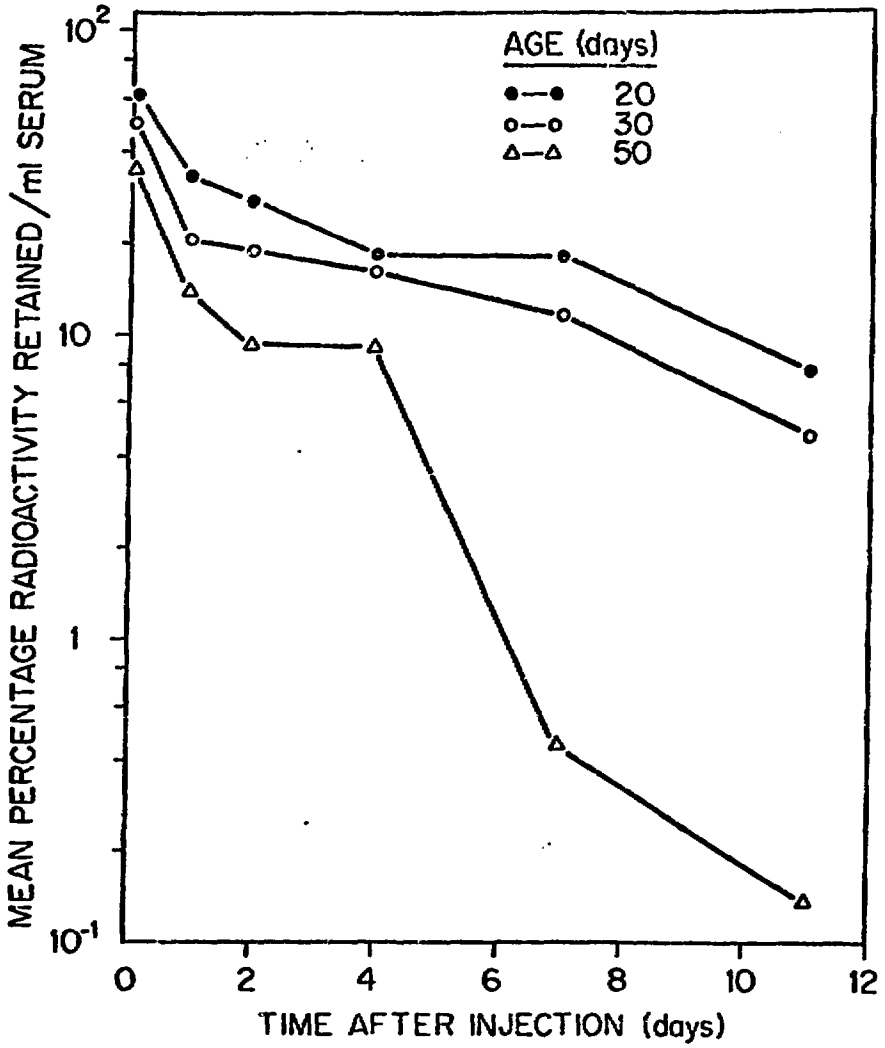
FIGURE LEGENDS

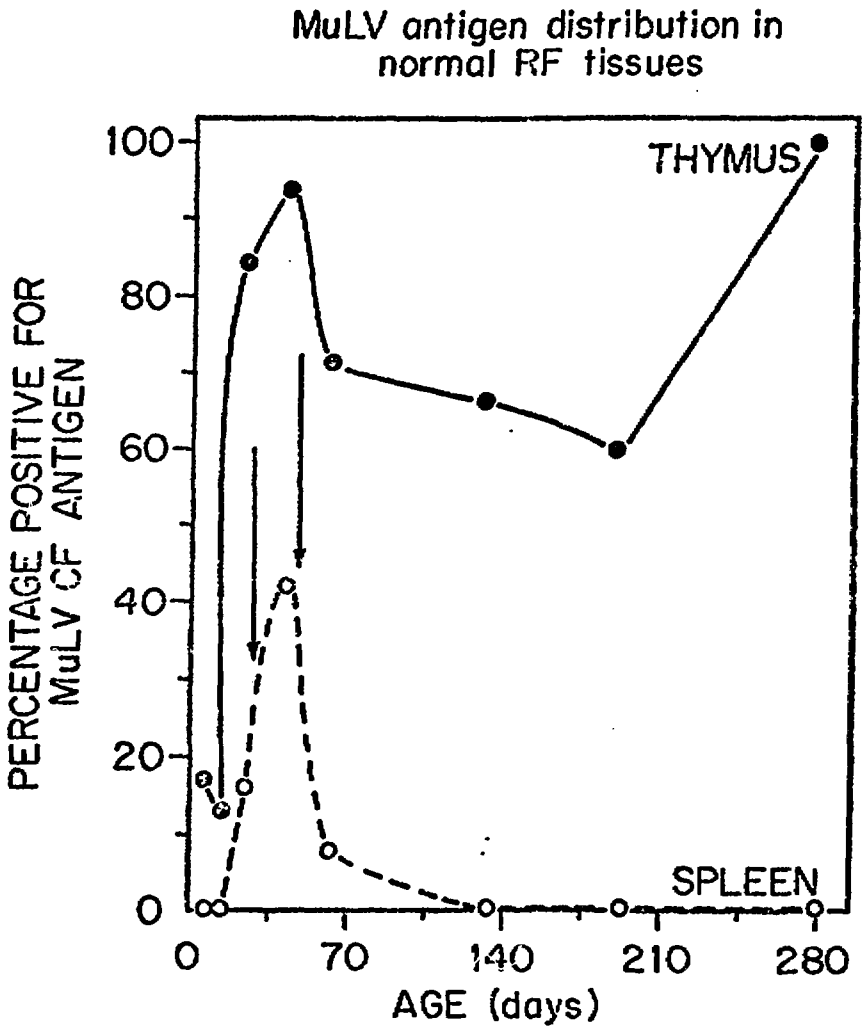
Figure 1. Residual radioactivity per ml of serum in 20, 30, and 50 day old RF mice as a function of time following injection of ^{125}I-labelled human gamma globulin.

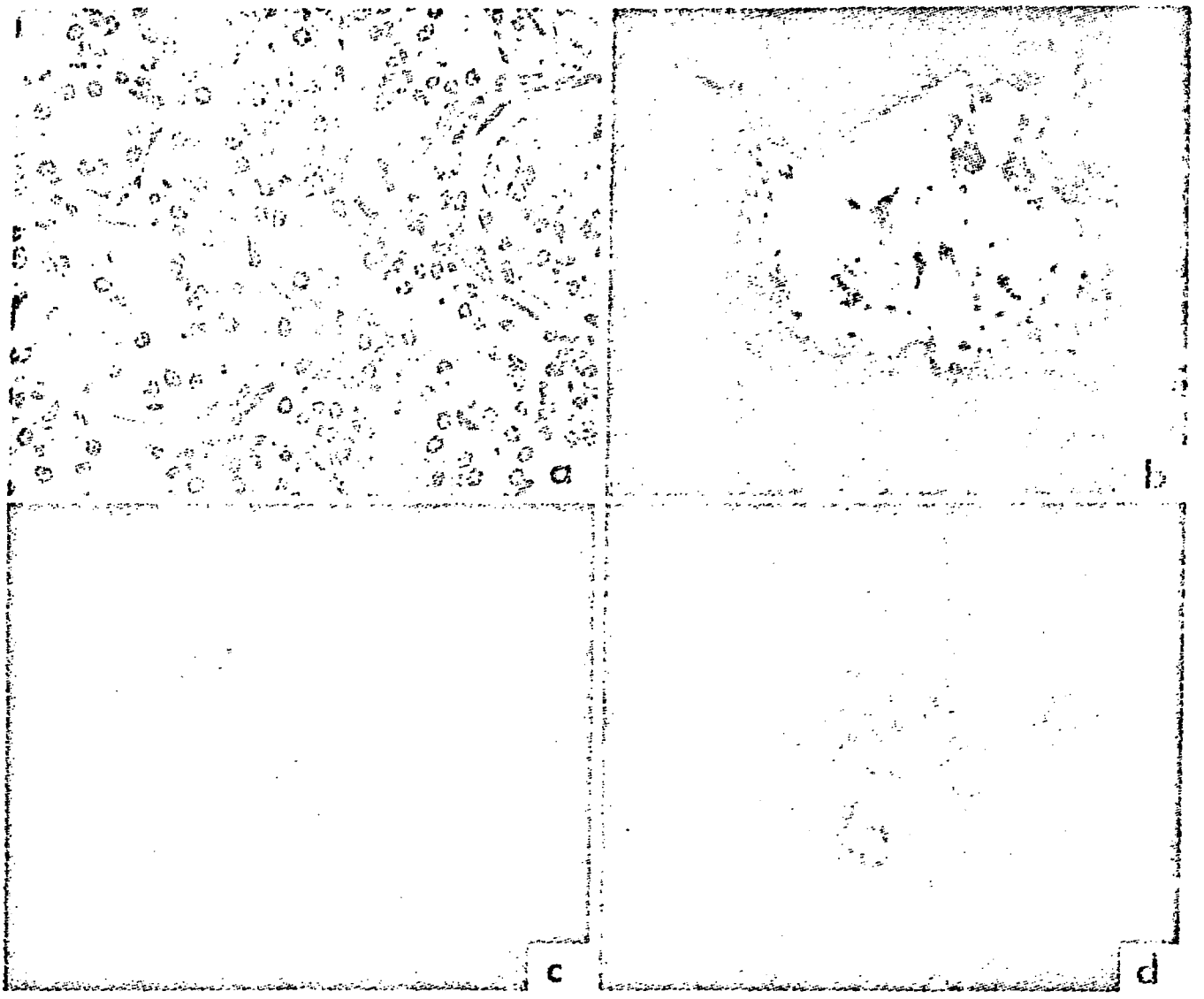
Figure 2. Frequency of MuLV positive spleens and thymuses as a function of age in the RF mouse.

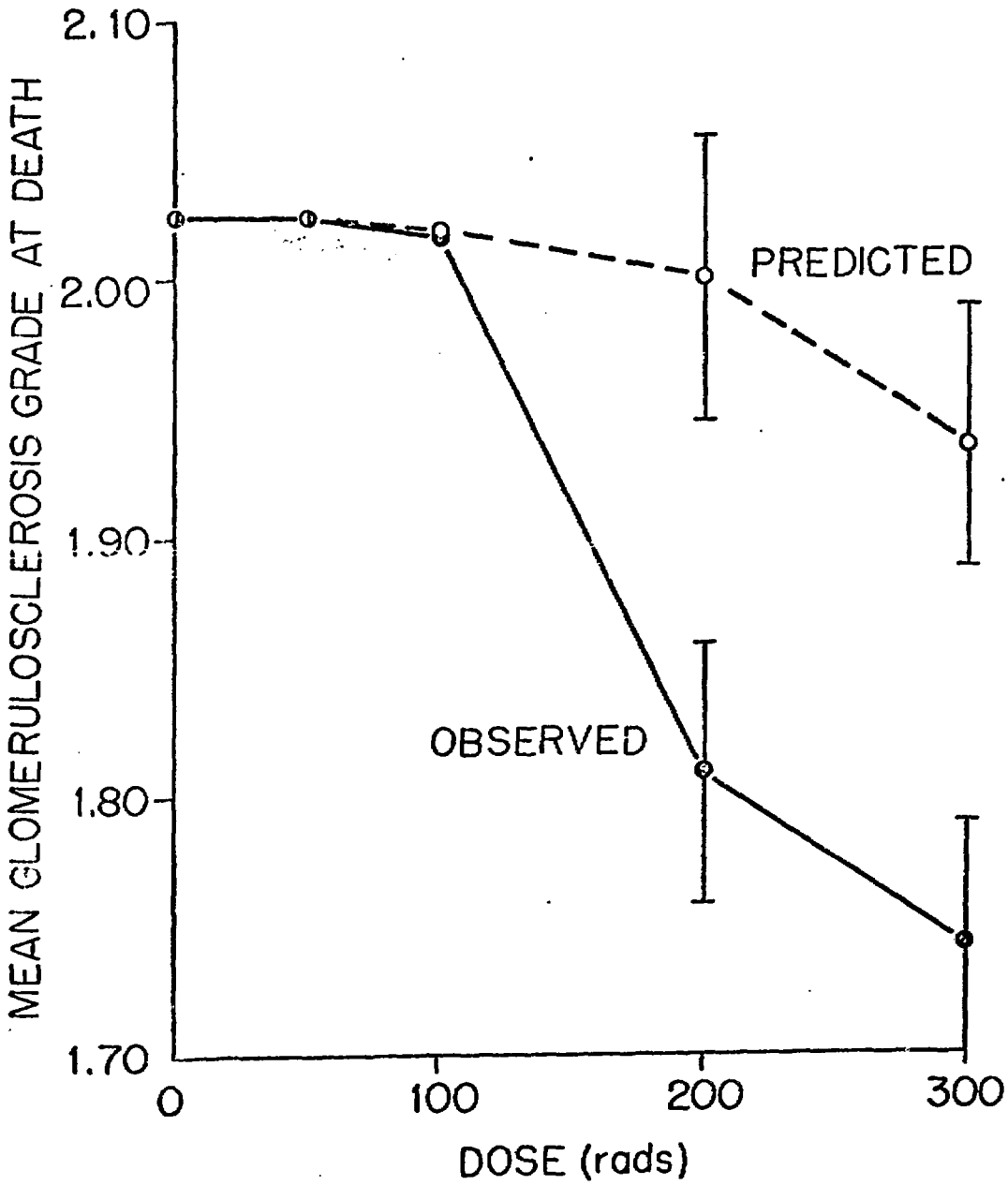
Figure 3. (a). Typical severe glomerulosclerosis in the RF mouse, (b) Glomeruli of 1.5 year old RF mouse stained with fluorescein-conjugated antibody to murine IgG, (c & d) same as (b) but from 1 year old mice.

Figure 4. Expected and observed mean glomerulosclerosis grade at death in RF mice as a function of the radiation received at the age of 70 days.


AGE (days)
20
30
50
10^2
10
1
10^{-1}
MEAN PERCENTAGE RADIOACTIVITY RETAINED/ml SERUM
0
2
4
6
8
10
12
TIME AFTER INJECTION (days)


MuLV antigen distribution in
normal RF tissues
PERCENTAGE POSITIVE FOR
MuLV CF ANTIGEN
100
80
60
40
20
0
THYMUS
SPLEEN
0
70
140
210
280
AGE (days)


a
b
c
d


2.10
2.00
1.90
1.80
1.70
MEAN GLOMERULOSCLEROSIS GRADE AT DEATH
PREDICTED
OBSERVED
0
100
200
300
DOSE (rads)