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INDUCTION OF TYPE 1 DIABETES IN DIABETES-RESISTANT (BB-DR) RATS

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ABSTRACT

The BioBreeding- Diabetes Prone (BB-DP) rat spontaneously develops an autoimmune diabetic syndrome that is dependent on the RT1^{u} Major Histocompatibility Complex (MHC) haplotype and homozygosity for an allele at the *Lymphopenia (Lyp)* locus. Lyp mutation is responsible for a peripheral T-lymphopenia. There are other genetic loci contributing to diabetes susceptibility in this strain. BB rats carrying wild-type *Lyp* alleles are not lymphopenic and are resistant to spontaneous diabetes (Diabetes Resistant [DR]). Our study shows that thymectomy and exposure to one sublethal dose of γ-irradiation (TX-R) at 4 weeks of age result in the rapid development of insulitis followed by diabetes in 100% of DR rats. Administration of CD45RC^{-}CD4^{+} TCRαβ^{+} T cells from unmanipulated syngeneic donors immediately after irradiation prevents the disease. Splenic T cells from TX-R induced diabetic animals adoptively transfer type 1 diabetes to T-deficient recipients. WAG, WF and LEW strains are resistant to TX-R induced insulitis/ diabetes. This novel model of TX-R induced diabetes in BB-DR rats can be used to identify environmental and cellular factors that are responsible for the initiation of antipancreatic autoimmunity.

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INTRODUCTION

The BB-DP rat spontaneously develops a T-cell mediated, autoimmune syndrome that is similar to that observed in Non-Obese Diabetic (NOD) mice and humans.[1,2] Two of the diabetes susceptibility loci of the BB rat have been identified, IDDM1, which maps to the *Lyp* locus on chromosome 4, and IDDM 2, which maps to the MHC class II haplotype RT1u of this animal.[3] The Lyp allele maps by the BB-DP rat shortens the life span of naïve T cells, resulting in a 5-10 fold decrease in the number of peripheral T cells.[4] This peripheral T-lymphopenia may contribute to the development of diabetes, through a multifactorial process.

Our demonstration that autoimmune diabetes can develop in nonlymphopenic BB-DR rats, complicates the pathogenic role of the BB-DP T-lymphopenia. BB-DR rats are genetically related to BB-DP rats, but are not lymphopenic and do not develop diabetes when maintained in a specific pathogen- free (SPF) environment.[5,6] Experimental induction of a peripheral T- lymphopenia in BB-DR rats, through the administration of a depleting monoclonal antibody, cyclophosphamide or sublethal γ-irradiation is followed by the rapid development of diabetes in a conventional environment.[7,10] However, diabetes can also occur in unmanipulated BB-DR rats after infection with Kilham's rat virus (KRV) or injection of polyinosinic-polycytidylic (poly [I: C]).[5,8] Importantly, susceptibility to these experimentally induced type 1 diabetic syndromes is not restricted to BB-related strains

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as long as the animals are haploidentical to BB rats at the MHC class II locus.[8,9,10] This observation suggests that diabetes susceptibility alleles are widespread among laboratory rats.

Adult thymectomy followed by four subsequent, sublethal doses of γ-irradiation given 2 weeks apart results in the development of another type 1 diabetic syndrome 10 weeks after irradiation in various strains of rats, including some that do not carry the RT1u MHC haplotype.[10] Therefore, it seems that most of the experimentally induced type 1 diabetic syndromes require the BB rat MHC class II haplotype, but none requires the BB rat genetic background exclusively. Here we introduce a novel model of experimentally induced diabetes that is restricted to non-lymphopenic, BB-related strains.

MATERIAL AND METHODS

Diabetes-resistant BB-DR and diabetes-prone BB-DP rats were purchased from BRM (Worchester, MA). Diabetes-prone BB.7^b rats that are congenic for the RT7^b allele of rat CD45 were derived in our laboratory by introducing the RT7^b allele of Wistar Furth (WF) rats into BB-DP rats, followed by >10 backcrosses to BB-DP rats.[11] Nonlymphopenic and hence diabetes-resistant (nonlyp BB/W) rats has been generated by introgressing the wild-type *Lyp* allele from BB-DR into BB-DP rats, followed by systematic backcrossing to BB-DP rats. Diabetes-prone and lymphopenic DR.*Lyp/Lyp* as well as diabetes-resistant and nonlymphopenic DR.*Lyp*/± congenic lines were obtained from Dr.A. Lernmark (Washington University, Seattle, WA). Other rat strains were purchased from Harlan Sprague- Dawley (Indianapolis, IN). All animals received autoclaved food and acidified water (SPF conditions). All sentinels remained serologically negative for the diabetogenic Kilham virus during the course of the study (virus antibody- free [VAF] conditions).

Thymectomy and sham thymectomy were performed surgically under general anesthesia using a mixture of Xylazine, Ketamine Hydrochloride and Acepromaxine Maleate, and Buprenorphine Hydrochloride as analgesic.[4] Importantly, the content of the thoracic cavity of all animals was examined both macroscopically and flowcytometrically at sacrifice to verify that the thymectomy was complete. Whole-body γ-irradiation of rats (5Gy) was performed from a ^{137}Cs source (Gamma cell 40). Rats were tested three times a week for the presence of glycosuria and ketonuria. Once the animals became glycosuric, the diagnosis of type 1 diabetes was made on the basis of hyperglycemia (blood glucose>16.7 mmol/l) for two consecutive days. Diabetic rats were treated with subcutaneous implants of insulin (Linplant; University of Toronto, ON, Canada). After the rats were killed, pancreas, lung, kidney and liver were fixed in 10% formalin for histology.

The monoclonal antibodies (mAbs) used in this study were affinity-purified from hybridoma culture supernatants on a Sepharose column coated with rat anti-mouse Ig or mouse anti-rat Ig and then conjugated with fluoresceinated isothiocyanate (FITC), biotin, allophycocyanin or phycoerythrin (PE) using standard procedures. These mAbs included anti-rat Ig (MARK1), anti-NKRP-1 (3.2.3), anti-CD8α (MRC-OX8), anti-CD4 (W3/25), anti-CD45RC (OX22), anti-CD5 (MRC-OX19), anti-TCRαβ (R73), anti-RT6β (6A5), anti-RT7a (NDS-58), anti-RT7b (8G6.1) and anti-CD3 (G4.18). Suspensions of mononuclear cells (MNCs) were incubated with biotinylated mAb, followed by streptavidin-PE/Texas Red Tandem. PE-labeled and FITC-conjugated mAbs were then added simultaneously. Viable cells were gated using forward and side angle scatter and were analyzed by flow cytometry with a FACS Calibur (BD Biosciences, San Jose, CA) or sorted using a Moflo (Cytomation, Denver, CO). At least 10^4 cells/sample were acquired for analysis.

T cells were enriched by negative selection using a resetting procedure.[11] Briefly, donor T cells were purified from pooled splenic and lymph node MNCs through the depletion of macrophages, B-lymphocytes and NK cells. The cell suspension was incubated with a mixture of mouse mAbs specific for the mentioned cell populations. MAb- coated cells were mixed with sheep red blood cells (SRBC)(Cederlane, Hornby, Canada) coated with rat anti- mouse Ig. The cell suspension was rotated for 30' at 4°C to allow rosette formation. Rosettes were separated by centrifugation at 200×g for 1'. The supernatant containing the T cells was recovered and analyzed. The degree of enrichment was assessed by FACS analysis. The purity of T cells obtained from nonlymphopenic animals by resetting was routinely>98%. Furthermore, different subsets of CD4$^+$ T cells and T-depleted splenocytes were purified by fluorescence-activated cell sorting. Adoptive transfer of these T cell subpopulations was done by intravenous (IV) injection into the different recipients.

RESULTS

Thymectomy and sublethal irradiation induce diabetes in BB-DR rats in an age-dependent manner

When 4-week-old BB-DR rats were thymectomized and one week later, received one sublethal dose of 5Gy, 100% of the animals (31 of 31) developed diabetes 21-35 days after irradiation with a mean onset of 28±6 days (Table I). Both sexes were susceptible. The diabetic syndrome was characterized by the acute development of polyuria, polydipsia, weight loss, hyperglycemia, glycosuria and ketonuria. Diabetic animals required daily

Table I. TX-R induces type 1 diabetes in nonlymphopenic rats with a BB genetic background.

Strain†	MHC haplotype	Type 1 diabetes	Insulitis	Mean day of onset after R
BB-DR	$RT1^{u/u}$	31/31	NA	28±6
DR.Lyp/+	$RT1^{u/u}$	6/6	NA	34±5
Non-lyp BB/W	$RT1^{u/u}$	7/7	NA	31±3
LEW	$RT1^{l/l}$	0/4	0/4	
WAG	$RT1^{u/u}$	0/3	0/3	
WF	$RT1^{u/u}$	0/9	0/9	

†Both sexes were equally represented in each of the phenotypic categories.

NA, not applicable; MHC, major histocompatibility complex; R, radiation.

Table II. TX-R induced type 1 diabetes is age-dependent in BB rats.

Age (weeks)		Type 1 diabetes	Insulitis†
TX	5Gy		
4	5	31/31	NA
4	8	0/5	0/5
4	6	0/5	0/5
6	8	0/5	0/5
8	9	0/4	0/5
4	-	0/4	0/4
-	5	0/5	0/5

†Insulitis in nondiabetic animals. NA, not applicable.

insulin injections to survive. Prospective, histological analysis of the pancreas performed weekly after irradiation showed that diabetes was preceded by the development of insulitis. No inflammation was observed in the exocrine pancreas, lungs, kidneys and liver of thymectomized and irradiated rats.

As shown in Table II, susceptibility to TX-R induced diabetes was age dependent. Specifically, thymectomy had to be done between 3 and 5 weeks, and sublethal irradiation had to be done within one week after thymectomy. Exposure to irradiation or thymectomy alone failed to induce diabetes.

TX-R induced diabetes is a T cell mediated autoimmune disease

The presence of insulitis before the development of the disease strongly suggests that TX-R-induced diabetes was autoimmune in nature. To determine whether this is the case, we performed an adoptive transfer of MNCs from acutely diabetic TX-R rats to nondiabetic, 4-week-old sublethally irradiated BB-7^b rats. Adoptively transferred populations of lymphocytes (2×10^6 cells intravenously) included sorted, CD^{3-} splenocytes and splenocytes enriched (70-80%) in T cells by resetting. All of the recipients of splenic T cells developed diabetes within 4 weeks after transfer (Table III), whereas none of the animals that received CD^{3-} splenocytes or were left untreated did. No insulitis was found in the recipients that had not become diabetic and were killed 8 weeks after transfer (data not shown). These results demonstrate that TX-R induced diabetes is a T cell mediated autoimmune disease.

Syngeneic CD45RC-CD^{4+} T cells prevent induction of TX-R induced diabetes

As expected, TX-R resulted in peripheral T-lymphopenia in BB-DR rats. Specifically, T cells accounted for 6±1.3% and 22±4% (n=7) of splenic and lymph node MNCs, respectively in flow cytometry, at the onset of diabetes. To assess the ability of various T cell subsets to modulate TX-R induced diabetes in BB-DR rats, they received unfractionated T cells or purified T cell subsets isolated from adult unmanipulated BB-DR donors immediately after irradiation. The T cell subsets consisted of $CD4^+$, $CD8^+$, $CD45RC^-CD4^+$ or $CD45RC^+CD4^+$ T cells. As shown in Table IV, as few as 2×10^5 unfractionated T cells, $CD4^+$ T cells and $CD45RC^-CD4^+$ T cells protected from diabetes in 100% of the recipients. In contrast, reconstitution of TX-R BB-DR rats with up to 5×10^6 $CD45RC^+CD4^+$ T cells or up to 2×10^6 $CD8^+$ T cells was not protective. Importantly, the ability of unfractionated T cells to prevent diabetes was lost when T cell reconstitution was delayed by 1-2 weeks, suggesting that the autoimmune process is initiated soon after irradiation and/or expansion of regulatory T cells is required before the initiation of the diabetogenic process. These results demonstrate that the diabetic syndrome induced by TX-R in BB-DR rats depends on T cell regulation.

TX-R induced diabetes is observed only in BB-DP re-

Table III. Adoptive transfer of type 1 diabetes by T cells from diabetic TX-R, RT7a, BB-DR rats.

Donor cells	Recipient	IDDM	Insulitis†
None	5Gy BB-7^b	0/5	0/5
Splenic T cells	5Gy BB-7^b	4/4	NA
T depleted splenocytes	5Gy BB-7^b	0/4	0/4

T cells were prepared by resetting and were injected intravenously into 4-week-old, sublethally irradiated BB-7b rats. †Insulitis in nondiabetic animals. NA, not applicable.

Table IV. TX-R induced diabetes is prevented by syngeneic T cells.

Cells injected	Cell number	Day of injection‡	Type 1 diabetes	Insulitis†
None	-	0	24/24	NA
T cells	$5*10^6$	0	0/6	0/6
	$2*10^6$	0	0/3	0/3
	$2*10^5$	0	0/3	0/3
	$5*10^6$	7	2/3	1/1
	$5*10^6$	14	3/3	NA
CD4$^+$ T cells	$2*10^6$	0	0/3	0/3
	$2*10^5$	0	1/3	2/2
CD4$^+$CD45RC$^-$	$5*10^6$	0	0/5	0/5
	$2*10^5$	0	0/3	0/3
CD4$^+$CD45RC$^+$	$5*10^6$	0	2/2	NA
CD8$^+$ T cells	$2*10^6$	0	2/3	1/1

After irradiation, TX-R BB-DR rats received an intravenous injection of the indicated T cell subset isolated from 3-month-old, unmanipulated BB-DR rats. ‡The day of irradiation is considered as day 0. †Insulitis in nondiabetic animals. NA, not applicable.

lated strains

To evaluate whether susceptibility to TX-R induced diabetes is genetically determined, we exposed non-lymphopenic BB-DP related strains (nonlyp BB/W and DR.*Lyp*/+) and three other BB-unrelated strains (WAG [RT1$^{u/u}$], WF [RT1$^{u/u}$], LEW [RT1$^{l/l}$]) to TX-R. All of the rats were followed for up to 3 months after TX-R.

After TX-R, 100% of non-lymphopenic BB-DP related animals developed diabetes (Table I). In contrast, none of the BB-DP un-related animals became diabetic and none of them had insulitis at the time they were killed. These results demonstrate that nonlymphopenic animals that are genetically related to the BB-DP strain are uniquely susceptible to TX-R induced diabetes, but the role of MHC genes in this case remains unknown.

DISCUSSION

This study describes a novel, autoimmune, type 1 diabetic syndrome that can be rapidly induced in BB-DP related strains with a very high incidence. Many features of this diabetic syndrome distinguish it from those induced in RT1u strains through viral infection, administration of poly I: C or other methods.[10,12] Susceptibility to diabetes induced by KRV and poly I: C is widely distributed in nonlymphopenic, RT1u expressing strains, whereas TX-R induced diabetes seems to be restricted to BB related strains.[5,8] Therefore, the latter syndrome may be helpful in identifying diabetes susceptibility factors that are related to the BB genetic background and possibly contribute to both spontaneous and experimentally induced diseases.

The diabetic syndrome could be induced in WAG rats and other strains by thymectomy and multiple low doses of irradiation,[10,12] but they were resistant to TX-R induced diabetes in this study (Table I). Adoptive transfer of diabetes induced by thymectomy and multiple low doses of irradiation to irradiated syngeneic recipients by splenic T cells was unsuccessful despite preactivation of donor T cells by

ConA in vitro in other experiments. Furthermore, only a small proportion of the T cell recipients developed lesions of insulitis.[10,12] In contrast, all recipients of splenic T cells freshly isolated from TX-R induced diabetic donors developed diabetes in a few weeks in our study (Table III). On the other hand, for induction of the diabetic syndrome in other experiments,[10,12] thymectomy must be performed between 3 and 6 weeks and irradiation must be initiated 2 weeks later, whereas in the case of our study, thymectomy has to be performed in animals that are <4 weeks old and irradiation can not be delayed beyond 1 week after thymectomy (Table II). Importantly, the experimental procedure seems to affect the development and function of regulatory T cells with the $CD45RC^{-}CD4^{+}TCR\alpha\beta^{+}$ membrane phenotype because reconstitution of this subset immediately after irradiation prevents the development of the disease[13] (Table IV).

It has been previously reported that type 1 diabetes can be induced in BB-DR rats by a single sublethal dose of irradiation.[10] In our study, this protocol remained unsuccessful, independent of the dose of irradiation and age of the animals (Table II). We believe that this discrepancy is related to environmental factors.

One of the interesting aspects of spontaneous and experimentally induced diabetes in BB-DP related strains is that the various manipulations that prevent or precipitate diabetes have to be done before 4 weeks. Reconstitution of diabetes-prone BB-DP rats with normal T cells protects the recipients from diabetes, provided that the protective T cells are injected in the first 4 weeks of life. Thymectomy of BB-DP rats prevents diabetes when performed in <4-week-old animals but does not affect the time course and incidence of the disease when delayed.[14] There is evidence in the NOD mouse that activation of diabetogenic T cells by their specific β cell antigens occurs in pancreatic lymph nodes around the age of 2 weeks.[15] Our study demonstrates that potentially diabetogenic T cells are present in the pool of recirculating T cells of BB-DR rats. Presentation of self antigens to their specific T cells in these rats must result in T cell tolerance or ignorance because these animals remain diabetes free.

By considering that potentially deleterious presentation of β cell antigens persists throughout the life of BB-DR rats, our study shows that TX-R has a differential effect on the homeostasis or repertoire of peripheral T cells in young and adult animals. $CD45RC^{-}CD4^{+}$ $TCR\alpha\beta^{+}$ T cells that prevent autoimmunity account for a low proportion of peripheral T cells in young animals.[16,17] The proportion of this regulatory T cell subset increases with age. It is possible that the differential effect of thymectomy and TX-R on diabetes susceptibility in young and adult animals is related to age- related changes in the repertoire of peripheral T cells. However, the peripheral T lymphopenia is so severe immediately after irradiation that we could not detect reliable differences in the proportions of naïve and memory T cells between young and adult rats.

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