Total Lymphoid Irradiation Prevents Diabetes Mellitus in the Bio-Breeding/Worcester (BB/W) Rat

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SUMMARY

Total lymphoid irradiation (TLI) at doses of 2200 rads or greater prevented diabetes in susceptible BB/W rats. Two of 29 (7%) treated rats became diabetic compared with 23 of 39 (59%) controls ($P < 0.001$). TLI did not, however, prevent insulitis or thyroiditis in nondiabetic rats, nor did it restore the depressed concanavalin-A responsiveness of BB rat lymphocytes. T-lymphocyte subset proportions were the same in both groups. TLI was associated with significant radiation-related mortality, and nondiabetic TLI-treated rats weighed significantly less than controls. We conclude that TLI is effective in the prevention of BB rat diabetes. However, TLI fails to correct the subclinical immunologic abnormalities of the model and is associated with significant morbidity. DIABETES *33*:543–547, June 1984.

The Bio-Breeding (BB) rat[1] develops a syndrome of spontaneous diabetes that shares many of the characteristics of human insulin-dependent diabetes mellitus (IDDM). The rats are lean and develop acute hyperglycemia with ketonemia between 60 and 120 days of age. Equal numbers of males and females develop the syndrome, and most affected animals die within 2 wk unless treated with insulin.

The presence of lymphocytic insulitis suggests a cell-mediated autoimmune pathogenesis of the syndrome. Additional data that support this hypothesis include the finding of circulating autoantibodies,[2–4] the passive transfer of BB diabetes[5] and insulitis,[6] and prevention of the disorder by immunosuppression.[7–9]

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We investigated the effect of total lymphoid irradiation (TLI) on young diabetes-prone BB rats. TLI is an immune intervention designed to deliver high doses of radiation to lymphoid tissues. Radiation is given in multiple small daily doses using lead shielding to protect radiosensitive nonlymphoid tissue. TLI has been used to prevent allograft rejection[10,11] and, more recently, to ameliorate autoimmune diseases in both humans[12,13] and animals.[14–17] In this study, we present data showing that TLI is effective in preventing diabetes in the BB/W rat. The data also support the hypothesis that BB rat diabetes has an autoimmune pathogenesis.

MATERIALS AND METHODS

Animals. All experiments used 35–43-day-old, diabetes-prone Bio-Breeding/Worcester (BB/W) rats from the colony maintained at the University of Massachusetts Medical School, Worcester, Massachusetts. The frequency of diabetes in these rats averages 40–60%. Male and female rats were used in approximately equal numbers. In the study of lymphocyte subsets, control rats were drawn from a subline of BB/W rats bred for the absence of diabetes. Spontaneous diabetes in these W-line rats has not occurred in 10 generations of brother-sister matings ($N > 1000$).

Radiation. Total lymphoid irradiation was given using a General Electric Maximar 250-III unit (200 kV, 15 mA), which was calibrated before each use with a Victoreen R-Meter. The source-to-skin distance was 30 cm, and the rate of administration averaged 30 rads/min. Before irradiation, rats were anesthetized with chloral hydrate (0.36 mg/kg body wt). The anesthetized rats were irradiated in lead shielding devices previously described by Slavin et al.[11] This shielding design permitted radiation of major lymph nodes (submandibular, cervical, axillary, inguinal, and mesenteric), the thymus, and the spleen while protecting radiosensitive nonlymphoid organs. The radiation schedule was 200 rads per day, 5 days per week, until the total dose was accumulated.

Protocols. Three experiments were performed. The first two experiments investigated the effect of TLI at different doses on the incidence of diabetes in the BB/W rat. In experiment 1, litters of rats were randomized into 3 groups. Twelve rats

TABLE 1
Effect of total lymphoid irradiation (TLI) on the frequency of diabetes in BB/W rats

Treatment	Diabetic	Nondiabetic
1600 rads	3	8
3400 rads*	0	11
Control	5	7
2200 or 2400 rads†	2	16
Control	18	9

*$P < 0.05$ vs. corresponding control.
†$P < 0.001$ vs. corresponding control.

received 1600 rads of TLI, 12 received 3400 rads of TLI, and 12 were sham irradiated. In experiment 2, 12 experimental rats received 2400 rads of TLI and 8 littermate controls were sham irradiated. The sham-irradiated control rats received only anesthesia. Throughout the study, the animals were given ad libitum access to food and water, were weighed weekly, and were tested for diabetes twice weekly between the ages of 60 and 120 days. Animals that appeared ill were tested more frequently. Diabetes was diagnosed on the basis of 4+ glucosuria (Tes-tape) and a plasma glucose ≥250 mg/dl.

The final experiment investigated the effect of TLI on the incidence of diabetes, insulitis, and thyroiditis; on subpopulations of lymphocytes; and on the lymphocyte response to the mitogenic activity of concanavalin-A (con-A). Seventeen experimental rats received 2200 rads of TLI and 19 littermate controls received anesthesia alone. After the TLI dose was complete, these rats were weighed weekly and tested twice weekly for diabetes. Rats that became diabetic were killed and not studied further. At 120 days of age, the surviving nondiabetic experimental and control rats were anesthetized with ether, and 4 ml of blood was collected by orbital puncture and used for the measurement of con-A responsiveness.

Approximately 7 days later, the rats were again anesthetized with ether, and 2 ml of blood was removed. Each rat was then killed, and its pancreas and thyroid were removed, fixed in Bouin's solution, and embedded in paraffin. Sections were later stained with hematoxylin and eosin and examined for the presence of insulitis and thyroiditis. The examinations were performed by a pathologist (A. A. L.) who was unaware of the treatment status of the rats. In addition, the spleens were also removed for use in the study of lymphocyte subsets.

Concanavalin-A stimulation. The response of lymphocytes to con-A was measured as previously described.[18] Briefly, blood was diluted in RPMI-1640 medium (Microbiological Associates), layered on Lympholyte M (Cedarline Laboratories), and centrifuged. The interface cells were counted with a hemocytometer and their viability was ascertained with trypan blue. Viability was greater than 90% in all experiments. Cells were cultured in Limbro 96-well, flat-bottom microtiter plates at 1×10^6 cells/ml in RPMI-1640 medium supplemented with 5% heat-inactivated fetal bovine serum, 5×10^{-5} M 2-mercaptoethanol, and, per milliliter, 3 mg of glutamine, 100 U of penicillin, and 100 μg of streptomycin. Concanavalin-A (Miles-Yeda, Rehovot, Israel) was prepared to provide a stock solution of 1 mg/ml in phosphate-buffered saline from which dilutions were then made. Appropriate amounts of con-A were added to lymphocytes to achieve doses of 0.03, 0.125, 0.5, or 1.0 μg/ml in a final volume of 200 μl/well. Measurements at each dose were performed in triplicate and subsequently averaged. The cells were cultured for 72 h in an incubator with 5% CO_2 in air at 37°C. After incubation, methyl-[^{3}H]thymidine (1 μCi/well, specific activity 6.7 Ci/mM, New England Nuclear) was added, and the cells were incubated for an additional 18 h. The cells were collected on glass fiber filters with a Mash II harvester, and [^{3}H]thymidine incorporation was measured with a Hewlett-Packard liquid scintillation counter.

Lymphocyte subsets. The blood samples obtained at the time of killing were used to measure total peripheral white blood cell counts and peripheral and splenic lymphocyte subset percentages. White cell counts were determined manually with a hemocytometer.

Monoclonal antibodies directed against T-helper cells (W 3/25) and T-suppressor cells (OX 8) were obtained from Accurate Chemical and Scientific, Westbury, New York. Monoclonal antibody specific for T-cells (OX 19) was a gift of D. Mason. F(ab')$_2$ goat anti-mouse IgG (Cappel Laboratories, West Chester, Pennsylvania) was absorbed with Sepharose 4B-rat IgG and fluoresceinated as previously described.[19] Normal mouse IgG was used as a negative control.

Spleen cell suspensions were prepared by teasing apart spleens in minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS) (Flow Laboratories, Rockville, Maryland) and filtering through nylon mesh. Red blood cells were lysed with Tris-buffered ammonium chloride (0.015 M Tris-0.73% ammonium chloride, pH 7.4). The spleen cells were pelleted through FBS, washed twice with MEM-FBS, and adjusted to a concentration of 3×10^7 cells/ml. The viability of the cells was 90–95% as determined by trypan-blue exclusions.

One million cells from peripheral blood buffy coats or 1 million splenic leukocytes in 100 μl of MEM, 10% newborn bovine serum (Flow Laboratories), and 0.1% sodium azide were incubated with 1:40 (final concentration) W 3/25, 1:400 OX 19, or 1:400 OX 8, for 30 min at 4°C. The cells were washed 3 times and then stained with 200 μg/ml fluoresceinated goat anti-mouse IgG. The cells were washed 3 times and resuspended in 0.5 ml of medium.

Cells were analyzed for light scatter and fluorescence intensity with an FACS III (Becton-Dickinson FACS Systems, Mountain View, California). For each sample, the fluorescence signal from 10,000 to 20,000 viable cells was measured.

Statistical procedures. Statistical analysis of 2×2 tables used the Fisher Exact Test for $N \leq 23$; all other tables were analyzed using the chi-squared statistic with Yates' correction where appropriate.[20] Comparisons among 3 means used one-way analyses of variance; comparisons of 2 means used the unpaired t test with pooled variance estimate.[21] In analyzing the frequency of diabetes, only those animals surviving either to the age of 120 days or to the onset of diabetes are included. Animals that died before 120 days of age or the diagnosis of diabetes are excluded. All parametric data

TABLE 2
Effect of 2200 rads of TLI on the frequency of insulitis and thyroiditis in BB/W rats

	2200 rads TLI	Control
No insulitis	5	2
Insulitis	6	7
No thyroiditis	3	3
Thyroiditis	8	6

Frequency of insulitis and thyroiditis in nondiabetic rats surviving to 120 days of age. There are no statistically significant differences between irradiated and control rats.

are presented as the mean ± standard error of the mean (SEM).

RESULTS

Radiation-related mortality and morbidity. No nondiabetic, sham-irradiated rats died before 120 days of age or the diagnosis of diabetes. Among the 53 rats that received TLI, 13 (25%) died before 120 days of age or the onset of diabetes. At each radiation dose the ratio of mortality to the total N was as follows: 1600 rads (1/12), 2200 rads (6/17), 2400 rads (5/12), and 3400 rads (1/12). The mean age at death for these rats was 87 ± 4 days. In addition to the increased mortality among irradiated rats, there was also a reduction in growth among nondiabetic animals treated with TLI when compared with nondiabetic controls. Because the number of controls that remained nondiabetic was small, the growth data from all three experiments were combined. Between the start of radiation and 120 days of age, TLI-treated male rats gained 156 ± 10 g (N = 17) while controls gained 237 ± 16 g (N = 8, $P < 0.001$). Similarly, TLI-treated female rats gained 76 ± 8 g (N = 17) while controls gained 116 ± 8 g (N = 7, $P < 0.01$).

Frequency of diabetes. The frequency of diabetes in rats receiving either 2200 rads or 2400 rads was similar, and these two groups were combined to form a single intermediate-dosage group. The results are shown in Table 1. There was no decrease in the frequency of diabetes at 1600 rads. TLI significantly reduced the frequency of diabetes at 2200 or 2400 rads and at 3400 rads.

Frequency of insulitis and thyroiditis. The frequency of insulitis and thyroiditis among nondiabetic animals in experiment 3 (2200 rads) is given in Table 2. There were no significant differences between irradiated and control animals.

Concanavalin-A-induced lymphocyte mitogenesis. Studies of the mitogenic response to con-A were performed on the nondiabetic rats in experiment 3 (2200 rads) that survived to 120 days of age. The results are shown in Table 3. At con-A doses of 0, 0.5, and 1.0 μg/well, there were no differences between irradiated and control rats. At doses of 0.03 and 0.125 μg/well, TLI-treated rats exhibited a slight but significant reduction in con-A responsiveness when compared with nonirradiated BB/W controls. It should also be noted that the response of both groups of BB rats is less than we have previously observed in the nondiabetic W-line BB rat.[22]

T-lymphocyte subsets. White blood cell counts and the percentages of OX 19-, W 3/25-, and OX 8-labeled peripheral blood and splenic lymphocytes are shown in Table 4. There were no significant differences between TLI-treated and nonirradiated BB/W rats. The percentages of peripheral and splenic lymphocytes labeled with OX 19, W 3/25, and OX 8 in both of these groups were all very much less than those observed in the nondiabetic W-line controls. There were no significant differences in total white cell count among the 3 groups, however.

TABLE 3
Concanavalin-A response of peripheral blood lymphocytes from TLI-treated and sham-irradiated BB/W rats

	Response (log_{10} counts/min ± SEM)	
Con-A dose (μg/well)	2200 rads TLI (N = 11)	Control (N = 9)
0	3.23 ± 0.18	3.10 ± 0.10
0.03	3.25 ± 0.11*	3.63 ± 0.09
0.125	3.36 ± 0.31*	4.27 ± 0.26
0.5	3.40 ± 0.40	4.16 ± 0.37
1.0	3.05 ± 0.36	3.20 ± 0.48

*$P < 0.05$ compared with BB/W sham-irradiated control.

DISCUSSION

Autoimmunity may be defined as a lack of tolerance to self. It can result from abnormalities of either humoral or cell-mediated immune mechanisms. The BB rat is an animal model of spontaneous diabetes thought to result from the autoimmune destruction of the pancreatic beta cell. Other well-recognized immunologic abnormalities of the BB rat include the presence of lymphocytic thyroiditis,[23] severe lymphopenia,[24,25] and a reduced T-cell response to concanavalin-A mitogenic stimulation.[22,26]

Diabetes in the BB rat can be prevented in several ways. Antilymphocyte serum,[7] cyclosporin-A,[8,9] glucocorticoids,[9]

TABLE 4
White blood cell counts and percentages of lymphocytes labeled by OX 19, W 3/25, and OX 8 monoclonal antibody T-cell markers in TLI-treated and control BB/W rats and in nondiabetic W-line rats

	2200 rads TLI (N = 11)	Sham irradiated (N = 9)	W-line (N = 8)
Peripheral blood lymphocytes (%)			
OX 19	7.7 ± 0.8	9.7 ± 0.8	55.7 ± 5.8*
W 3/25	14.0 ± 3.6	9.6 ± 1.7	40.1 ± 2.7*
OX 8	11.6 ± 2.4 (N = 8)	7.9 ± 0.5 (N = 7)	20.6 ± 1.0*
Splenic lymphocytes (%)			
OX 19	3.9 ± 0.9	5.6 ± 1.2	46.3 ± 3.0*
W 3/25	13.1 ± 1.3	11.7 ± 1.7	29.5 ± 1.5*
OX 8	3.6 ± 0.8	4.8 ± 0.9	15.6 ± 1.1*
White blood cell count ($cells/mm^3$)	9200 ± 2738	5933 ± 1268	6500 ± 1297

*$P < 0.01$ compared with both TLI- and sham-irradiated groups. The number (N) of rats tested in the three groups is that indicated at the top of each column, except where otherwise noted.

total body irradiation,[7] and neonatal thymectomy[28] are effective immunosuppressive methods. Neonatal bone marrow transplantation[26,27] and the transfusion of whole blood[22] are additional nonimmunosuppressive methods of prevention. The last two treatments not only prevent diabetes, but also ameliorate the depressed responsiveness of BB rat lymphocytes to concanavalin-A.

In this study, we demonstrate that TLI is another effective method for preventing diabetes in the BB rat. It is, in addition, an effective method of immunosuppression. TLI has previously been shown to suppress nonspecifically the mixed lymphocyte reaction and to inhibit the response of T-cells to concanavalin-A,[10,11] and our results are consistent with these previous observations.

The mechanism by which TLI works is unknown, but there are data to suggest that it causes depletion of immunocompetent lymphocytes as well as suppressor cell predominance.[10,29,30] This may occur on the basis of a differential radiosensitivity, recovery of different cell subsets, or some influence on cell maturation. In any event, the balance of various subsets of lymphocytes is altered.

The effects of TLI last only a few weeks, after which time stem cells located in the protected bone marrow repopulate the lymphocyte compartment. Thus, it is unclear how the prolonged protective effect in the present experiments has been achieved. It has previously been demonstrated, however, that if an allogeneic bone marrow transplant is performed immediately after TLI treatment in mice, the graft will not reject.[10,11] This implies that tolerance was induced and chimeric mice were formed. This and other studies show that TLI can produce long-lasting effects in the lymphoid system in experimental animals and man.[10,12,31]

We have demonstrated that TLI prevents diabetes, but not insulitis or thyroiditis, in the BB/W rat. The most plausible explanation of this result would seem to be an effect of TLI on T-effector cells. The presence of insulitis and thyroiditis suggests that autoimmune target organ recognition is not impaired by TLI, while the reduction in clinical diabetes implies that target organ (islet) cell killing is impeded. We suggest that TLI either reduced the number of such effector cells, blocked their effects by active cell-mediated suppression, altered the recruitment of other T-cells needed to complete beta cell destruction, or enhanced suppression so as to reduce effector-cell killing of beta cells. Alternatively, one may also speculate that TLI altered the production, release, or activity of lymphokines.

While TLI did prevent diabetes in the BB/W rat, it should be noted that it was not a benign procedure. TLI produced significant mortality and morbidity in the form of growth retardation, although no such effects were previously noted in nondiabetic strains of mice and rats.[10,11]

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