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A Major Histocompatibility Complex Class II Restriction for BioBreeding/Worcester Diabetes-inducing T Cells

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Summary
Inbred diabetes-prone (DP) BioBreeding/Worcester (BB/Wor) (RT1u) rats develop spontaneous autoimmune diabetes, which, like human insulin-dependent diabetes mellitus, is mediated by autoreactive T lymphocytes. Breeding studies have shown an absolute requirement for at least one copy of the major histocompatibility complex (MHC) RT1u haplotype for spontaneous diabetes expression. Concanavalin A-activated spleen cells from acutely diabetic DP rats adoptively transfer diabetes only to recipients that express at least one RT1u haplotype. To investigate the basis for the MHC requirement in BB/Wor autoimmunity, diabetes-inducing T cell lines were derived from the spleens of acutely diabetic DP rats. Upon activation in vitro with islet cells, the T cell lines adoptively transfer insulinitis and diabetes into young DP recipients and non-diabetes-prone RT1 congenic rat strains that are class II u. Recipients that are RT1u at only the class I A or C locus, but not at the class II B/D loci, do not develop diabetes after T cell transfer. The adoptive transfer of diabetes by Concanavalin A-activated diabetic DP spleen cells also requires that donor and recipient share class II B/D u gene products. Furthermore, the adoptive transfer of diabetes into MHC class II congenic rats is independent of the class I haplotype; i.e., it occurs in the presence of class I A a C or A u C a gene products. BB/Wor T cells can be activated in vitro for the transfer of diabetes with islet cell antigens and class II-positive but not class II-negative antigen-presenting cells. The inductive phase of BB diabetes is therefore MHC class II restricted, and this appears to operate at the level of interaction between inducing T cells and class II u antigen-presenting cells. These results may explain the well-documented, but not yet understood, MHC class II genetic contribution to insulin-dependent diabetes mellitus pathogenesis, and they may facilitate the development of protocols designed to prevent diabetes onset in susceptible individuals.

Diabetes-prone (DP)1 BioBreeding/Worcester (BB/ Wor) rats develop spontaneous autoimmune diabetes, in which the frequency of insulin-dependent, ketosis-prone hyperglycemia is 80–95% in both sexes. BB diabetes is characterized morphologically by a β cell-specific mononuclear cell infiltrate (insulitis) within the pancreatic islets of Langerhans. The autoimmune attack selectively destroys the insulin-producing β cells, with sparing of glucagon-, somatostatin-, and pancreatic polypeptide-synthesizing islet cells (1). These clinical features of BB diabetes are identical to those of human insulin-dependent diabetes mellitus (IDDM) (2).

BB/Wor rat diabetes is T cell dependent; the development of hyperglycemia is prevented by neonatal thymectomy (3) and in vivo treatment with mAbs directed against CD5+ (pan T) or CD8+ (cytotoxic) T cells (4). Spontaneous BB diabetes is an MHC-linked disease. Breeding studies have shown an absolute requirement for at least one copy of the MHC RT1u haplotype for the appearance of spontaneous diabetes in crosses between BB and non-BB strains of rat (5–7). Genetic susceptibility, however, has not been previously assigned to single genes of the MHC, nor have the immune effector mechanisms leading to β cell destruction been identified as being class I– or class II-restricted events.

BB/Wor diabetes can be adoptively transferred with acutely diabetic DP spleen cells that have been activated in vitro with Con A, a polyclonal mitogen (8), or staphylococcal enterotoxin E (SEE; Toxin Technology, Sarasota, FL) (9), a T cell receptor Vβ family-specific stimulus (10). Adoptive transfer has been demonstrated using young DP (8), cyclophosphamide-treated histocompatible RT1u non-BB (11) or athymic nude RT1u rats (12) as recipients. Transfer studies using young DP recipients have implicated both the CD4+ (13) and CD8+ (14) T cell subsets as being necessary for adoptive transfer. Since DP recipients possess

1Abbreviations used in this paper: BB/Wor, BioBreeding/Worcester; CAS, Con A-activated Lewis rat splenocytes; DP, diabetes prone; DR, diabetes resistant; IDDM, insulin-dependent diabetes mellitus; NOD, nonobese diabetic; SEE, staphylococcal enterotoxin E.
an endogenous cohort of autoreactive CD4+ and CD8+ T cells, these studies do not permit a precise determination of the cell type required for initiating the diabetogenic process. Experiments designed to discriminate between inductive versus effector events and to map each arm of the diabetogenic immune response to a specific MHC locus would require the use of non-BB, non-diabetes-prone RT1a class I or class II congenic recipients in adoptive transfer studies. In non-BB RT1a congenic recipients, one can measure de novo disease induction rather than the acceleration of a spontaneous disorder that would eventually occur in diabetes-prone BB recipients.

To investigate the basis for the MHC requirement in BB autoimmune diabetes, diabetes-inducing T cell lines were generated from the spleens of acutely diabetic DP rats. The diabetes transfer capabilities of the T cell lines were tested in syngeneic and class I or class II congenic non-diabetes-prone recipients. The ability of class I or class II congenic APC to activate the T cells in vitro for the adoptive transfer of diabetes in vivo was also assessed. The results presented herein explain, at least in part, the MHC genetic contribution to IDDM pathogenesis in the rat and shed light on the pathogenesis of β cell destruction.

Materials and Methods

Animals. BB/Wor DP and BB/Wor diabetes-resistant (DR) rats and inbred PVG.R8, PVG.R23, LEW1.AR2, and LEW1.WR1 rats were raised at the University of Massachusetts Medical Center (Worcester, MA) under viral antibody–free conditions. Viral antibody–free LEW1.AR2 and LEW1.WR1 breeding stock were obtained from the Central Institute for Laboratory Animal Breeding (Hannover, Germany).

Islet Cell Preparation. Islets were isolated from 90-d DR rats by the method of Gotot et al. (15). Pancreatic tissue was digested with 4 mg/ml collagenase P (Boehringer Mannheim Corp., Indianapolis, IN) for 25 min at 37°C. Islets were enriched on Histopaque 1077 (Sigma Chemical Co., St. Louis, MO) under viral antibody–free conditions. Viral antibody–free LEW1.AR2 and LEW1.WR1 breeding stock were obtained from the Central Institute for Laboratory Animal Breeding (Hannover, Germany).

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Results

Generation and Characterization of BB/Wor Diabetes-Inducing T Cell Lines. BB/Wor autoimmune diabetes can be adoptively transferred into syngeneic recipients with acutely diabetic DP spleen cells that have been activated in vitro by SEE, a TCR VB family–specific stimulus (9). T cell lines were generated from the spleens of acutely diabetic DP rats by first culturing them in vitro with SEE. After 3 d, SEE-reactive lymphoblasts (~9% of input spleen cells) were en-

Induction of BB Diabetes Is Class II Restricted
riched on density gradients and recultured in CAS as a source of T cell growth factors. At the second round of antigen-specific selection, T cells were stimulated with whole BB/Wor islet cells and APC. Islet cell-reactive T lymphocytes were density gradient purified and passaged further in CAS. At the third cycle of antigen activation, T cell lines were restimulated with either SEE and APC or with islet cells and APC. This protocol generates a population of T cells with potent diabetes transfer activity. A single intraperitoneal injection of 12-34×10⁶ T cells adoptively transferred diabetes into 21–28-d-old DP recipients in as few as 5 d after cell injection (Table 1), with a mean time to hyperglycemia of 7.9 d (n = 48). DP rats also develop spontaneous thyroiditis, although at a low and variable incidence (22). Diabetic recipients of T cell lines, however, did not develop thyroiditis (n = 48). Hyperglycemia was always accompanied by a β cell-destructive insulitis. Only antigen-activated T cells transfer disease; unstimulated (resting) T cells transferred >5 d after antigen activation (with islet cells or SEE) did not induce adoptive diabetes (Table 1, line 4) or insulin. By single-color flow cytometry, the cell lines were comprised (at all time points tested) of 70–90% CD4⁺ and 10–30% CD8⁺ T cells (data not shown). Although DP rats have low, barely measurable levels of CD8⁺/CD5⁺ CTLs and increased numbers of CD8⁺/CD5⁻/3.2.3⁺ NK cells (23, 24), the T cell lines did not contain 3.2.3⁺ NK cells at any time. The cell lines proliferate in vitro in response to both islet cells and SEE (data not shown). One cell line (A91-1), also derived from acutely diabetic DP spleens, was initially selected and then repetitively stimulated in vitro with whole islet cells and APC, but it did not transfer diabetes or insulin to any of 28 DP recipients (21–25 d old) given 19–57×10⁶ T cells (data not shown). The initial in vitro activation step with SEE appears to select for T cells with both islet cell reactivity and diabetes transfer capabilities.

Adoptive Transfer of T Cell Line-mediated Diabetes is MHC Class II⁺ Restricted and Independent of Class I Haplotype. To exclude the possibility that the T cell lines were simply accelerating or costimulating an endogenous immune process genetically present in DP recipients, T cell lines were transferred into RT1 congenic non-BB strains of rat. These congenics contain non-BB RT1u genes, in different allelic combinations on the genetic background of parental Lewis (RT1b) and PVG (RT1f) strains. Neither the RT1b congenics nor their parental strains spontaneously develop insulitis or diabetes. To prepare congenic rats for BB/Wor T cell injections, recipients were treated with cyclophosphamide 24 h before transfer. Cyclophosphamide depletes T cells and enables RT1 congenic rats to accept the BB/Wor T cells, with which they share only partial genetic identity (11). Cyclophosphamide alone does not induce diabetes or insulitis in RT1 congenic rats (see Table 3).

Table 1. BB/Wor Diabetes-inducing T Cell Lines

| Cell line | Incidence of diabetes | Stimulus  | Cell No. | Mean time to diabetes | Mean blood glucose |
|-----------|-----------------------|-----------|----------|-----------------------|--------------------|
| BF-1      | 6/6                   | Islet cells | 16–20    | 7                     | 26.4               |
| BF-1      | 3/3                   | SEE       | 32       | 5                     | 25.0               |
| BB-3      | 4/4                   | Islet cells | 19       | 6                     | 19.8               |
| BB-3      | 0/4                   | None      | 22       | —                     | —                  |
| BB-3      | 2/2                   | Islet cells | 12       | 8                     | 15.7               |
| BB-5      | 3/3                   | SEE       | 34       | 12                    | 28.9               |
| J193      | 2/3                   | Islet cells | 17       | 7                     | 27.9               |

T cell lines were activated for 3 d with islet cells/APC or SEE/APC as described in Materials and Methods. Blast-transformed cells were harvested on Histopaque 1077 and cultured for a further 2 d in 10% CAS. Resting T cells (no stimulus) were transferred after 2 passages in CAS. T cells were injected intraperitoneally into 21–28-d female DP rats. Recipients were monitored for 21 d for the development of hyperglycemia (blood glucose ≥13.8 mmol/liter). A total of 45 out of 48 DP rats injected with T cell lines developed diabetes, with a mean time to hyperglycemia of 7.9 d.
Table 2. Adoptive Transfer of Diabetes into PVG.R8 Rats by Islet Cell-activated BB/Wor T Cell Lines

| Cell line | Cell No. | Incidence of diabetes $\times 10^6$ | Mean time to diabetes | Mean blood glucose mmol/liter |
|-----------|----------|-------------------------------------|-----------------------|-----------------------------|
| Ju92      | 31       | 3/4                                 | 7                     | 23.8                        |
| BB-5      | 39       | 6/6                                 | 7.5                   | 25.2                        |
| 092       | 16       | 3/4                                 | 8                     | 17.9                        |
| 092       | 29       | 2/3                                 | 7                     | 24.6                        |

T cell lines were activated for 3 d with islet cells and APC as described in Materials and Methods. Blast-transformed cells were harvested on Histopaque 1077 and cultured for a further 2 d in 10% CAS. T cells were injected intraperitoneally into 21-30-d female PVG.R8 rats treated 24 h earlier with cyclophosphamide, 180 mg/kg body weight. None of eight female PVG.R8 rats given 180 mg/kg cyclophosphamide alone developed diabetes or insulitis. All rats were monitored for 21 d for the development of glycosuria and hyperglycemia (blood glucose $\geq 13.8$ mmol/liter).

Table 3. Adoptive Transfer of Diabetes into MHC Congenic Rat Strains by Islet Cell-activated BB/Wor T Cell Lines

| Strain       | RT1 haplotype | Cell No. | Incidence of diabetes $\times 10^6$ | Incidence of insulin |
|--------------|---------------|----------|-------------------------------------|---------------------|
| PVG.R8       | A' B/D'C      | 16-39    | 14/17                               | 14/17               |
| PVG.R8       | A' B/D'C'     | 0        | 0/8                                 | 0/8                 |
| PVG.R23      | A' B/D'C'm    | 27-36    | 0/14                                | 0/14                |
| PVG.R23      | A' B/D'C'm    | 0        | 0/11                                | 0/11                |
| LEW1.AR2     | A' B/D'C     | 36       | 0/7                                 | 0/7                 |
| LEW1.AR2     | A' B/D'C     | 0        | 0/10                                | 0/10                |
| LEW1.WR1     | A' B/D'C     | 28       | 9/10                                | 9/10                |
| LEW1.WR1     | A' B/D'C     | 0        | 0/7                                 | 0/7                 |

21-30-d-old male and female RT1 congenic rats received cyclophosphamide, 180 mg/kg body weight intraperitoneally, 24 h before cell transfer. Control rats received cyclophosphamide alone. Rats were monitored for 21 d for glycosuria and hyperglycemia (blood glucose $\geq 13.8$ mmol/liter).

Adoptive Transfer of Diabetes by Con A-activated Acutely Diabetic DP Spleen Cells Also Requires that Donor and Recipient Share Class II gene Products. To confirm the in vivo MHC restriction pattern obtained with islet cell-activated T cell lines, Con A-activated acutely diabetic DP spleen cells were also injected into cyclophosphamide-treated RT1 congenic rats. One spleen equivalent of Con A-activated cells transferred diabetes and insulitis into PVG.R8 and LEW1.WR1 but not PVG.R23 and LEW1.AR2 rats (Table 4). 13 out of 14 LEW1.AR2 recipients developed fulminant GVHD (manifested by wasting, anemia, and massive splenomegaly) 10 d after transfer. Two out of three DP rats concurrently receiving the same Con A-activated spleen cells developed diabetes at 10-11 d after transfer. Thus, cells with diabetogenic potential survived in LEW1.AR2 recipients, but without inducing hyperglycemia or insulitis.

As assessed by adoptive transfer, the cognitive or inductive phase of BB diabetes is MHC class II restricted and can proceed in the presence of either class I A u C a or A a C u gene products. Thus, the induction of transferred diabetes is dependent first upon CD4 + T cell recognition of B cell autoantigen in the context of class II u in recipient target tissue (islet of Langerhans). These experiments do not, however, rule out a role for CD8 + CTL in the autoimmune attack leading to B cell destruction.

Class II Restriction of BB Diabetes Operates at the Level of Interaction between Inducing T Cells and APC. To examine the APC as a potential locus for the class II restriction, BB T cell lines were activated in parallel with sonicated BB islet cells and BB or RT1 congenic APC (irradiated spleen

Table 4. Adoptive Transfer of Diabetes into MHC Congenic Rat Strains by Con A-activated Spleen Cells from Acutely Diabetic Diabetes-prone BB/Wor Rats

| Strain       | RT1 haplotype | Diabetes | Insulitis |
|--------------|---------------|----------|-----------|
| PVG.R8       | A' B/D'C     | 19/23    | 21/23     |
| PVG.R23      | A' B/D'C'    | 0/17     | 0/17      |
| LEW1.AR2     | A' B/D'C'    | 0/14     | 0/11      |
| LEW1.WR1     | A' B/D'C'    | 5/5      | 5/5       |
| BB/Wor DP    | A' B/D'C'    | 24/27    | 25/27     |

Recipient rats were 21-30-d-old rats of both sexes. Each rat received one spleen equivalent of Con A-activated acutely diabetic DP spleen cells intraperitoneally or intravenously. Congenic rats received cyclophosphamide, 150-180 mg/kg body weight i.p. 24 h before spleen cell injections. For each experiment, rats were concurrently transferred into both congenic and BB rats. Recipients were monitored for 3 wk (BB rats) or 4 wk (congenic rats) for the development of hyperglycemia (blood glucose $\geq 13.8$ mmol/liter).
Figure 1. Photomicrographs show adjacent pancreatic islet sections taken from PVG.R8 rats treated with cyclophosphamide alone (A, B) or cyclophosphamide followed 24 h later by an injection of islet cell-activated BB/Wor T cells (C, D). Tissues were fixed in Bouin’s solution, and an immunoperoxidase technique was used for identification of insulin (A, C) and glucagon (B, D). The islets of cyclophosphamide control (A, B) reveal no evidence of insulitis. Insulin-positive β cells (A) and surrounding glucagon-positive α cells (B) are normal in appearance. The islets of the diabetic rat reveal an intraislet mononuclear cell infiltrate with almost complete destruction of the pancreatic β cells (C). Peripheral glucagon-positive α cells (D) are preserved in the diabetic PBV.R8 rats. A and B, ×200; C and D, ×170.

cells). The sonication step was added to deplete the islet cells of intact class II+ APCs of BB origin, as such cells are a normal component of the islets of Langerhans. After activation, equal numbers of T cells were injected into 21-25-d DP recipients, which were monitored for hyperglycemia for 15 d after cell injection. BB T cells activated in vitro with islet cell antigens and BB, PVG.R8 (B/Dr), or LEW1.WR1 (B/Da) APC, but not PVG.R23 (B/Da) or LEW1.AR2 (B/Da) APC, adoptively transferred diabetes (Table 5). Thus, BB diabetes–inducing T cells are class IIa restricted both in vivo and in vitro. The class II restriction appears to operate at the level of the interaction between inducing T cells and class IIa APC.

Discussion

BB autoimmune diabetes is an MHC-linked disease with a requirement for both CD4+ (13) and CD8+ (4, 14) T cells. To investigate the basis for the MHC requirement, diabetes-inducing T cell lines were generated from the spleens of acutely diabetic DP rats. When activated in vitro with whole islet cells and APC, the T cell lines have potent diabetes transfer activity, wherein a single injection of T cells adoptively transferred hyperglycemia into 21-28-d-old DP recipients in as few as 5 d after injection. Hyperglycemia was always accompanied by a β cell–destructive insulitis with sparing of the glucagon- and somatostatin-secreting islet cells. Although DP rats may also develop spontaneous thyroiditis (22), diabetic recipients of T cell lines never manifested any signs of intrathyroid lymphocytic infiltrates. Interestingly, the T cell lines can also be activated for diabetes transfer with the superantigen, SEE. The ability of a superantigen to activate diabetes-inducing T cells is reminiscent of a recent report suggesting a role for superantigen in human IDDM etiology (25).

To determine which type of T cell initiates the development of diabetes, BB/Wor T cell lines were injected into non–diabetes-prone RT1u class I or class II congenic recipients. Upon islet cell activation in vitro, the T cell lines rapidly transferred insulitis and diabetes into class IIa congenic rats. Recipients that are RT1u at only the class I A or C locus, but not at the class II B/D loci, did not develop diabetes after T cell transfer. The adoptive transfer of dia-
with class I hyperexpression and infiltrating CD8+ T cells process and then present antigen to 13 ceil peptide-specific protein that is taken up by class II + intraislet APC, which being invariant concomitants of the autoimmune attack BB rat 13 cells do, however, express class I products in vivo, producing 13 cell targets do not express detectable MHC important in BB diabetes. Anti-CD8 mAb treatment re-

The MHC contains the predominant genetic susceptibility factors for IDDM in humans (41, 42), the BB rat (7), and the NOD mouse (43, 44). In particular, MHC class II genes are associated with disease susceptibility in all three species (45-47). The manner in which the products of the IDDM-associated MHC genes influence the pathogenesis of diabetes is still unknown. The mechanism could be either at the level of thymic T cell selection or during peripheral immune response activation, both of which require appropriate peptide presentation by MHC molecules. Our data indicate that, in the BB rat, the MHC class II ge-

Table 5.  **BB/Wor Diabetes-inducing T Cells Require Activation with MHC Class II-positive APC and Islet Cell Antigens for Disease Transfer**

| Experiment No. | Source of APC | Class II loci | Incidence of diabetes | Mean time to diabetes |
|---------------|---------------|---------------|-----------------------|----------------------|
| 1             | BB/Wor        | B/D^a         | 7/8                   | 11                   |
|               | PVG.R23       | B/D^a         | 0/8                   | —                    |
| 2             | BB/Wor        | B/D^a         | 6/7                   | 9                    |
|               | LEW1.WR1      | B/D^a         | 4/5                   | 10                   |
| 3             | BB/Wor        | B/D^a         | 7/8                   | 9                    |
|               | PVG.R8        | B/D^a         | 8/9                   | 9                    |
| 4             | PVG.R8        | B/D^a         | 1/2                   | 11                   |
|               | LEW1.AR2      | B/D^a         | 0/3                   | —                    |
| 5             | BB/Wor        | B/D^a         | 4/4                   | 12                   |
|               | LEW1.AR2      | B/D^a         | 0/2                   | —                    |

BB/Wor T cells (4 × 10^5/ml) were activated in parallel with DP or RT1 congenic 3,000-R spleen cells (2 × 10^6/ml), 5% FCS, 5% CAS, and sonicated BB islet cells. The sonicates were always checked microscopically for the absence of whole cells. Islet cell sonicates were used at a ratio of 1 cell equivalent per 21 T cells. After 3 d, blasts were harvested on Histopaque 1077 and cultured for 2 d in 10% CAS. Equal numbers of T cells (usually 25 × 10^5) were then injected intraperitoneally into 21–25-d female DP rats, which were monitored for 15 d for development of glycosuria and hyperglycemia (blood glucose ≥13.8 mmol/liter).
nhetic contribution to IDDM pathogenesis may be explained by binding of β cell peptide to permissive class IIa molecules, resulting in the activation of diabetes-inducing T cells. Specifically, BB T cells can be activated in vitro for the transfer of diabetes with islet cell antigens and class IIα-positive, but not class IIα-negative, APC (Table 5). The MHC class II restriction of BB diabetes thus operates at the level of interaction between inducing T cells and class IIα APC. These data lend support to the peptide affinity model for the class II genetic contribution to IDDM susceptibility: Susceptibility is caused by peptide presentation by a class II gene product that binds diabetogenic peptide, resulting in the activation of β cell-specific autoreactive T cells (48).

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