# REVERSAL OF NZB/NZW DISEASE WITH
# TOTAL LYMPHOID IRRADIATION*

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During an investigation of the cellular basis of the immunodeficiency in patients with Hodgkin's disease, long-lasting immunologic alterations were demonstrated after lymphoid irradiation (1). Alterations which were not present in unirradiated patients included a profound T lymphopenia and marked reduction in the in vitro mixed leukocyte reaction (MLR)[1] of peripheral blood lymphocytes to allogeneic cells. It was subsequently demonstrated that fractionated total lymphoid irradiation (TLI) produced potent immunosuppression in normal laboratory animals (2). Preparation of recipients with TLI allowed for successful bone marrow transplantation across major histocompatibility barriers in mice, rats, and dogs (3–5). Permanent chimerism developed without graft-versus-host disease. Experiments designed to look at the mechanism of graft survival revealed that allogeneic marrow cells, as well as heterologous soluble antigens, injected into animals after TLI evoked a state of antigen-specific tolerance rather than immunity (3, 6). An active role for suppressor T cells has been demonstrated (6).

NZB/NZW $F_1$ mice spontaneously develop an autoimmune disease characterized by anti-nuclear antibodies and a fatal immune complex glomerulonephritis (7). These features are similar to those seen in human systemic lupus erythematosus (SLE). These $F_1$ mice offer an excellent model in which to study therapeutic regimens possibly applicable to human disease. Immunologic abnormalities including spontaneous B-cell activation, age-related T-cell suppressor defects, and resistance to tolerance induction have been implicated in the pathogenesis (8–11). Many studies have demonstrated the efficacy of immunosuppressive drug therapy before clinical NZB/NZW disease appears, but few have been effective in mice with established or advanced disease (12–23). The object of the present study was to determine whether TLI could produce a remission in mice with moderate or advanced disease. Preliminary studies carried out in a single small group of animals showed that TLI can suppress the renal disease and markedly increase survival (S. Slavin and S. Strober, unpublished observations). The experimental results reported here using large groups of animals show that TLI was able to decrease the level of proteinuria and anti-DNA antibodies, and increase the 1-yr survival from 25% to >90%.

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## Materials and Methods

*Mice.* Parent NZB and NZW mice were obtained from Dr. M. E. Gershwin (University of California, Davis, Calif.) and Dr. H. O. McDevitt (Stanford University Medical Center). NZB/NZW hybrids were bred in the animal facilities of the Stanford University Medical Center. Only female hybrids were used.

*Irradiation.* TLI was administered using a regimen described previously (3). Briefly, mice were anesthetized with pentobarbital for proper positioning in an apparatus that exposed the major lymph nodes, thymus, and spleen to the x-ray source. The skull, lungs, long bones, and tail were shielded with lead. A Philips unit (250 kV, 15 mA, Philips Medical Systems, Inc., Shelton, Conn.) was used to deliver 17 fractions of 200 rad each to achieve a total dose of 3,400 rad during a 3- to 4-wk period.

*Proteinuria.* Proteinuria was measured with tetrabromphenol paper (Albustix, Ames Co., Inc., Elkhart, Ind.) on freshly expressed urine samples. This colorimetric assay which is relatively specific for albumin was graded $1-4^+$ and approximates protein concentration as follows: $1^+ \sim$ 30 mg%, $2^+ \sim$ 100 mg%, $3^+ \sim$ 300 mg%, and $4^+ \geq$ 1,000 mg%. Low grade proteinuria was defined as $1^+-2^+$, and high grade proteinuria was defined as $>2^+$ ($>100$ mg%).

*Anti-DNA Antibodies.* Antibodies to native DNA were determined using a modification of the Farr technique (24). Native DNA from *Escherichia coli* labeled with $^{125}$I (Amersham Corp., Amersham, England) was added to 50 $\mu$l of serum, diluted 1:10, and preincubated at 56°C for 30 min to decrease nonspecific binding. The specimens were incubated for 1 h at 37°C, and then for 16 h at 4°C. An equal volume of 90% saturated ammonium sulfate (SAS) was added, and the precipitate was collected by centrifugation and counted in a gamma counter (Beckman Instruments, Inc., Fullerton, Calif.) Results were expressed as arbitrary binding units as determined initially by reference standards (Amersham Corp.), and subsequently by standardized NZB/NZW sera. Binding units (units per milliliter) were similar to percent DNA bound when counts bound by the 0 U/ml standard are subtracted from total bound counts. (10 U/ml = 8.4% DNA bound, 15 U = 13.4%, 25 U = 25.5%, 60 U = 59.2%). Equal numbers of treated and control sera were measured in each assay.

A group of 31 nonexperimental mice including BALB/c, C57Bl/Ka, and young 3-mo-old NZB/NZW females had a mean ($\pm$ 2 SD) anti-DNA binding equal to 7.3 U/ml $\pm$ 7.9 U/ml. Values greater than 2 SD above the mean ($>24$ U/ml) in experimental mice were considered elevated above normal.

*Collection of Serum Samples.* Mice were bled at the initiation of the experiment and then monthly from the retro-orbital sinus. Blood was allowed to clot for 1 h, and serum was removed and stored at $-20$°C.

## Results

*Mice Treated With TLI at 6 Mo of Age.* 48 NZB/NZW female mice, aged 5–7 mo (mean age = 6.0 mo) were documented to have at least low grade proteinuria. 7 of 55 tested showed no proteinuria and were excluded from the study. Littermate animals were randomly allocated to treatment (24 animals) or control (24 animals) groups. During irradiation, both groups were taken from the housing area daily and anesthetized whereas only the treated animals were placed under the radiation machine. Both groups were housed in the same area and given similar diets. Mice were checked for proteinuria and bled monthly. When possible, kidneys were obtained after death for histology.

NZB/NZW mice tolerated the irradiation extremely well and there were no deaths during irradiation in the treated group. During the irradiation period, mean weight loss of treated animals was 9% of total body weight compared to 7% in the control group. An occasional irradiated animal developed transient diarrhea. All side effects cleared rapidly in the week after irradiation. To date, there have been no deaths directly attributable to the irradiation, and, one animal with tumor has been identified
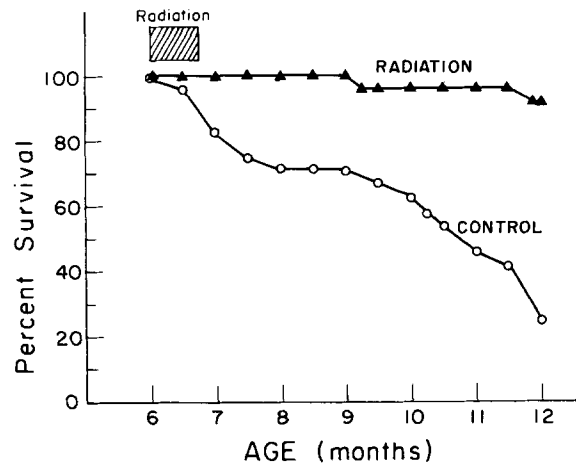
Fic. 1. Effect of treatment on the survival of NZB/NZW mice up to 1 yr of age. ▲—▲—▲, radiation group, O—O—O, control group. Hatched area is the time period of irradiation.

in both the irradiated and control groups.

*Survival.* Fig. 1 shows the improved survival rate in irradiated NZB/NZW mice. By 1 mo after the initiation of irradiation, there were 4 deaths in the control group and no deaths in the treated group. At age 8 mo, (2 mo after irradiation) the difference in the survival of the two groups became statistically significant ($P < 0.005$ by Fisher's exact test.) The difference continued to widen so that by 12 mo of age, 2 of the 24 treated animals and 18 of the 24 control animals had died ($P < 0.00001$). Neither death in the treated group was associated with significant prior proteinuria or clinical illness characteristic of the NZB/NZW kidney disease. Renal histology was not available in these two mice secondary to postmortem autolytic changes, and no obvious cause for death was found at autopsy. Of the 18 control group deaths, 16 had prior high grade proteinuria and characteristic clinical illness. Renal histology available from 10 of these animals showed extensive end-stage glomerular damage by light microscopy.

*Proteinuria.* Fig. 2 shows a significant reduction in the incidence of high grade proteinuria in the treated group by age 8 mo (2 mo after irradiation) ($P < 0.0001$). The difference became even more apparent as the study progressed and by 12 mo of age, 75% of the control group had progressed through a stage of high grade proteinuria compared to <10% in the irradiated group. High grade proteinuria present in one animal in the treatment group at initiation of the study was suppressed by 2 mo after irradiation, and did not reappear through the duration of the study.

*Anti-DNA Antibodies.* Fig. 3 demonstrates the suppression of anti-DNA antibodies in the treated group. 46% of the treated group initially had levels of anti-DNA antibodies above normal (>24 U/ml), but by 2 mo after irradiation, only 17% had elevated levels. Treated mice had median values equal to 5 U/ml both at 7 and 8 mo of age and equal to 11 U/ml at 9 mo of age. In contrast, the control group showed a rise in anti-DNA antibodies over this time period such that 60% were above normal by 2 mo (median = 36 U/ml) and 75% were above normal by 3 mo after irradiation (median = 44 U/ml). The treated group maintained their lowered levels through 12 mo of age. The levels of antibody in treated and control group are significantly
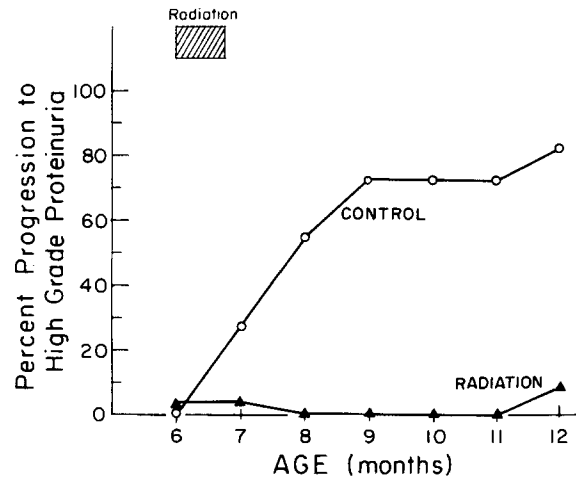
Fig. 2.   Effect of treatment on the cumulative progression to high grade proteinuria ($>2^+$, $>100$ mg%) up to 1 yr of age. Mice that died without high grade proteinuria are not included in the denominator after their point of death. Two control animals died before 7 mo of age without proteinuria. Two treated animals also died without proteinuria, one at 10 mo and one at 12 mo of age. ▲—▲—▲, radiation group; O—O—O, control group.
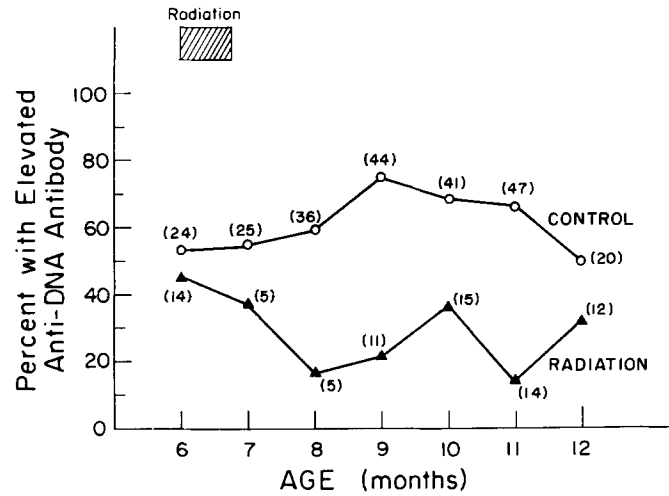


Fig. 3.   Effect of treatment on anti-DNA antibodies up to 1 yr of age. The data is expressed as percentage of mice with levels elevated above normal. The mean normal value ($\pm$SD) was 7.3 U/ml $\pm$ 7.9. Values $>2$ SD above this mean ($>24$ U/ml) were considered elevated. The numbers in parentheses are the median values in units per milliliter for the group of mice at that time. ▲—▲—▲, radiation group; O—O—O, control group.

different at 8, 9, 10, and 11 mo of age ($P < 0.01$ by Wilcoxon rank test). A comparison after 11 mo is difficult because of the small number of control animals remaining alive.

*Mice With Advanced Proteinuria.*   18 7-mo-old female NZB/NZW mice were selected for advanced proteinuria ($3^+$ or $4^+$) and placed in treatment (12 animals) or control (6 animals) groups. Several mice which were clinically ill (ascitic or wasted) were
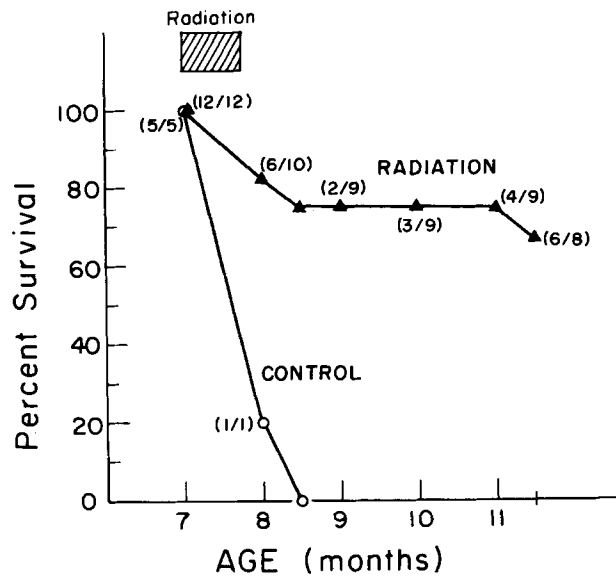
FIG. 4. Effect of treatment on advanced NZB/NZW disease. The numbers in parentheses indicate the fraction of mice with high grade proteinuria of those alive at that time. ▲—▲—▲, radiation group; O—O—O, control group.

included in the study. One animal in the control group died before the onset of the study and therefore is not included in the analysis. In this experiment, control mice were not anesthetized as a result of their poor clinical state.

Fig. 4 shows the prolongation of survival and reversal of proteinuria in the treated group. All 5 control animals died within 6 wk of beginning the study, whereas 3 of 12 irradiated animals died during this time period (2 anesthetic deaths) ($P < 0.01$). Thereafter, the nine treated animals remained alive for more than 4 mo after irradiation. Several clinically ill mice actually showed clinical improvement during the irradiation period. At 1 mo after irradiation, 4 of the 10 remaining animals had reversal of high grade proteinuria and at 2 mo after irradiation, 7 of 9 animals had reversal of high grade proteinuria. Subsequently, high grade proteinuria reappeared in some of these animals.

## Discussion

Many studies with NZB/NZW mice have begun treatment at an age (<4 mo) when the autoimmune disease has not yet been expressed (12, 13, 15, 18, 19, 21, 23). These studies are properly termed prophylactic and have limited application to human SLE. Some therapeutic studies used animals 4-5 mo old, when anti-DNA antibodies and gamma globulin deposition in the kidney are present but histologic glomerular damage and onset of proteinuria have not yet occurred (25, 16, 17, 20, 26). Various immunosuppressive and anti-inflammatory treatment regimens begun at the time of early disease can prevent proteinuria and renal death (12-23).

Few studies have shown actual suppression of disease after the development of proteinuria. Continuous high dose, weekly cyclophosphamide has been effective in

suppressing already present renal disease and prolonging survival when begun after the age of 5–6 mo (12, 13, 21, 22). In contrast to these studies, a trial of combination therapy with cyclophosphamide, azathioprine, and methylprednisolone which was markedly effective at 5 mo of age was unsuccessful in prolonging survival at 8 mo of age (20). When 8-mo-old mice were divided into high ($\geq 3^+$) and low ($<2^+$) proteinuria groups, only the low proteinuria animals showed a response to therapy. Cyclosphosphamide treatment has been associated with an increased tumor incidence in NZB/NZW mice (12, 19, 21, 23). Another drug capable of suppressing NZB/NZW renal disease is the anti-viral agent, ribavirin (26). NZB/NZW mice treated at 20 wk of age developed high grade protenuria and then lost proteinuria while treatment was continued. In addition, survival of the mice was considerably prolonged.

TLI is a routine, safe radiotherapy regimen used to treat Hodgkin's disease (27). Severe side effects are unusual and all severe complications requiring hospitalization are <1% (including infectious complications). No cases of acute leukemia have been observed in several hundred Hodgkin's disease patients treated with radiotherapy alone at Stanford during the past 10 yr (28). We have demonstrated the ability of TLI to produce potent immunosuppression in humans and normal laboratory animals (1, 2), and therefore attempted to treat NZB/NZW disease. The experimental results show that NZB/NZW hybrid females with proteinuria (aged 5–7 mo) had no mortality and minimal (mean 9%) weight loss during the administration of 3,400 rad (TLI). Over 90% of the treated animals, but only 25% of the untreated controls were alive at 12 mo. The treated group showed a decrease in both proteinuria and anti-DNA antibodies. TLI was also able to markedly prolong survival even in animals with advanced proteinuria such that 67% were alive at 11.5 mo of age. Although only one animal with a tumor has been identified in both the treated and control groups, we are continuing to follow the treated animals carefully for late tumor formation.

The mechanism by which TLI suppresses NZB/NZW disease is unknown at present. The decrease in anti-DNA antibodies suggests that is not just an anti-inflammatory effect or a local effect of radiation on the kidney. It is likely that the potent immunosuppression induced by TLI plays an important role. There is considerable evidence that the qualitative aspects of the immunosuppression produced by TLI are unique as compared to known immunosuppressive drugs and single dose, whole body irradiation. Only TLI causes long-lasting T lymphopenia and B lymphocytosis (2), and allows for the induction of tolerance to alloantigens and heterologous proteins (3, 6). The antigen specific tolerance has been shown to be dependent on the generation of suppressor T cells (6). Furthermore, there is evidence for the generation of antigen nonspecific suppressor cells shortly after TLI in the BALB/c mice (6).

NZB/NZW mice have an age related loss in the induction and maintenance of antigen-specific tolerance as well as a loss in antigen nonspecific T-suppressor function (9, 10). These immune abnormalities may contribute to the immunopathology and expression of autoimmune disease in these mice (11). We are presently investigating whether the suppression of NZB/NZW disease with TLI is associated with increased susceptibility to tolerization and the generation of suppressor T cells.

In summary, we have demonstrated a reversal of NZB/NZW disease without significant side effects with TLI. There is already an extensive experience with TLI in humans, and it has been well tolerated with only rare serious complications (27). This study suggests that TLI may have potential application to human SLE.

## Summary

NZB/NZW mice spontaneously exhibit autoimmune disease similar to that seen in human systemic lupus erythematosus (SLE). We demonstrated that total lymphoid irradiation (TLI) reversed well expressed disease in 6-mo-old NZB/NZW females with a prolongation in survival, decrease in proteinuria, and decrease in anti-DNA antibodies as compared to control animals. Few side effects were observed in the treated group. TLI also prolonged survival in animals with advanced renal disease. These findings suggest that TLI may have application to the treatment of human SLE.

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