MAJOR HISTOCOMPATIBILITY COMPLEX RESTRICTION FRAGMENT LENGTH POLYMORPHISMS DEFINE THREE DIABETOGENIC HAPLOTYPES IN BB AND BBN RATS

By JOHN B. BUSE,*‡‡ RANIA RIFAI-HADDAD,* SARAH LEES,* HIROSHI TANIGUCHI,* DAVID CHAPLIN,** EDGAR M. MILFORD,‡,‖ J. G. SEIDMAN,¶ GEORGE S. EISENBARTH,*,‡,§,‖ AND RICHARD A. JACKSON*,‡,§,‖

*From the *Joslin Diabetes Center, ‡Brigham and Women's Hospital, §New England Deaconess Hospital, and the Departments of ‖Medicine and ¶Genetics, Harvard Medical School, Boston, Massachusetts; the **Department of Medicine, Washington University School of Medicine, St. Louis, Missouri; and the ‡‡Department of Physiology, Duke University Medical Center, Durham, North Carolina*

BB rats spontaneously develop insulin-dependent, ketosis-prone diabetes mellitus (DM).[1] Their disease is of autoimmune etiology, since it is characterized by: (*a*) lymphocytic and macrophage inflammation of pancreatic islets (1), (*b*) circulating autoantibodies that bind to pancreatic islets of Langerhans (2), (*c*) increased incidence of Ia antigen–bearing T lymphocytes associated with the development of diabetes (3), (*d*) progressive and selective destruction of insulin-producing beta cells (1, 4), (*e*) the ability to transfer disease to immunodeficient animals with concanavalin A–activated splenic lymphocytes (5), and (*f*) selective destruction of beta cells in transplanted islets (6); in addition, it can be prevented with many forms of immunotherapy (7). BB rats also exhibit a profound T cell lymphocytopenia with an almost total lack of circulating T lymphocytes, although their thymocytes are normal in number and cell surface phenotype (8). In breeding studies, we and others (9, 10) have shown that at least two genes contribute to the development of diabetes in the BB rat. One is autosomal recessive and determines the profound T cell immunodeficiency present in affected rats at birth. The second is linked to RT1, the rat major histocompatibility complex (MHC).

To further define the BB rat's RT1-linked diabetogenic gene, we asked whether there are MHC differences between BB rats and control strains at the genomic level (as determined by studying restriction fragment length polymorphism [RFLP]) that are related to MHC diabetogenicity, as determined in breeding studies. Using a class II H-2 A-alpha probe, we previously reported (11) the

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R. A. J., Research Division, Joslin Diabetes Center, 1 Joslin Place, Boston, MA 02215.

[1] *Abbreviations used in this paper:* AZ, sodium azide; DM, diabetes mellitus; MHC, major histocompatibility complex; PBS, phosphate-buffered saline; RFLP, restriction fragment length polymorphism; SDS, sodium dodecyl sulfate; WF, Wistar Furth.

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existence of four polymorphic MHC types in the control BBN population, which reflects the colony from which the BB rat was derived, only one of which was found in BB rats. We also reported preliminary results (12) using class I, II, and III MHC probes to find polymorphisms that further subdivide the BB and BBN population. In this paper we extend those preliminary findings and characterize five RFLP-defined MHC types in the BBN population and a distinct RFLP-defined MHC type present only in BB rats, using a class I MHC probe. We evaluated the diabetogenicity of these RFLP-defined MHC chromosome types in a breeding study and determined the serologic RT1 phenotypes encoded by these chromosomes using monoclonal antibodies. These findings allow us to provide preliminary localization of the MHC-linked diabetogenic gene of rats as well as insight into the organization of class II genes in the RT1 complex. The organization of these studies is schematized in Fig. 1.

Materials and Methods

Animals. BB/O rats and Wistar control rats (here termed BBN) were kindly provided by Dr. Pierre Thibert (Department of National Health and Welfare, Ottawa, Canada) or bred from his stock in the animal facility at the Joslin Diabetes Center. BB/W rats from sublines with a high incidence of diabetes (NC, PA, FA, BF) or low incidence sublines (WA, WC) were provided by Dr. A. A. Like (University of Massachusetts Medical Center, Worcester, MA). Animals from a new strain of diabetic rats (BB-Hooded) with a 95% incidence of diabetes, developed by Dr. J. Logothetopoulos (Best and Banting Institute, Toronto, Canada), were also provided by Dr. Thibert. BB/O and BBN rats were co-derived from the same original outbred colony by selection for (BB/O) or against (BBN) the development of diabetes. Both strains are maintained as outbred closed colonies. BB/W rats have been inbred by brother-sister mating for 16+ generations. Low-incidence BB/W sublines were split off from the high incidence BB/W lines after six generations of inbreeding by selecting against the development of diabetes, and are often referred to as W-line rats. BBN and W-line rats do not exhibit the T cell immunodeficiency present in the diabetes-prone BB/O, BB/W (high incidence sublines), and BB-Hooded rats. Wistar Furth (WF) Buffalo, Lewis, and ACI rats were obtained from Harlan Sprague Dawley, Inc. (Indianapolis, IN). Animals were housed according to NIH guidelines at the animal facility at the Joslin Diabetes Center and provided ad libitum commercial rat chow and water. Diabetes-prone animals were screened every 2–3 d for glycosuria and were determined to be diabetic if they had serum glucose values >300 mg/dl on two consecutive days. Diabetic animals were maintained with daily doses of Lente insulin (Eli Lilly & Co., Indianapolis, IN).

Probes. The A-alpha probe was isolated by Dr. A. Ben-Nun (Weitzman Institute, Rehovot, Israel) and is a 1.3 kb HindIII genomic fragment of the A-alpha region of C57BL/10 encoding part of the alpha 1 and all of the alpha 2, transmembrane and intracytoplasmic domains as well as intervening introns. The DC-beta probe was provided by Dr. P. Peterson (University of Uppsala, Uppsala, Sweden) and is a cDNA clone of a HLA-DC-like gene (13). The E-beta probe (subclone 8.48, kindly provided by Dr. L. Hood, California Institute of Technology, Pasadena, CA) is a 7.2 kb genomic fragment encoding the beta-1 and beta-2 domains of E^d (14). The E-alpha probe used is a 1.4 kb BglII genomic fragment of a PstI subclone of cosI^d-alpha-1 (kindly provided by Dr. R. Flavell, Biogen, Cambridge, MA) encoding the alpha-1 domain of E^d (15). Three class III genomic probes were used: probe f as well as a 6.7 kb BamHI fragment and a 2.5 kb BamHI fragment of cosmid E7 from the "sex-limited protein" area of H-2 (16, 17). The class I probe (S16) is a 500 basepair genomic fragment of the third coding block (C-2 domain) of H-2L^d (18). Because of high sequence homology in MHC genes among mammals, it is assumed though not proved that these probes hybridize with genes in rats homologous to the MHC genes in mice and humans (19–22).

DNA Isolation and Determination of RFLP. ~1 cm of rat tail was frozen in liquid nitrogen and stored at −80°C until use. The tail was cut into 1–2-mm slices, placed on ice in 2 ml 0.1 M NaCl, 0.2 M sucrose, 0.01 M EDTA, 0.03 M Tris-HCl, pH 8, and homogenized using a Polytron (Brinkman Instruments Co., Westbury, NY). Then, 0.5 ml of 0.25 M EDTA, 2.5% sodium dodecyl sulfate (SDS), 0.5 M Tris-HCl, pH 9.2 was added and the mixture incubated at 65°C for 30 min. 0.3 ml 8 M potassium acetate was added, the mixture incubated for 1 h on ice, and the aqueous solution was extracted twice with 5 ml 100:1 chloroform/isoamyl alcohol. DNA was precipitated from the aqueous phase with a final concentration of 50% ethanol for 10 min at room temperature. The methods for RFLP analysis have been previously described (11) except as noted below. For class I RFLP analysis, 25 μg of DNA were digested to completion using restriction enzyme EcoRI (New England BioLabs, Beverly, MA) in appropriate buffer. DNA fragments were separated on 0.7% agarose gels, transferred to nitrocellulose filters, and hybridized with ^{32}P-labeled DNA probe. All hybridizations were done at high stringency, with final washes at 50°C in 30 mM NaCl, 3 mM trisodium citrate, and 0.1% SDS. Autoradiography was used to visualize bands of specifically bound, labeled probe.

Determination of T Cell Lymphopenia. A rapid screening assay for lymphopenia had been developed and found (9) to be both sensitive and specific compared with our standard assay. Briefly, 0.125 ml of blood was collected into heparinized Natelson tubes and mixed with 0.125 ml RPMI 1640 plus 4 mg/ml gentamycin sulfate (RPMI plus G) in 96-well V-bottomed plates. The plates were centrifuged at 1,000 *g* and the supernatants were aspirated. The anti-rat T cell monoclonal antibody OX19 (Accurate Chemical & Scientific Corp., Westbury, NY) was diluted 1:100 with RPMI plus G and 0.5% bovine serum albumin; 0.05 ml was added to each well, incubated for 45 min at 4°C, and washed twice with 0.2 ml RPMI plus G. A second incubation, with 0.05 ml fluorescein-conjugated $F(ab')_2$ goat anti-mouse IgG (diluted 1:10 in rat serum; Tago, Inc., Burlingame, CA), was for 45 min at 4°C. The cells were washed three times with RPMI plus G. The red cell/white cell pellet was diluted 10-fold with phosphate-buffered saline, and 10^5 cells were evaluated for the presence of T lymphocytes using an Epics V flow cytometer (Coulter Electronics Inc., Hialeah, FL) as previously described (9). Typically, nonlymphopenic animals have a 5–10-fold higher percentage of OX19-positive cells than do lymphopenic animals by this assay. Known lymphopenic and nonlymphopenic animals were included in each assay as positive and negative controls.

RT1 Typing. Class I MHC typing (RT1.A) was determined by indirect immunofluorescence on Ficoll-Hypaque–isolated peripheral blood mononuclear cells as previously described (9) using monoclonal antibodies LWF.415 (anti-RT1.A^u) and AP.1.1.13 (anti-RT1.A^a). Class II MHC typing (RT1.B and RT1.D) was determined on Ficoll-Hypaque density gradient (specific gravity, 1.090)-purified splenic mononuclear cells as follows. Surface immunoglobulin was capped in the presence of excess fluorescein-labeled rabbit anti-rat IgG (Cappel Laboratories, Cochranville, PA), diluted 1:25 in RPMI plus G containing 20% fetal bovine serum, for 1 h at 37°C. Capped cells were washed three times with phosphate-buffered saline plus 0.1% sodium azide (PBS plus Az) to prevent further capping. A second incubation was carried out in the presence of monoclonal antibody supernatants specific for RT1.B^u (LW2.3C9) or RT1.D^a (4D11) for 1 h at 4°C. The splenocytes were again washed three times in PBS plus Az and then incubated with an excess of rhodaminated rabbit anti-rat IgG (Cappel Laboratories) diluted 1:25 in PBS plus Az. Cells were washed three times with PBS plus Az and examined with a Leitz-Diavert microscope equipped for epifluorescence. A reaction was considered positive if >90% of cells exhibiting fluorescein capping (surface immunoglobulin–positive B cells) also exhibited rhodamine rim fluorescence (class II antigen). WF (RF1^u), AC1 (RT1^a), Lewis (RT1^l), and Buffalo (RT1^b) rats were used as positive or negative controls. These antibodies were either produced in Dr. E. Milford's laboratory (LWF.415, LW2.3C9, and 4D11) or kindly provided by Dr. E. Blankenhorn (AP.1.1.13) (University of Pennsylvania Medical School, Philadelphia, PA). The method used for class II RT1 typing was kindly provided by J. Kut, Brigham and Women's Hospital.

Data were analyzed using a CLINFO computer sytem (NIH GCRC No. 5M01 RR00888-10CLR.

Results

RFLP Analysis Using Class II MHC Probes. We have previously reported (11) the existence of class II MHC polymorphisms in the BB (diabetes-prone) and BBN (control strain "BB normal") population using an A-alpha probe. To summarize, in the rats studied using restriction enzyme BamHI, the A-alpha (class II) probe hybridizes with two bands, designated "1" and "2". Using restriction enzyme EcoRI, the A-alpha probe also hybridizes with two bands, designated "a" and "b". Thus, using the A-alpha probe with BamHI and EcoRI, four polymorphic patterns are seen, which we have defined as type 1a (BamHI 2.9 kb, EcoRI 8.2 kb), type 1b (BamHI 2.9 kb, EcoRI 1.7 kb), type 2a (BamHI 2.4 kb, EcoRI 8.2 kb), and type 2b (BamHI 2.4 kb, EcoRI 1.7 kb). These RFLP patterns are inherited in a classic Mendelian fashion. The results of our RFLP analysis using the A-alpha probe in BB, BBN, and Wistar Furth rats are presented in Table I. All diabetes-prone BB strain animals (BB/O, BB/W, and BB-Hooded rats [n = 30]) are 2a/2a homozygotes. In the BBN population, 1a, 1b, 2a, and 2b chromosomes exist and most animals are heterozygotes (Table I). Analyzed in terms of chromosomes, the population of BBN rats we studied consisted of 48% 2a, 35% 1b, 16% 1a, and 1% 2b chromosomes. (Since 2b rats are very rare and because we were unable to breed them, they have not been studied further.) Wistar Furth rats (WF), an inbred strain of rat bearing the RTL^u haplotype, share A-alpha RFLP type 2a–defined homozygosity with BB rats. We used 10 other restriction enzymes (ApaI, BglII, EcoRV, HindIII, KpnI, PstI, PvuII, SacI, SmaI, XhoI) to try to subdivide 2a rats using the A-alpha probe, but, with all enzymes, BB/O, BBN 2a, and WF rats showed identical banding patterns. The same was true using enzyme MspI for WF, BB/W, BB/O, and BB-Hooded rats.

Other class II probes were used in an effort to further subdivide the BB and BBN rat population. A DC-beta probe with restriction enzymes BamHI and

TABLE I

A-Alpha-defined RFLP Types in BB, BBN, and WF Rats

Chromosome type	BBN (n)	BB* (n)	WF (n)
2a/2a	30	30	3
2b/2b	0	0	0
1a/1a	2	0	0
1b/1b	13	0	0
2a/1a	10	0	0
2a/1b	22	0	0
2a/2b	0	0	0
1a/1b	16	0	0
1b/2b	2	0	0
1b/2a‡ or 1a/2b	32	0	0

* BB rats included 18 BB/O rats, 6 BB hooded, 4 BB/W from high incidence litters, and 5 BB/W from low incidence litters.

‡ These heterozygous types cannot be distinguished except in family studies. 22 of 22 of animals with this RFLP pattern are 1b/2a according to such studies.

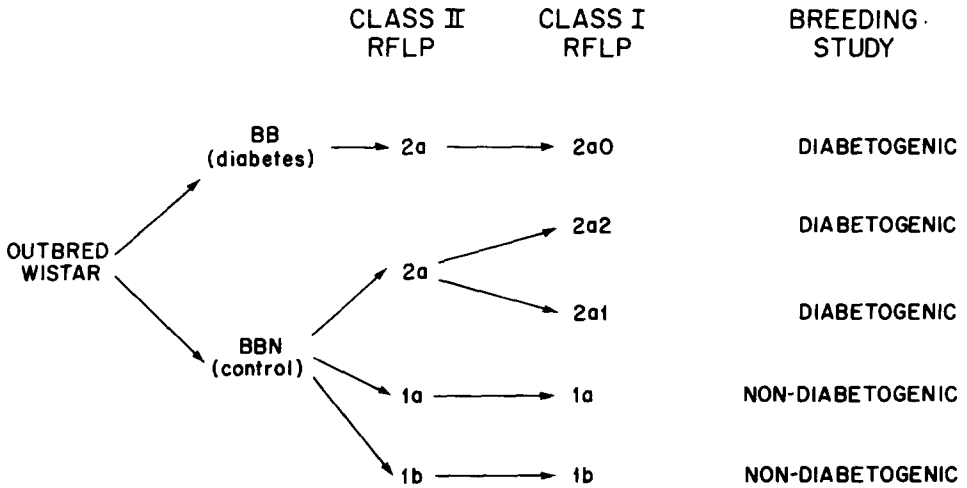

FIGURE 1. Schematization of our experiments to define diabetogenic MHC haplotypes in the BB and BBN rat populations. Both strains were derived from the same outbred Wistar rat colony by selecting for the development of diabetes (BB) or against the development of diabetes (BBN). Class II RFLP analysis subdivides the control BBN rats into four subgroups (1a, 1b, 2a, and 2b), while BB rats exhibit one of these (2a). (2b BBN rats are not included in this figure since they are rare and we have not been able to breed them.) Class I RFLP analysis absolutely distinguishes BB (2a0) from BBN rats (2a1, 2a2, 1a, 1b). A breeding study was carried out to evaluate the diabetogenicity of the various RFLP-defined MHC haplotypes.

EcoRI generated the same data (though different band molecular sizes) as did the A-alpha probe with restriction enzyme BamHI (distinguishing "1" BBN rats [1a and 1b] from 2a rats [BB/O, WF, and 2a BBN]). However, there was no polymorphism analogous to that seen with A-alpha and EcoRI (12). An E-alpha probe also generated the same pattern of polymorphisms (though, again, different molecular sizes), with restriction enzymes BamHI and EcoRI, distinguishing 1a and 1b rats from 2a rats (BB/O, WF, and BBN). However, no RFLP was evident with enzyme HindIII and PvuII using the E-alpha probe (data not shown). An E-beta probe showed no polymorphisms between BB/O, WF, and 1a, 1b, and 2a BBNs using restriction enzymes BamHI and EcoRI. In addition, we were unable to subdivide 2a rats using the E-beta probe and the panel of 11 restriction enzymes mentioned above for A-alpha (data not shown).

To summarize (Fig. 1), the A-alpha class II probe, in concert with restriction enzymes BamHI and EcoRI, generates RFLPs that subdivide the BBN population into 1a, 1b, 2a, and 2b rats. However, all BB rats exhibit the 2a A-alpha RFLP, as do all Wistar Furth rats.

RFLP Analysis Using Class III MHC Probes. We were unable to detect any polymorphisms between BB/O, WF, and 1a, 1b, and 2a BBN rats using three H-2 S-region probes from the area coding for the "sex-limited protein" (Slp) of H-2 with restriction enzymes BamHI, EcoRI, HindIII, MspI, and PvuII (data not shown). These probes have demonstrated limited polymorphisms in mice (16).

RFLP Analysis Using Class I MHC Probes. In contrast to other MHC probes, the S16 class I MHC probe allowed us to absolutely distinguish all BB from BBN

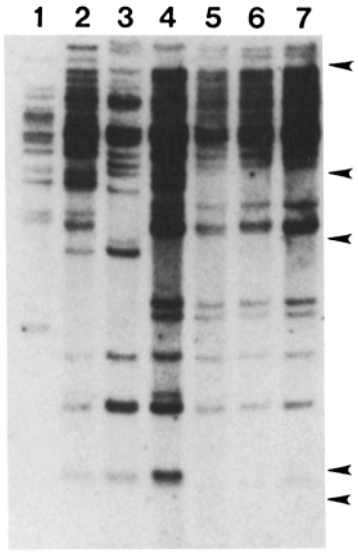

FIGURE 2. RFLP analysis using restriction enzyme EcoRI and the S16 class I probe in a 1b BBN rat (lane *1*), 1a BBN (*2*), 2a2 BBN rat (*3*), 2a1 BBN rat (*4*), BB-Hooded rat (*5*), BB/W W-line rat (*6*), and a Wistar Furth rat (*7*). All BB substrains as well as Wistar Furth rats exhibited the same class I RFLP (termed 2a0) and could be distinguished from all BBN rats. Markers at right designate molecular sizes in kilobases 23.7, 9.5, 6.7, 2.3, and 2.0, from top to bottom.

rats. At least five different patterns were evident (Fig. 2) using the S16 probe and restriction enzyme EcoRI. BBN rats that exhibit the A-alpha 1b RFLP had a distinctive band pattern (Fig. 2, lane *1*) which we call class I RFLP 1b. BBN rats with the A-alpha 1a RFLP had another pattern (Fig. 2, lane *2*) which we designate 1a. The A-alpha–defined type 2a RFLP can be divided into 2a1 and 2a2 forms using the S16 probe in concert with EcoRI (Fig. 2, lanes *4* and *3*, respectively). However, BB/O, BB/W (including high-incidence sublines and the control W line), BB-Hooded, and Wistar Furth rats all shared a class I RFLP that we call form 2a0 (2a because they exhibited the 2a A-alpha RFLP, and 0 because they lacked bands in the 8.0–9.5 kb range where all other rats have at least one band). The 2a0 RFLP is shown in lanes *5* (BB-Hooded), *6* (BB/W low-incidence subline) and *7* (WF) of Fig. 2. Similar distinguishing polymorphisms are evident using restriction enzymes Xh01, PvuII, SmaI, PstI, ApaI, SacI, EcoRV, HindIII, BglII, and KpnI. The 2a1 RFLP (Fig. 2, lane *4*) may have resulted from a crossover event between the 1a class I RFLP (lane *2*) and the 2a0 RFLP (lanes *5*–*7*), since it shares bands with both of these patterns. Table II gives a breakdown of S16 class I–defined EcoRI RFLPs in a population of BB, WF, and BBN rats that also have the A-alpha–defined type 2a RFLP. All BB and BB-derived rats ($n = 37$) have the 2a0 class I (S16)–defined RFLP (as do WF rats), while all BBN rats sharing the type 2a A-alpha RFLP ($n = 20$) can be distinguished as having the 2a1 or 2a2 class I RFLP, distinct from the 2a0 rats. In contrast to our findings with the class II probes, where both BB and some BBN rats shared the 2a A-alpha RFLP, the class I probe absolutely distinguished BB rats (2a0) from BBN rats (2a1, 2a2, class I 1a and 1b) (Fig. 1).

TABLE II
Class I MHC Probe (S16)-defined RFLP Types in BB, BBN (2a), and WF Rats

Chromosome type	BBN (2a) (*n*)	BB* (*n*)	WF (*n*)
2a0/2a0	0	36	8
2a1/2a1	12	0	0
2a2/2a2	1	0	0
2a1/2a2	7	0	0
2a0/2a1	0	0	0
2a0/2a2	0	0	0

* BB rats include 21 BB/O rats, 5 BB-Hooded rats, 4 BB/W rats from high incidence sublines, and 6 BB/W W-line rats.

Breeding Study to Determine Diabetogenicity of RFLP-defined Chromosomes. BBN female rats with 1a, 1b, and 2a A-alpha–defined RFLPs were mated with a single diabetic BB-Hooded male rat for a study of the segregation of MHC polymorphisms with respect to the development of diabetes. This strain of diabetic rat was chosen because of its 95% incidence of diabetes. In the F_1 animals derived from these matings, we determined that the class I and II MHC RFLPs were inherited in a Mendelian fashion, as expected. Namely, the offspring of a 1a/1a by 2a0/2a0 mating were all 1a/2a0, *et cetera*. F_1 rats were brother-sister mated to produce F_2 rats.

F_2 rats were screened for lymphopenia when 25–35 d old using monoclonal antibody OX19, as described in Materials and Methods. Overall, 131 of 557 (23%) F_2 rats were lymphopenic, not statistically different from the 25% expected inheritance of a recessive trait. The inheritance of lymphopenia was independent of the inheritance of MHC RFLPs. In lymphopenic F_2 rats in which RFLPs were determined (n = 131), 17% were homozygous for BBN-derived RFLPs, 56% were heterozygous for BB-H and BBN-derived RFLPs, and 27% were homozygous for the BB-derived 2a0 RFLP (not statistically different from the expected 1:2:1 normal distribution of a recessive trait). In nonlymphopenic F_2 rats in which RFLPs were determined (n = 96), the percent distribution was 22:55:23 (again, not statistically different from that expected).

To evaluate the diabetogenicity of the MHC RFLPs, we followed lymphopenic F_2 animals for the development of glycosuria and hyperglycemia (>300 mg/dl serum glucose). Of those that survived to 120 d of age, 13 developed DM. The breakdown of the incidence of diabetes as a function of BBN grandparental A-alpha–defined RFLP type demonstrated (Table III) a significantly higher incidence of diabetes in lymphopenic F_2 rats derived from 2a BBN matings than in those from 1a or 1b BBN matings, suggesting a diabetogenic effect of the 2a BBN MHC compared with the 1a or 1b BBN MHC.

To further define the MHC association of diabetes, we studied the class I (S16) RFLPs in lymphopenic F_2 rats who survived to 120 d of age. The incidence of diabetes as a function of class I RFLP types is presented in Table IV. There was no statistical difference in the incidence of diabetes among the various 2a subtypes defined by the class I probe. Among lymphopenic F_2 rats that were homozygous for the 2a0 (BB derived) class I RFLP, 28% (5 of 18) developed DM. Among

TABLE III

Incidence of Diabetes Among Lymphopenic F_2 Rats as a Function of BBN Grandparental Class II (A-Alpha) RFLP Type

BBN Grandparent A-alpha RFLP	*n*	DM incidence		
		No.	Percent	
1a	20	1	5.0	$\chi^2 = 4.0, P < 0.05$
2a	29	8	27.6	
1b	45	4	8.9	$\chi^2 = 4.5, P < 0.05$

TABLE IV

Class I (S16) RFLPs of Lymphopenic F_2 Rats Surviving to 120 d of Age vs. Percent of Diabetes Mellitus

	Class I RFLP	*n*	Incidence of DM	
			No.	Percent
BB MHC homozygotes	2a0/2a0	18	5	27.8
BB/2a BBN MHC heterozygotes	2a0/2a1	8	3	37.5
	2a0/2a2	9	2	22.2
		17	5	29.4
2a BBN MHC homozygotes	2a1/2a2	3	2	66.7
	2a2/2a2	3	0	0
		6	2	33.3
BB/1a or 1b BBN MHC heterozygotes	2a0/1a	7	0	0
	2a0/1b	25	1	4.0
		32	1	3.1
1a or 1b BBN MHC homozygotes	1a/1a	10	0	0
	1b/1b	11	0	0
		21	0	0

lymphopenic 2a0/2a1 and 2a0/2a2 rats (heterozygotes for BB[2a0]-and BBN [2a1 or 2a2]-derived MHC chromosomes), 29% (5 of 17) developed DM. And among lymphopenic 2a1/2a2 or 2a2/2a2 rats (homozygotes for BBN-derived MHC chromosomes), 33% (2 of 6) developed DM. These findings indicate that the diabetogenic effect of the BBN-derived 2a1 and 2a2 MHC is similar to that of BB-derived 2a0 MHC. In contrast, of 21 lymphopenic F_2 rats homozygous for the BBN-derived 1a or 1b chromosome, none developed DM, indicating a lack of diabetogenicity of the 1a or 1b MHC compared with the 2a MHCs ($\chi^2 = 7.6$; $P < 0.01$).

Furthermore, the MHC-linked diabetogenic gene seems to be functionally recessive, as only 3% (1 of 32) lymphopenic F_2 rats heterozygous for the nondiabetogenic 1a or 1b MHC and the BB-derived 2a0 MHC developed diabetes, compared with 29% (12 of 41) rats homozygous for either the BB- or BBN-derived diabetogenic 2a MHC RFLP ($\chi^2 = 8.4$; $P < 0.005$). The one 1b/

TABLE V
Serologic Typing of MHC RFLPs in BB and BBN Populations

Class I (S16) RFLP	Anti-RT1.A^u	Anti-RT1.A^a	Anti-RT1.B^u	Anti-RT1.D^a
2a0/2a0	+	−	+	−
2a1/2a1	+	−	+	−
2a2/2a2	+	−	+	−
1a/1a	−	+	−	+
1b/1b	−	−	−	−

2a0 rat that developed diabetes could not be bred, so we cannot exclude a recombination event in this animal. To summarize (see Fig. 1), the A-alpha class II probe allowed us to distinguish some of the control BBN rats from the diabetes-prone BB rats. The class I probe absolutely distinguished BB rats (2a0 RFLP) from BBN rats (2a1, 2a2, 1a, and 1b class I RFLPs), enabling us to follow the segregation of BB vs. BBN MHC chromosomes in matings of BB and BBN rats. The breeding study demonstrates that there is an MHC-linked diabetogenic gene associated with the 2a A-alpha RFLP in the BB (2a0) as well as the BBN (2a1 and 2a2) control rat populations, but not with the 1a or 1b RFLP.

Serologic Determination of RTl Type of RFLP-defined BBN MHC Types. Using indirect immunofluorescence with monoclonal antibodies directed against class I MHC antigens (RT1.A^a and RT1.A^u) and class II MHC antigens (RT1.B^u and RT1.D^a), we determined the serologic RTl type corresponding to the MHC RFLPs in the BB and BBN population (Table V). Briefly, 1a/1a rats expressed RT1.A^{a}D^a; all 2a rats (2a0/2a0, 2a1/2a1, and 2a2/2a2) expressed RT1.A^{u}B^u but not RT1.D^a; and RFLP 1b/1b rats did not express RTl^a nor RTl^u at the loci tested.

Discussion

The BB rat develops type I diabetes of autoimmune etiology. Previous studies have identified two genes as contributing to the development of diabetes. One determines the T cell lymphopenia found in BB rats. The other is linked to RTl, the MHC of rats. To further define the molecular basis of the diabetogenic MHC-linked gene of BB rats, we studied RFLP in BB and BBN rats. BBN rats are an outbred strain of rats maintained as a closed colony in Ottawa, Canada and reflect the original commercial colony in which diabetes appeared and from which the various BB strains were developed.

Using a class II A-alpha probe, we were able to distinguish four RFLP in the BBN population, only one of which (2a) has been found in three independently derived BB colonies. A DC-beta probe detected no additional polymorphisms that showed only linked polymorphisms homologous to the A-alpha RFLPs. An E-alpha probe also detected the same pattern of polymorphisms but only with two of four enzymes tested. Using an E-beta probe we were unable to detect any polymorphisms in the BBN or BB population. Mouse, human, and rat class II MHC genes exhibit considerable sequence homology (20) and these probes should recognize RT1.B-alpha, RT1.B-beta, RT1.D-alpha, and RT1.D-beta sequences, respectively (19, 22). Other investigators have detected limited poly-

morphism by RFLP analysis using class II probes (23). Our findings indicate otherwise, since four class II RFLP were found in a closed population using a single genomic probe (A-alpha) and two restriction enzymes. The degree of RFLP among class II gene sequences in the population studied seems to be: RT1.B-alpha ≥ RT1.B-beta > RT1.D-alpha ≥ RT1.D-beta. In the H-2 system of mouse, the class II region has been divided into a relatively conserved I-E subregion and a more polymorphic I-A subregion. E-beta sequences span the boundary of these two subregions and exhibit considerable RFLP, while E-alpha is in the conserved subregion and exhibits little RFLP (24). Therefore, it is surprising that in our rat population we could detect no polymorphisms using an E-beta probe; this suggests that the boundary between I subregions of rats may be shifted such that the E-beta equivalent (RT1.D-beta) is in the more conserved I-E subregion. The E-beta gene of mouse is associated with a recombination "hot spot" that accounts for all intra–I region recombinants (24). It is interesting to note that the only I region recombinant in rats has mapped between the A-beta and A-alpha equivalents (23), supporting the notion that intra–class II region dynamics may be difficult in the rat than in the mouse.

We were unable to find any polymorphisms using mouse class III MHC probes. A class I probe (S16), however, identified many polymorphisms and could be used to absolutely distinguish BBN rats from BB rats. BBN rats that share A-alpha–defined 2a RFLPs can be subdivided into 2a1 and 2a2 forms, while all BB rats and BB-derived strains have a distinct form that we designated 2a0. The S16 class I probe should hybridize with both RT1.A and RT1.C/E sequences, areas coding for both classical class I major histocompatibility antigens and Qa- and T1-like molecules. As in the mouse, most class I genes have been mapped to the right of the class II region, the RT1.C/E loci (23). These class I– and class II–defined RFLPs are inherited as tightly linked alleles (no evidence of recombination using A-alpha and S16 probes in 123 F_2 rats derived from 1a or 1b BBN × 2a0 BB matings [two meiotic events]).

A breeding study was carried out to evaluate the diabetogenicity of the various MHC RFLP in the BB and BBN population. As expected, the BB-derived 2a0 RFLP exerted a diabetogenic effect: 28% of 2a0 homozygous lymphopenic rats developed DM. Additionally, the 2a1 and 2a2 BBN-derived RFLP are diabetogenic while the 1a and 1b BBN-derived RFLP are nondiabetogenic. Whether the diabetogenic gene in the BBN population is identical to that in BB rats is not known. The age of onset of diabetes in 2a0/2a0 homozygous rats ($n = 5$) was 115.4 ± 7.7 d (mean ± SEM); in 2a0/2a1, 2a0/2a2, or 2a1/2a2 diabetic rats ($n = 7$), the age of onset was 88.4 ± 8.0 d ($P < 0.05$ by two-tailed t test). These results suggest a form of synergism between the diabetogenic 2a0, 2a1, and 2a2 MHC-bearing chromosomes, analogous to the earlier age of onset of diabetes in patients with type I diabetes and having the HLA DR3/DR4 haplotype (Dr. N. Maclaren, personal communication). However, there was no analogous higher incidence of DM in 2a heterozygotes compared with 2a0/2a0 lymphopenic F_2 rats.

The incidence of diabetes in lymphopenic F_2 rats homozygous for diabetogenic MHC-bearing chromosomes (type 2a0, 2a1, or 2a2) is ~10-fold higher (29%) than in lymphopenic F_2 rats heterozygous for a nondiabetogenic halplotype (1a

or 1b) and the 2a0 diabetogenic haplotype (3%). We do not know if the one 1b/2a0 rat that developed diabetes was the result of a crossover between the diabetogenic loci and the 1b polymorphisms detected by RFLP analysis. In that case, the MHC-linked diabetogenic gene may be truly recessive. Alternatively, there may be a much lower incidence of diabetes among heterozygotes. In either case, the diabetogenic gene linked to the 2a MHC RFLP neither functions in the classic codominant fashion of MHC gene allele expression nor as a classic immune response gene, which usually confers responsiveness to foreign antigens in a dominant fashion. In the NOD mouse, another animal model of spontaneous autoimmune type I DM, there is also an MHC-linked diabetogenic gene that acts in a recessive manner (26). The same may be true of man since there is at least a fourfold increase in the incidence of overt diabetes in HLA identical siblings of patients with type I diabetes as compared with siblings having only one HLA haplotype in common with their diabetic sibling (27).

The "recessiveness" of the diabetogenic influence of MHC genes can be explained by at least three mechanisms. If the MHC-linked diabetogenic gene is a class II immune response gene, a decreased incidence of diabetes in heterozygotes may be due to decreased cell surface expression of the diabetogenic class II molecule on antigen-presenting cells, lymphocytes, or, possibly, the target tissue. Class II molecules are expressed as alpha:beta heterodimers. In F_1 animals (heterozygotes for MHC genes), it has been shown that the alpha and beta chains can associate randomly, resulting in one-half to one-eighth the level of expression of a parental type heterodimer compared with the parental strain (28); furthermore, the responsiveness of MHC-restricted T cell clones as proportional to the concentration of responder class II molecules (29). Alternatively, the anti-islet immune response involved in type I diabetes may be controlled by a class II immune "suppressor" gene. In such a system, low responsiveness is inherited as a dominant trait and high responsiveness as a recessive trait (30, 31). Finally, the MHC-linked gene involved in the pathogenesis of type I DM may code for deletion of an enzyme. For example, congenital adrenal hyperplasia results from a deficiency in a 21-hydroxylase involved in cortisol biosynthesis and is inherited as an MHC-linked autosomal recessive trait often associated with HLA-Bw47, C4A-null. Recent studies have defined the molecular basis of this disease association. The defect in 21-hydroxylation appears to be due to a deletion involving a P-450 21-hydroxylase in the MHC. This deletion may have also deleted part of the neighboring HLA-B and C4A regions, converting the common HLA-B13, C4A3 haplotype to the rare HLA-Bw47, C4A-null haplotype (32).

Serologic typing of class I (RT1.A) and class II (RT1.B and RT1.D) antigens in BB and BBN rats showed that the diabetogenic 2a0, 2a1, and 2a2 MHC RFLPs code for the expression of $RT1.A^uB^u$ while the nondiabetogenic 1a RFLP codes for $RT1.A^aD^a$ and the nondiabetogenic 1b RFLP codes for neither $RT1^a$ nor $RT1^u$, at the loci tested. Others have reported that BB rats express serologically defined $RT1.A^u$ antigens (6, 10) and that, among BBN rats, animals express $RT1.A^a$ and/or $RT1.A^u$ (33). Examination of the class I (S16) RFLPs in Fig. 1 shows that 2a1 RFLP (lane *4*) may result from a crossover event between a 2a0 (lanes 5–7) and a 1a (lane *2*) MHC-bearing chromosome. It is significant that the 2a1 RFLP codes for $RT1.A^uB^u$ and not D^a. Therefore, the hypothesized cross-

TABLE VI

Comparison of the Incidence of Diabetes Among Lymphopenic F_2 Rats as a Function of Grandparental Control Strain and MHC Type

Control strain:	L*	BN*	WF*	BBN 1a	BBN 1b	BBN 2a
RT1 type	l	n	u	a	Non-u	u
n	88	237	63	20	45	29
Percent DM	9.1	7.6	9.6	5.0	8.9	27.6‡

* L, BN, and WF refer to Lewis, Brown Norway, and Wistar Furth strains. Data for these rats were derived from reference 8 by dividing *n* by 4 to correct for 25% incidence of lymphopenia, and recalculating the percent DM.

‡ Significantly different from WF ($\chi^2 = 5.0$, $P < 0.05$). All other differences from WF are not statistically significant.

over event that may have "created" the 2a1 MHC-bearing chromosome would have occurred on the RT1.C side of RT1.D, and the diabetogenic MHC-linked gene would be on the class II side of the crossover.

A possible interpretation of the results of the breeding program is that the serologically defined $RT1^u$ contributes to the development of diabetes in BB rats. However, data from our previous breeding study suggests otherwise (Table VI). In that study, the incidence of diabetes in F_2 rats derived from WF × BB crosses was similar to the incidence in Brown Norway × BB and Lewis × BB F_2 rats as well as in F_2 rats derived from 1a × BB and 1b × BB crosses (9), although WF rats are serologically and MHC RFLP identical to BB rats using both class I– and II–specific reagents. The incidence of diabetes in F_2 rats derived from 2a1 or 2a2 BBN × BB crosses (when both grandparents carried diabetogenic MHC genes) was three- to fivefold higher. Previously, it was impossible to directly test the diabetogenicity of the WF $RT1^u$ because it is indistinguishable from the BB $RT1^u$. With the availability of class I–defined 2a1 and 2a2 diabetogenic MHC RFLPs, the diabetogenicity of the potentially nondiabetogenic WF (2a0) MHC can now be directly assessed in breeding studies.

The availability of three diabetogenic MHC types should be of value in localizing and defining the diabetogenic MHC-linked gene in the rat. Because of its similarities to the MHC-linked diabetogenic gene in the NOD mouse and man, these studies may provide data with more general implications for the role of the MHC in DM and other autoimmune diseases.

Summary

Class I and II major histocompatibility complex (MHC) probes can be used to subdivide diabetes-prone BB rats and their BBN control strain, coderived from the same outbred colony by selection against diabetes. Class II probes (A-alpha in particular) distinguish four restriction fragment length polymorphisms (RFLP), termed 1a, 1b, 2a, and 2b, in the BBN population, only one of which (2a) is found in BB rats. The degree of class II RFLP in the population studied is RT1.B-alpha ≥ RT1.B-beta > RT1.D-alpha ≥ RT1.D-beta, suggesting that intra–class II region dynamics may be different in rats compared with mice. A class I probe (S16) absolutely distinguished BB from BBN rats, since all BB rats exhibit an RFLP pattern termed 2a0, while 2a BBN rats can be subdivided into

2a1 and 2a2 forms. Serologic evaluation has shown that 2a0, 2a1, and 2a2 rats express RT1.A^uB^u, 1a rats express RT1.A^aD^a, and 1b rats express neither $RT1^a$ nor $RT1^u$ at the loci tested. A breeding study was carried out to determine the diabetogenicity of the MHC-defined RFLP's. As expected, the BB-derived 2a0 is diabetogenic. The BBN-derived 2a1 and 2a2 RFLPs are also diabetogenic, while 1a and 1b rats do not carry MHC-linked diabetogenic genes. The MHC-linked diabetes gene acts in a functionally recessive manner, since there is a 10-fold higher incidence in homozygotes than in heterozygotes. Analysis of the RFLP patterns leads us to hypothesize that the 2a1 RFLP results from a crossover between 1a and 2a0 MHCs and that the diabetogenic MHC-linked gene is on the class II side of Qa and Tl. The availability of three diabetogenic MHC haplotypes should help localize the MHC-linked diabetogenic gene of rats.

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