# Diabetes Prevention in BB Rats by Frequent Blood Withdrawal Started at a Young Age

JEAN-FRANÇOIS YALE, MARK GROSE, THOMAS A. SEEMAYER, AND ERROL B. MARLISS

**The BB rat diabetic syndrome has been prevented by various immunosuppressive and reconstitution measures. We observed an effect of multiple blood samplings on diabetes incidence and examined its immunological correlates. Individual litters were divided into two groups; one was sampled and the other was sham sampled as the control group. Sixty-four diabetes-prone and 59 non–diabetes-prone rats were studied. The sampled rats had blood removed at 15 (28% of total blood volume), 30 (30%), 50 (21%), 75 (16%), and 120 days of age. The sham-sampled control rats had blood removed only at 120 days of age. The incidence of diabetes in the sampled group was markedly lower than that of their sham-sampled littermates (22 vs. 78%). This result was associated with a correction of their OX19$^+$ (pan–T-lymphocytes) and W3/25$^+$ (helper/inducer) T-lymphocyte–number defects. An increase in lymphocyte subsets was also seen in the non–diabetes-prone BB rats, significant for all but the OX19$^+$ cells. Islet pathology and pancreatic insulin content were consistent with metabolic outcomes. The effect of blood withdrawal thus has implications for understanding the pathogenesis of both the diabetes syndrome and the lymphopenia of the BB rat. Furthermore, it suggests that a stimulation of lymphopoiesis by blood withdrawal (analogous to that of erythropoiesis) may be a hitherto unrecognized physiological response in normal animals. *Diabetes* 37:327–33, 1988**

Type I (insulin-dependent) diabetes in the BB rat shows many analogies to the human syndrome at the metabolic (1) and immunologic (2) levels. Both immunosuppression and immune reconstitution have prevented diabetes in BB rats. The successful immunosuppression protocols include use of antilymphocyte serum (3), neonatal (4) and adult thymectomy (5), total lymphoid irradiation (6), use of OX8 and OX19 monoclonal antibodies (7), and use of various immunosuppressant drugs alone or in combination (8–12). The successful reconstitution protocols include bone marrow transplantation in the neonatal period (13), lymphocyte transfusions (14), and spleen cells sequestered in diffusion chambers (15). None of these has yet explained the fundamental mechanisms of the disease process.

In the course of a study designed to elucidate the early time course of the immunodeficiency (16), we developed methods to study very young rats that involved sampling a relatively large proportion of their blood volumes. We observed a lower-than-expected incidence of diabetes in two litters followed to the usual age of onset of diabetes. This led to our study in which we report prevention of type I diabetes in BB rats by frequent large blood withdrawals starting at a young age, associated with correction of lymphopenia.

From the McGill Nutrition and Food Science Centre and the Department of Pathology (T.A.S.), McGill University, Montreal, Quebec, Canada.
Address correspondence and reprint requests to Dr. E.B. Marliss, McGill Nutrition and Food Science Centre, Royal Victoria Hospital, 687 Pine Avenue West, Montreal, QC, Canada H3A 1A1.
Received for publication 22 December 1986 and accepted in revised form 2 September 1987.

## MATERIALS AND METHODS

**Animals.** P. Thibert (Animal Resources Division, Health and Welfare Canada, Ottawa, Canada) provided pregnant rats, which gave birth to seven litters of diabetes-prone (BBdp; $n$ = 64) and five litters of non–diabetes-prone (BBn; $n$ = 59) rats in our laboratory. The rats were kept in metabolic cages in laminar-flow hoods in humidity- (70%) and temperature- (20°C) controlled rooms with 12-h light-dark cycles. They were fed ad libitum (Purina Rat Chow, Ralston-Purina, St. Louis, MO), except for periods of fasting before oral glucose tolerance tests (OGTTs).

**Experimental protocol.** When 15 days old, each litter was divided into two groups; one group was sampled at frequent intervals ($n$ = 46 BBdp and 46 BBn), and the other was only sham sampled ($n$ = 18 BBdp and 13 BBn). All the sampled rats had blood withdrawn by cardiac puncture with a sterile 25-gauge needle under ether anesthesia at 15, 30, 50, 75, and 120 days (Table 1). The blood removed was measured for peripheral blood lymphocyte subsets. The sham-punctured rats were anesthetized in the same way, and the needle

TABLE 1
Blood volume removed in sampled diabetes-prone and non–diabetes-prone BB rats in relation to age

| | Age on day of blood sample (days) | | | |
|---|---|---|---|---|
| | 15 | 30 | 50 | 75 |
| Diabetes-prone rats | | | | |
| Diabetic* | | | | |
| Blood removed (% of total vol) | 27 ± 2 | 36 ± 3 | 20 ± 1 | 14 ± 1 |
| Body wt (g) | 30 ± 2 | 82 ± 6 | 187 ± 13 | 279 ± 26 |
| Nondiabetic | | | | |
| Blood removed (% of total vol) | 28 ± 1 | 28 ± 1† | 21 ± 1 | 16 ± 1 |
| Body wt (g) | 34 ± 1 | 79 ± 4 | 195 ± 6 | 301 ± 12 |
| All diabetes-prone rats | | | | |
| Blood removed (% of total vol) | | 30 ± 1 | 21 ± 1 | 16 ± 1 |
| Body wt (g) | 33 ± 1 | 80 ± 3 | 190 ± 5 | 294 ± 11 |
| Non–diabetes-prone rats | | | | |
| Blood removed (% of total vol) | 25 ± 1 | 24 ± 1 | 20 ± 1 | 16 ± 1 |
| Body wt (g) | 33 ± 1 | 87 ± 2 | 212 ± 6 | 306 ± 12 |

The percentage of total blood volume removed was calculated from the theoretical total blood volume estimated according to body weight (17). Data are means ± SE.
*Rats were considered diabetic if glycosuria persisted for 3 days.
†$P < .05$ vs. diabetic BB rats.

was inserted in the heart, but no blood was withdrawn except at 120 days of age. Dams were also lightly anesthetized with ether immediately before reexposing them to their pups to reduce the chance that pups would be rejected. The amounts of blood that could be removed from pups and still ensure their survival had been established in studies of Wistar rat pups (unpublished data).

Body weight, urine volume, qualitative glycosuria, and ketonuria (Chemstrip uG 5000 K, Boehringer, Mannheim, FRG) were recorded daily. On appearance of glycosuria for 3 consecutive days, rats were considered diabetic and treated according to a fixed protocol. At diabetes onset, rats received 1 U s.c. heat-treated Ultralente insulin (40 U/ml, Novo, Copenhagen) at 1600 h. Each day the insulin dosage was adjusted according to glycosuria: the dosage was decreased 0.5 U/day if glycosuria was negative, increased 0.5 U/day if >5 g/dl, and not changed otherwise.

OGTTs were performed after overnight removal of chow in all nondiabetic rats when 90 and 120 days old. Glucose (2.5 g/kg body wt, 50 g/dl) was given by gavage, and plasma was sampled for glucose from the tail vein of nonanesthetized rats before and 60 min after the glucose load. Rats were considered glucose intolerant if their 60-min postgavage plasma glucose was >180 mg/dl.

All rats were killed when between 120 and 180 days old. Under light ether anesthesia, their blood was removed by cardiac puncture and centrifuged, and the plasma was kept at −20°C for future analysis. Lymphocyte counts and subsets were determined. The pancreas was carefully dissected, weighed, and either immediately frozen at −20°C for pancreatic insulin measurements or fixed in Bouin's solution for pathological examination. The pancreas of some rats was sliced longitudinally, with half fixed in Bouin's solution for subsequent pathological examination and the other half immediately frozen at −20°C for pancreatic insulin measurements.

**Assays.** Plasma glucose was measured on a Beckman Glucose Analyzer II (Fullerton, CA). Each frozen whole or half pancreas was homogenized by ultrasonic disintegration at 4°C in 8 ml of an acid-ethanol solution (75% ethanol, 1.5% vol/vol 12 M HCl, and 23.5% vol/vol distilled water), and the extracts (supernatant after centrifugation at 600 × *g* for 15 min) were kept at −20°C until assay. Insulin was measured on appropriate dilutions of pancreatic extracts with anti-beef insulin antibody (P. Wright), $^{125}$I-labeled porcine insulin, and rat insulin standards (24.5 IU/mg; Novo), and dextran-coated charcoal separation of bound from free insulin.

The peripheral blood lymphocyte subsets were measured on 1 ml of blood diluted in phosphate-buffered saline (PBS) with 2.5% fetal calf serum. The cell suspension was then layered on Ficoll-Hypaque [165 ml of 9% Ficoll (Pharmacia, Uppsala, Sweden) with 69 ml 34.5% Hypaque Sodium (Winthrop, Aurora, Canada), sp gr 1.077]. After centrifugation at 400 × *g* for 30 min, cells were extracted and washed twice with PBS (each centrifugation at 100 × *g* for 10 min). The final pellet of cells was resuspended in PBS, and cell concentration was measured with a hemocytometer, with cell viability evaluated by trypan blue exclusion.

Six samples of $2.5 \times 10^5$ cells were deposited into the wells of microtiter plates and centrifuged (200 × *g* for 1 min). Twenty-five microliters of the appropriate dilutions of antibodies were then added to each well, and 25 μl of culture medium RPMI-1640 without glutamine (Flow, McLean, VA) was added in the background wells. The antibodies were the mouse monoclonals W3/13 (pan–T-lymphocytes, but also thymocytes, natural killer cells, neutrophils, and plasma cells), OX19 (pan–T-lymphocytes and thymocytes), W3/25 (helper/inducer T-lymphocytes, macrophages), OX8 (suppressor/cytotoxic T-lymphocytes, natural killer cells), and OX6 (B-lymphocytes, Ia$^+$ T-lymphocytes) (Serotec, Bicester, UK). After a 30-min incubation at 4°C, the cells were washed three times with RPMI-1640. Fluorescein-conjugated F(ab′)$_2$-fragment goat anti-mouse IgG (heavy- and light-chain specific, no cross-reactivity to rat IgG) (Cappel, Malvern, PA) was then added to all wells, and the mixture was again incubated for 30 min at 4°C. After three more washes with RPMI-1640, 5 μl of the mixture was placed on a 12-well microscope slide, and all wells were read on a fluorescence

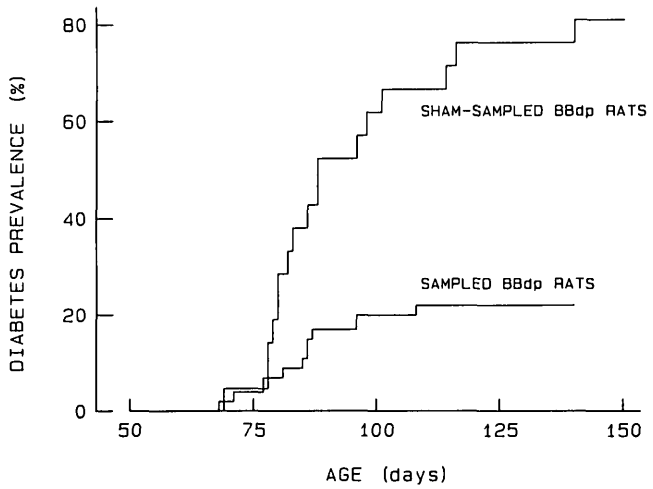


**FIG. 1. Time course of diabetes appearance in sampled and sham-sampled diabetes-prone BB rats. Data are presented as cumulative percentage of the total number of rats studied in each group that developed diabetes.**

microscope (Leitz Orthoplan, Wild Leitz Canada, Willowdale, Canada) by two readers. The results were expressed as the percentage of total lymphocytes showing fluorescence and calculated in cells per milliliter of blood according to the number of cells extracted for each sample. Recovery of lymphocytes from whole blood, comparing cells extracted with those calculated from white blood cell counts and differentials, was 60–80%. When subjected to cytocentrifugation and stained with Wright's Giemsa, essentially all cells (99%) were determined to be mononuclear by light-microscopic differential counts. The background fluorescence was <5% in all samples.

The fixed pancreases were embedded in paraffin. Step sections were prepared with hematoxylin-phloxine-saffron (HPS), Congo red, and Prussian blue stains. Serial sections were prepared for immunocytochemical study with the peroxidase-antiperoxidase method to detect insulin- and glucagon-reactive cells as previously described (18).

**Statistics.** Statistical analysis was performed with the SAS/STAT software (SAS, Cary, NC) on a Hewlett-Packard Vectra microcomputer (Sunnyvale, CA). The data were analyzed with the Waller-Duncan *k*-ratio *t* tests when multiple means were compared and unpaired *t* tests when only two means were compared. Incidence of diabetes between groups was compared by $\chi^2$-analysis. Results were considered significant at $P < .05$.

## RESULTS

**Metabolic state.** When subjected to blood withdrawal at a young age, all rats became cold to the touch and slower in their movements for ~30 min, but they improved when suckled by their dams. All rats but one survived the blood samplings. There were no infections in any of the groups. There was no significant effect of blood sampling on body weight in either the BBdp or the BBn rats when metabolic outcome was taken into account. The rats that became diabetic tended to weigh less at the end of the experiment than the nondiabetic rats (Table 1), but the weights were similar for sampled and sham-sampled groups (data not shown). Similarly, there were no significant differences in body weights of sampled and sham-sampled BBn and nondiabetic BBdp rats (data not shown).

A marked effect of blood withdrawal on diabetes occurrence was seen soon after the age that diabetes usually appears (Fig. 1). The sham-sampled rats showed a rapidly increasing onset of diabetes, reaching 83% (15 of 18) diabetes incidence at the end of the follow-up period. In contrast, the incidence in their sampled littermates reached only 22% (10 of 45; $P < .001$). In addition, one sampled BBdp rat remained glucose intolerant without progressing to overt diabetes. This result raised the proportion of sampled rats with a metabolic abnormality to 24% (Table 2). None of the sham-sampled rats remained glucose intolerant without progressing to overt diabetes. The glucose-tolerant BBdp rats whose pancreatic insulin content was measured at death showed normal values, with no difference between those sampled or not. The diabetic rats all had markedly reduced pancreatic insulin contents of <2.0 μg/g pancreas. The glucose-intolerant rat sampled also had a low pancreatic insulin content of 1.08 μg/g wet wt of pancreas.

Study of the morphology of the pancreases revealed in the sampled rats a redistribution in the occurrence of recognizable pathology from end-stage islets and/or insulitis toward the periductular lymphocytic infiltration (PLI) lesion alone (Table 2). Table 2 also shows the relationship between

TABLE 2
Metabolic outcome, pancreatic insulin content, and pancreatic morphology in sampled and sham-sampled diabetes-prone BB rats

| | Sampled diabetes-prone rats | | Sham-sampled diabetes-prone rats | |
|---|---|---|---|---|
| | Diabetes or glucose intolerance | Normal glucose tolerance | Diabetes | Normal glucose tolerance |
| *n*/total pancreases studied* | 11/45 (24) | 34/45 (76) | 15/18 (83) | 3/18 (17) |
| Pancreatic insulin | | | | |
| *n* | 11 | 20 | 15 | 3 |
| Content (μg/g pancreas) | 0.95 ± 0.77 | 140.85 ± 11.95 | 0.43 ± 0.22 | 160.47 ± 16.21 |
| Pancreatic morphology* | | | | |
| *n* | 9 | 16 | 14 | 3 |
| End-stage islets | 5 (56) | 0 (0) | 5 (36) | 0 (0) |
| Insulitis ± PLI | 2 (22) | 0 (0) | 4 (29) | 0 (0) |
| PLI | 2 (22) | 11 (69) | 5 (36) | 0 (0) |
| Normal | 0 (0) | 5 (31) | 0 (0) | 3 (100) |

PLI, periductular lymphocytic infiltration.
*Percents (in parentheses) indicate proportion of those pancreases studied showing the finding(s) indicated.

the morphology and the metabolic outcome. The diabetic rats showed mainly end-stage islets or insulitis, whereas all the glucose-tolerant BBdp rats showed either PLI alone or normal morphology. Four of the 24 pancreases examined from BBn rats showed PLI alone, whereas all the others had no inflammatory lesion. Some of the sampled nondiabetic BBdp and BBn rats showed a proliferative sclerotic lesion invading the islets. These lesions have been described elsewhere (19).

**Lymphocyte subsets.** The results of blood sampling on the peripheral blood lymphocyte subsets were assessed when the rats reached 120 days of age (Fig. 2). In BBdp rats, large-volume blood withdrawal started at an early age resulted in a marked increase in T-lymphocytes ($OX19^+$) and helper T-lymphocytes ($W3/25^+$) compared with their sham-sampled littermates (Fig. 2). The trend for such an increase in $W3/13^+$ and $OX8^+$ cells was not significant. Figure 3 shows that the sampled BBn rats also showed a significant increase (compared with their sham-sampled littermates) in $W3/25^+$ cells as well as in $W3/13^+$, $OX8^+$, and $Ia^+$ cells. The trend for the $OX19^+$ cells did not reach significance.

The time course of these lymphocyte subset changes was assessed in the blood removed from the sampled rats. When 15 days old, the BBdp rats showed significantly fewer $W3/25^+$ cells than the BBn rats (Fig. 4). The trend toward lower values of the other subsets did not reach statistical significance. The blood withdrawal was greatest in amount (relative to total blood volume) and frequency early in the time course, when there was a rise of all subsets in both groups. As the blood withdrawal was subsequently reduced in relative volumes as well as frequency, the two curves tended to separate. Nonetheless, the only significant differences between these two groups were lower $OX19^+$ cells at 75 days (Fig. 4*A*), and lower $Ia^+$ cells at 120 days in the BBdp rats (Fig. 4*D*).

These changes resulted in an increase of the $W3/25^+$ and $OX19^+$ cells in the sampled BBdp rats to levels similar to those of the nonsampled BBn rats (Fig. 4, *A* and *B*). The most characteristic defects in lymphocyte subsets, the

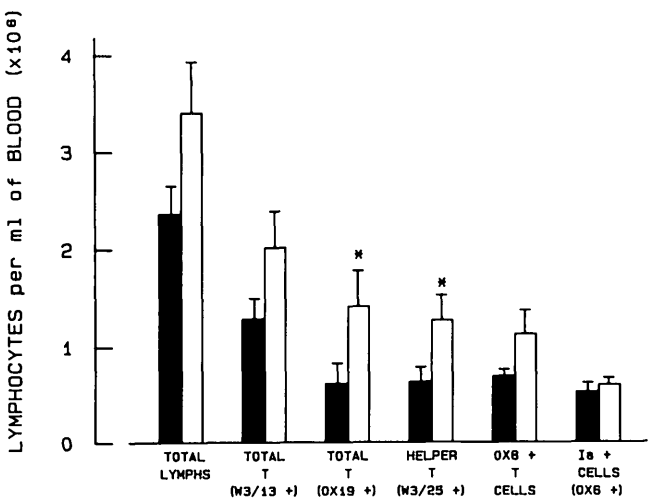


**FIG. 2. Blood lymphocyte counts and subsets in sampled (*open bars*) and sham-sampled (*closed bars*) diabetes-prone BB rats 120 days old. Data are means ± SE. *$P < .05$ vs. sham-sampled rats.**

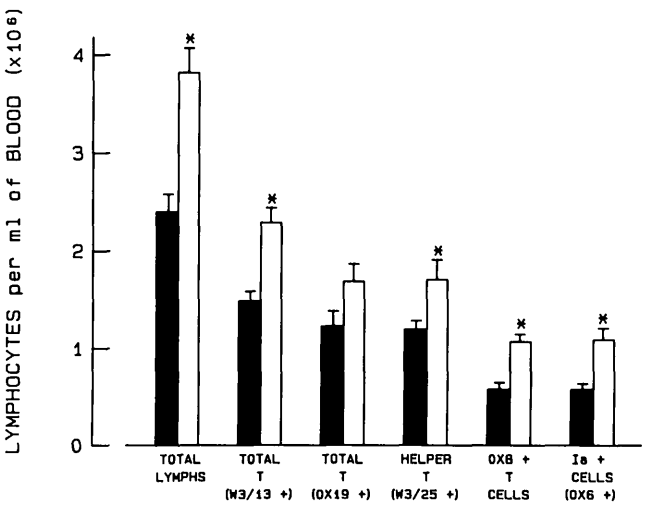


**FIG. 3. Blood lymphocyte counts and subsets in sampled (*open bars*) and sham-sampled (*closed bars*) non–diabetes-prone BB rats 120 days old. Data are means ± SE. *$P < .05$ vs. sham-sampled rats.**

$OX19^+$ and $W3/25^+$ T-lymphopenia (the lowest values obtained, as shown in the *open circles* of Fig. 4, *A* and *B*), were therefore corrected, which occurred in conjunction with the marked decrease in diabetes incidence.

The association between the metabolic outcome and the correction of the lymphopenia was even more evident when the lymphocyte subsets of the sampled BBdp rats that became diabetic were compared with those that did not (Table 3). When rats reached 120 days of age, there was no significant difference between the diabetic rats in the sampled group and those in the sham-sampled group. Similarly, the nondiabetic rats from the sampled and sham-sampled groups did not differ. Blood sampling was therefore associated with a shift from the lymphopenic diabetic outcome (which decreased from 83 to 22%) to the nonlymphopenic nondiabetic outcome (which increased from 17 to 76%) (Table 3).

Analysis of the lymphocyte subsets from age 15–120 days in the sampled rats in relation to their metabolic outcome revealed a progressive widening of the differences between the BBdp rats that became diabetic and the BBdp rats that did not. Figure 5 shows this result for total lymphocyte counts. When the rats were only 30 days old, the $W3/13^+$, $OX19^+$, and $W3/25^+$ cells were significantly higher in the rats that did not subsequently become diabetic (data not shown). At 75 days, the $OX8^+$ cells and the $OX6^+$ (B-lymphocytes and $Ia^+$ T-lymphocytes) cells also became higher in the nondiabetic group (data not shown). The final outcome at 120 days, in which all but the $OX6^+$ cells in the sham-sampled rats were different in diabetic versus nondiabetic, is shown in Table 3.

## DISCUSSION

We report that frequent removal of large amounts of blood started at a young age resulted in a dramatic decrease in the incidence of type I diabetes in the sampled rats, compared with sham-sampled littermates. This prevention occurred with preservation of the β-cells and a reduction in the occurrence of insulitis, although PLI still appeared. The relationship of PLI to insulitis remains uncertain, although it

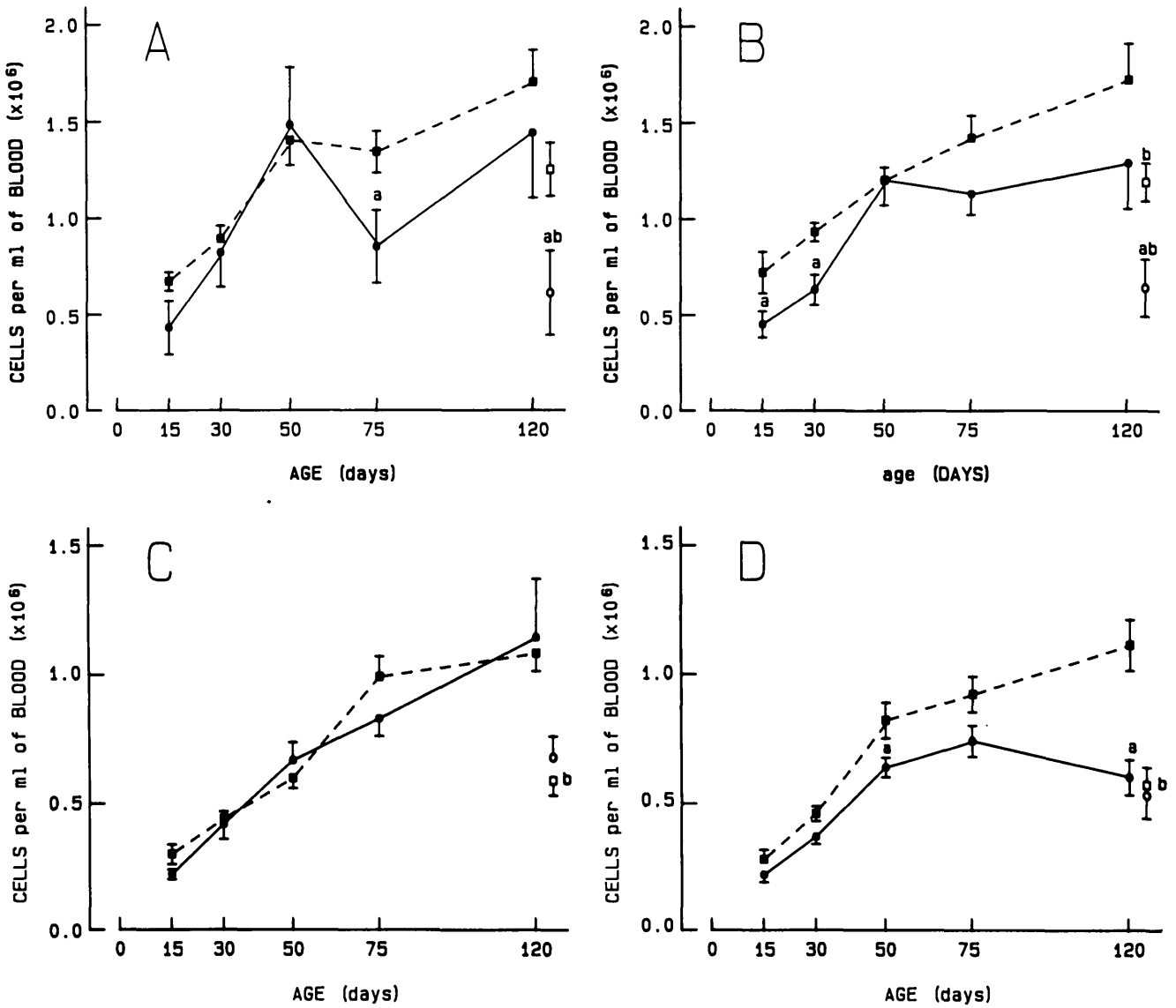


**FIG. 4. Time course of total lymphocyte counts and subsets in sampled diabetes-prone (●) and non–diabetes-prone (■) BB rats. Sham-sampled diabetes-prone (○) and non–diabetes-prone (□) BB rats were only assessed at 120 days of age. *A*: OX19⁺ T-lymphocytes. *B*: W3/25⁺ (helper/inducer T-lymphocytes and monocytes). *C*: OX8⁺ cells (suppressor/cytotoxic T-lymphocytes and natural killer cells) *D*: OX6⁺ cells (B- and Ia⁺ T-lymphocytes). Data are means ± SE. *a*, $P < .05$ vs. corresponding non–diabetes-prone BB rats; *b*, $P < .05$ vs. corresponding sampled rats.**

TABLE 3
Lymphocyte subsets in sampled and sham-sampled diabetes-prone BB rat littermates 120 days old, in relation to metabolic outcome

| | Sampled diabetes-prone BB rats | | Sham-sampled diabetes-prone BB rats | |
|---|---|---|---|---|
| Lymphocyte subset | Diabetic | Nondiabetic | Diabetic | Nondiabetic |
| | *n* = 10 (22%) | *n* = 35 (76%) | *n* = 15 (83%) | *n* = 3 (17%) |
| W3/13⁺ | 0.76 ± 0.18* | 2.41 ± 0.53† | 0.97 ± 0.12* | 2.73 ± 0.43† |
| OX19⁺ | 0.14 ± 0.03* | 2.00 ± 0.59† | 0.21 ± 0.03* | 2.16 ± 0.39† |
| W3/25⁺ | 0.27 ± 0.05* | 1.51 ± 0.37† | 0.39 ± 0.07* | 1.86 ± 0.25† |
| OX8⁺ | 0.58 ± 0.22* | 1.09 ± 0.22† | 0.56 ± 0.04* | 1.40 ± 0.30† |
| OX6⁺ | 0.30 ± 0.07* | 0.72 ± 0.10† | 0.48 ± 0.10*† | 0.77 ± 0.08† |

Data are means ± SE, $\times 10^6$ cells/ml of blood.
Means sharing the same symbol (* or †) in each row are not significantly different ($P > .05$).

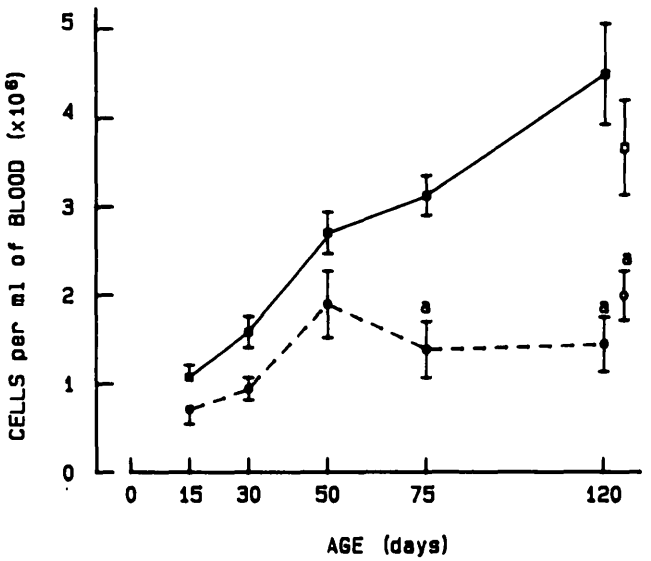


**FIG. 5. Time course of total lymphocyte counts in sampled diabetes-prone BB rats according to diabetic (●) or nondiabetic (■) outcome. Sham-sampled diabetes-prone rats were only assessed at 120 days of age and are shown according to their diabetic (○) or nondiabetic (□) outcome. Data are means ± SE. *a*, $P < .05$ vs. corresponding nondiabetic BB rats.**

could represent an "index lesion" of the pathophysiologic process (18). Its presence in BBn rats confirms previous reports in the literature and is in contrast to the absence of insulitis in the BBn rat. In some of the rats that did not become diabetic, we have recently reported an invasive fibrotic islet lesion, which we hypothesized was related to severe hypotension in these rats at the earliest sampling ages (19).

Frequent blood withdrawal was related to a marked increase in lymphocyte counts in both the BBdp and BBn rats compared with sham-sampled littermate controls. The most affected subsets in the BBdp rats were the $OX19^+$ and $W3/25^+$ cells. These are the cell subsets that are markedly deficient in the typical BBdp rat (2). The increase was sufficient to restore these mean levels for the whole group of sampled BBdp rats to values similar to those of normal sham-sampled BBn rats. The relationship between the lymphocyte subset numbers and the metabolic outcome is strengthened by the observation that of the sampled rats, those that did become diabetic were the ones that appeared not to respond to blood sampling by an increase in their lymphocyte counts. This difference between the two outcomes in the BBdp rats was not due to less blood being sampled in those that became diabetic. The volumes removed (in relation to their theoretical blood volume) were similar despite the outcome (Table 1) except at 30 days, when slightly more blood was removed from the rats that became diabetic.

The increase in lymphocyte numbers was not specific to the BBdp rats, because the BBn rats also showed significant increases in lymphocyte counts, which affected both T- and B-lymphocyte subsets. We are unaware of such a finding having been reported previously. The mechanism by which this effect occurred can only be hypothesized at this point. The most probable explanation would be a nonspecific stimulation of lymphopoiesis induced by the blood withdrawal, with a sustained "overshoot" in response to this stimulation. This stimulation could have overcome the defect responsible for the BBdp rats' lymphopenia and corrected their lymphocyte phenotypes. Whether the associated diabetes prevention occurs through this immune correction or through other mechanisms is unknown. Further research is thus needed to identify the mechanism(s) of the increase in lymphocytes in both normal and BBdp rats. The duration of the effect has not been established in either normal or BBdp rats, and it is conceivable that with longer follow-ups of BBdp rats the effect might revert and be associated with onset of the disease.

The immunosuppression and immune-reconstitution protocols that have been shown to prevent diabetes in BB rats have not always been associated with a correction of the lymphocyte defects. Nonetheless, at least two studies have shown prevention of diabetes in association with an improvement of the T-lymphopenia. Naji et al. (13) observed a decreased incidence of diabetes among BB rats rendered tolerant by neonatal bone marrow transfer from Wistar-Furth donors, with a concomitant return to normal of the total number of T-lymphocytes, the responses to skin graft, and the mixed lymphocyte responses. Absolute numbers of helper T-lymphocytes were not restored (13). Rossini et al. (14) reported attenuation of the syndrome by weekly lymphocyte transfusion, which produced an increase in $OX19^+$ and $W3/25^+$ lymphocyte counts and a restoration of concanavalin A responsiveness. This effect occurred in the presence of the persistence of the donor cells in the recipients for up to 5 mo (20). Our report appears to be novel in showing restoration of normal lymphocyte subsets in BB rats without the need for lymphocytes from non–diabetes-prone strains. It suggests that the lymphopenia in the BB rat is related to a severe regulation defect, rather than to an absolute incapacity to develop a normal cellular immune system.

Another possible effect of blood withdrawal would be the depletion of iron stores. We did not address the issue in our study. It has been shown that the iron-chelating agent desferrioxamine has an immunosuppressive action (21). Desferrioxamine has been shown to inhibit chronic islet allograft damage in CBA/J mice, and it was suggested that this effect may be related to an inhibition of hydroxyl radical formation, which is dependent on iron (22). An analogous mechanism may have resulted from our frequent large blood samplings started at an early age. Such an immunosuppressive action could theoretically have been responsible for the prevention of diabetes independently of its effects on lymphocyte numbers.

Finally, it could be argued that if part of the lymphopenia is due to an autoimmune attack, as suggested by the presence of antilymphocyte antibodies in BBdp rats (23), an immunosuppressive action, however mediated, could in fact have caused an increase in lymphocyte counts by attenuating such an "antilymphocyte" autoreactivity. This appears less likely an explanation because we observed that the BBn rats that did not have lymphopenia also responded to frequent large blood withdrawals by an increase in their lymphocyte counts.

In conclusion, frequent and large blood withdrawals resulted in a dramatic prevention of the diabetes syndrome in BB rats in our study. This maneuver was associated with an increase and/or correction of lymphocyte subset abnormalities. The exact mechanisms of both actions and their

relationship remain to be investigated. Apart from the pathogenic implications of our findings for both the diabetes and the lymphopenia of the BB rat, the findings also show the importance of controlling the blood volumes removed in any study of prevention of diabetes in the BB rat. Furthermore, they raise a fundamental question regarding the regulation of the number of circulating lymphocytes, which appears to be augmented even in normal rats in response to blood withdrawal.

**ACKNOWLEDGMENTS**

We express profound appreciation to Dr. P. Thibert for the supply of BB rats, Marie Montambault and Madeleine Faucher for technical assistance, and Jessie Blais for secretarial assistance.

This study was supported by Grant MS-6540 from the Medical Research Council of Canada.

J.-F.Y. received a postdoctoral fellowship from the Medical Research Council of Canada and currently holds a Diabetes Canada scholarship.

This work was presented in part at the 46th annual meeting of the American Diabetes Association, 19–24 June 1986, Anaheim, California.

**REFERENCES**

1. Marliss EB: Workshop on the BB rat: its potential for insight into human diabetes. *Metab Clin Exp* 32 (Suppl. 1):1–166, 1983
2. Yale J-F, Marliss EB: Altered immunity and diabetes in the BB rat. *Clin Exp Immunol* 57:1–11, 1984
3. Like AA, Rossini AA, Guberski DL, Appel MC: Spontaneous diabetes mellitus reversal and prevention in the BB/W rat with antiserum to rat lymphocytes. *Science* 206:1421–23, 1979
4. Like AA, Kislauskis E, Williams RM, Rossini AA: Neonatal thymectomy prevents spontaneous diabetes mellitus in the BB/W rat. *Science* 216:644–46, 1982
5. Like AA, McGill PD, Sroczynski E: Adult thymectomy prevents diabetes mellitus in BB/W rats (Abstract). *Diabetologia* 29:565A, 1986
6. Rossini AA, Slavin S, Woda BA, Geisberg M, Like AA, Mordes JP: Total lymphoid irradiation prevents diabetes mellitus in the Bio-Breeding/Worcester (BB/W) rat. *Diabetes* 33:543–47, 1984
7. Like AA, Biron CA, Weringer EJ, Byman K, Sroczynski E, Guberski DL: Prevention of diabetes in BioBreeding/Worcester rats with monoclonal antibodies that recognize T lymphocytes or natural killer cells. *J Exp Med* 164:1145–59, 1986
8. Like AA, Anthony M, Guberski DL, Rossini AA: Spontaneous diabetes mellitus in the BB/W rat: effects of glucocorticoids, cyclosporin-A, and antiserum to rat lymphocytes. *Diabetes* 32:326–30, 1983
9. Stiller CR, Laupacis A, Keown PA, Gardell C, Dupre J, Thibert P, Wall W: Cyclosporine: action, pharmacokinetics, and effect in the BB rat model. *Metab Clin Exp* 32 (Suppl. 1):69–72, 1983
10. Laupacis A, Gardell C, Dupre J, Stiller CR, Keown P, Wallace AC, Thibert P: Cyclosporine prevents diabetes in BB Wistar rats. *Lancet* 1:10–11, 1983
11. Jaworski MA, Honore L, Jewell LD, Mehta JG, McGuire-Clark P, Schouls JJ, Yap WY: Cyclosporin prophylaxis induces long-term prevention of diabetes, and inhibits lymphocytic infiltration in multiple target tissues in the high-risk BB rat. *Diabetes Res* 3 (Suppl. 1):1–6, 1986
12. Yale J-F, Grose M, Seemayer TA, Marliss EB: Immunological and metabolic concomitants of cyclosporin prevention of diabetes in BB rats. *Diabetes* 36:749–57, 1987
13. Naji A, Silvers WK, Kimura H, Anderson AO, Barker CF: Influence of islets and bone marrow transplantation on the diabetes and immunodeficiency of BB rats. *Metab Clin Exp* 32 (Suppl. 1):62–68, 1983
14. Rossini AA, Faustman D, Woda BA, Like AA, Szymanski I, Mordes JP: Lymphocyte transfusions prevent diabetes in the Bio Breeding/Worcester rat. *J Clin Invest* 74:39–46, 1984
15. Handler ES, Mordes JP, Nakamura N, Rossini AA: Prevention of spontaneous diabetes in the BB/W rat by Wistar Furth (WF) spleen cells sequestered in diffusion chambers (Abstract). *Diabetes* 35 (Suppl. 1):75A, 1986
16. Yale J-F, Grose M, Marliss EB: Time course of the lymphopenia in BB rats: relation to the onset of diabetes. *Diabetes* 34:955–59, 1985
17. Yale CE, Torhorst JB: Critical bleeding and plasma volumes of the adult germfree rat. *Lab Anim Sci* 22:497–502, 1972
18. Seemayer TA, Tannenbaum GS, Goldman H, Colle E: Dynamic time-course studies of the spontaneously diabetic BB Wistar rat. III. Light-microscopic and ultrastructural observations of pancreatic islets of Langerhans. *Am J Pathol* 106:237–49, 1982
19. Seemayer TA, Yale J-F, de Chadarevian JP, Grose M, Marliss EB: Obliterative segmental sclerosis of pancreatic islets: a possible consequence of hypertensive shock in young BB rats. *Am J Pathol* 125:327–31, 1986
20. Rossini AA, Mordes JP, Greiner DL, Nokano K, Appel MC, Handler ES: Spleen cell transfusion in the Bio-Breeding/Worcester rat: prevention of diabetes, major histocompatibility complex restriction, and long-term persistence of transfused cells. *J Clin Invest* 77:1399–401, 1986
21. Bowern N, Ramshaw IA, Badenoch-Jones P, Doherty PC: Effect of an iron-chelating agent on lymphocyte proliferation. *Aust J Exp Biol Med Sci* 62:743–54, 1984
22. Bradley B, Prowse SJ, Bauling P, Lafferty KJ: Desferrioxamine treatment prevents chronic islet allograft damage. *Diabetes* 35:550–55, 1986
23. Dyrberg T, Nakhooda AF, Baekkeskov S, Lernmark Å, Poussier P, Marliss EB: Islet cell surface antibodies and lymphocyte antibodies in the spontaneously diabetic BB Wistar rat. *Diabetes* 31:278–81, 1982