

Non-Major Histocompatibility Complex-Linked Diabetes Susceptibility Loci on Chromosomes 4 and 13 in a Backcross of the DP-BB/Wor Rat to the WF Rat

Anne-Marie Martin, Elizabeth P. Blankenhorn, Mark N. Maxson, Meng Zhao, Jean Leif, John P. Mordes, and Dale L. Greiner

BB rats are used as models of autoimmune human IDDM. Genetic control of IDDM in both species is complex, including both major histocompatibility complex (MHC)-linked and non-MHC-linked genes. DP-BB rats develop IDDM spontaneously. Expression of disease in these animals requires homozygosity at the *lyp* locus, which causes lymphopenia. All genetic analyses of BB rat diabetes to date have backcrossed to the DP-BB strain or used (DP-BB × non-BB)F₂ animals to ensure that a fraction of progeny are homozygous for *lyp*. Here we report the analysis of a backcross of the DP-BB rat to the histocompatible WF rat. Neither WF nor (WF × DP-BB)F₁ animals develop spontaneous IDDM. However, 95% of (WF × DP-BB)F₁ rats and a fraction of (WF × DP-BB) × WF backcross animals readily develop IDDM after treatment with polyinosinic:polycytidylic acid and a cytotoxic anti-RT6.1 monoclonal antibody. Using simple sequence length polymorphism analysis, we have mapped loci on chromosomes 4 and 13 that show significant linkage to IDDM expression and insulinitis. The susceptibility locus on chromosome 4 is linked to, but not identical to, *lyp*. We propose a disease model for the BB rat that requires 1) the RT1^u MHC haplotype for disease susceptibility, 2) a new locus on chromosome 4 for disease initiation (as measured by insulinitis), 3) a new locus on chromosome 13 for disease progression in response to environmental perturbation, and 4) *lyp* for spontaneous expression of disease. *Diabetes* 48:50–58, 1999

Human IDDM is familial, but its mode of inheritance is non-Mendelian and only partially understood (1–4). It is most clearly associated with certain permissive HLA haplotypes (5–7). The major histocompatibility complex (MHC)-associated locus is termed IDDM1. Additional data indicate the existence of a

non-MHC-associated locus (IDDM2) spanning the insulin gene on chromosome 11p15.5 (8). This region contains both the insulin gene and at least 10 polymorphisms in strong linkage disequilibrium. Results of a genome-wide search for human IDDM genes based on semiautomated fluorescence scanning have also been reported (1). In addition to confirming the large effect associated with IDDM1 and IDDM2, 18 distinct chromosome regions revealed some evidence of linkage to the disease. The data are strongly indicative of polygenic inheritance with a major locus at the MHC.

BB rats are used as models of IDDM. Approximately 90% of viral antibody-free (VAF) DP-BB rats develop spontaneous autoimmune hyperglycemia (9,10). Diabetes in the DP-BB rat is prevented by immunosuppression (9,11), thymectomy (12), and bone marrow allografts (13,14). Spleen cells from diabetic DP-BB rats adoptively transfer the disease (15–18). Insulinitis (19) and islet autoantibodies (20) are observed in all affected animals. Unlike humans with IDDM, DP-BB rats are lymphopenic and severely deficient in CD8⁺ and RT6⁺ T-cells (9). RT6 is a rat T-cell maturational alloantigen acquired post-thymically and expressed on regulatory T-cells (10).

Inheritance of diabetes in BB rats appears to involve at least one gene, designated *iddm2*, associated with the rat MHC. The BB rat expresses the class I RT1A^u, class II RT1B^u RT1D^u MHC haplotype (21). Expression of diabetes is independent of class I haplotype, but it requires the presence of at least one class II RT1^u allele (22–25). Intercross studies indicate that the RT1^u allele of the BB rat is not a unique diabetogenic variant allele, since RT1^u alleles derived from normal rat strains also confer susceptibility (22,26,27).

An autosomal recessive locus (termed *iddm1*) determining T-cell lymphopenia (*lyp*) is strongly associated with diabetes in DP-BB rats (28–30). This locus has been mapped to chromosome 4 (RNO4) (31,32). Neither the genetic basis for the T-cell lymphopenia in the DP-BB rat nor its specific role in the development of diabetes is known with certainty (9). Lymphopenia and diabetes susceptibility can be independently inherited traits (24,33), but deficiency in peripheral T-cells appears to be tightly linked with the expression of spontaneous IDDM in BB rats (28,29,34,35). For example, a congenic nonlymphopenic BB rat does not develop IDDM (36).

Evidence for the role of *lyp* in diabetes also comes from studies with DR-BB rats. This subline was developed from DP-BB forebears selected for normoglycemia. They are nonlymphopenic (i.e., *lyp*^{+/+}) and immunocompetent (9). Under VAF conditions, no DR-BB rats develop spontaneous autoimmunity (37), but IDDM can be induced by many interventions.

From the Department of Microbiology and Immunology (A.-M.M., E.P.B., M.N.M., M.Z.), Allegheny University of the Health Sciences, Philadelphia, Pennsylvania; and the Department of Medicine (J.L., J.P.M., D.L.G.), University of Massachusetts Medical School, Worcester, Massachusetts.

Address correspondence and reprint requests to Dr. John Mordes, Diabetes Division, 373 Plantation St., Biotech 2, Suite 218, Worcester, MA 01605. E-mail: john.mordes@ummed.edu.

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ANOVA, analysis of variance; cM, centimorgan; IL, interleukin; KRV, Kilham rat virus; LOD, logarithm of odds; LRS, likelihood ratio statistic; mAb, monoclonal antibody; MHC, major histocompatibility complex; PCR, polymerase chain reaction; poly I:C, polyinosinic:polycytidylic acid; QTL, quantitative trait locus; VAF, viral antibody-free.

These include low-dose irradiation, cyclophosphamide, high-dose polyinosinic:polycytidylic acid (poly I:C), viral infection, and in vivo depletion of RT6⁺ T-cells in combination with low-dose poly I:C (9). In early studies using crosses between DP-BB and DR-BB rats, diabetes segregated as a single autosomal recessive trait and was always accompanied by lymphopenia (29). In other studies, the association between diabetes and lymphopenia was confirmed, but not all lymphopenic animals developed diabetes (31).

Because spontaneous diabetes in the BB rat appears to depend on the presence of *iddm1*, it has been hypothesized that *lyp* itself might explain completely the genetic predisposition of these animals to diabetes (29,31). This hypothesis, however, does not address the possibility that autoreactive T-cells are generated, but not detected, in the absence of *lyp*. This possibility cannot be evaluated in backcrosses to the susceptible DP-BB rat, because disease expression is dependent on homozygosity for *lyp*. There is, however, evidence that other genes modify the development of diabetes in DP-BB-derived rats. In two separate reports, the presence of a resistance gene has been inferred from crosses between diabetic DP-BB and resistant nonlymphopenic non-BB rats. In one study, the RT1^u and *lyp* genes were placed on the ACI strain background (38). A total of 72 backcross animals were followed to 140 days of age in a clean facility. Among animals from the N4F1, N5F1, and N6F1 generations that were homozygous for RT1^u and *lyp*, none developed diabetes. The second study reported results from F₂ offspring of (DP × F344)F1 hybrids. The authors found six F2 rats homozygous for both RT1^u and *lyp* (31). Because none of these rats developed diabetes, the authors proposed that there was a third diabetes-modifying locus that conferred resistance to diabetes. They designated this locus *iddm3*. Proof of the existence of *iddm3* was sought by Klöting et al. (39), who discovered a candidate location for *iddm3* on chromosome 18.

These mapping results and the phenotype of the *lyp* gene have led us to hypothesize that in (DP-BB × non-DP)F1 hybrid rats, autoreactive T-cells are generated but do not lead to spontaneous disease in the absence of homozygosity for *lyp*. To test this hypothesis, we analyzed a backcross of (DP-BB × WF)F1 rats to the normal histocompatible WF rat. (WF × DP-BB)F1 rats do not develop spontaneous IDDM, but the disease is inducible in >95% of F1 animals after combined treatment with low-dose poly I:C and depletion of the regulatory RT6⁺ T-cell population (40). Our strategy was designed to facilitate the detection of non-MHC genes 1) by ensuring the presence of a uniform susceptible RT1^u haplotype in all animals and 2) by eliminating the requirement for homozygosity at *lyp* for disease detection. Using this strategy, we have mapped new loci on chromosomes 4 and 13 that show significant linkage to insulinitis and IDDM expression. The locus on chromosome 4 is linked to, but not identical to, *lyp*.

RESEARCH DESIGN AND METHODS

Animals. DP-BB/Wor rats from the BB subline were obtained from the VAF colony maintained at the University of Massachusetts Medical Center, Worcester, MA. Animals from this colony express the RT1^{u/u} MHC haplotype, express the RT6.1 T-cell alloantigen, and are certified to be serologically free of Sendai virus, pneumonia virus of mice, sialodacryoadenitis virus, rat corona virus, Kilham rat virus, H1 (Toolan's virus), GD7, Reo-3, *Mycoplasma pulmonis*, lymphocytic choriomeningitis virus, mouse adenovirus, Hantaan virus, and *encephalitozoon cuniculi*. About 90% of VAF DP-BB/Wor rats of the BB subline

develop spontaneous diabetes (37), and about 10% also develop lymphocytic thyroiditis that does not progress to frank hypothyroidism (41). WF rats (RT1^{u/u}) were purchased from Charles River Breeding Laboratories, Wilmington, MA. WF rats express the RT6.2 T-cell alloantigen (42). (DP-BB/Wor × WF)F1 and (DP-BB/Wor × WF) × WF backcross rats were bred in our facilities. T-cells of (DP-BB/Wor × WF)F1 animals coexpress both RT6.1 and RT6.2 antigens (42). All animals were maintained under VAF conditions in accordance with recommendations in the *Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, National Research Council, National Academy of Sciences, 1996) and the guidelines of the institutional animal care and use committee of the University of Massachusetts Medical Center.

Induction of diabetes. For the induction of autoimmune diabetes, all (WF × DP-BB) × WF backcross animals were screened for expression of the RT6.1 allotype. It was necessary to do so because of the lack of a depleting anti-RT6.2 monoclonal antibody (mAb) comparable to the depleting DS4.23 anti-RT6.1 mAb used in all prior analyses of diabetes induction in the DR-BB rat. Screening was done by flow microfluorometry as previously described (43). As expected, ~50% of the animals were RT6.1⁺ RT6.2⁺ heterozygotes (data not shown). At 30 days of age, the RT6.1⁺ RT6.2⁺ heterozygous backcross rats were treated with the cytotoxic DS4.23 anti-RT6.1 mAb (2 ml hybridoma supernatant 5 times/week) and poly I:C, a non-specific immune system activator (5 µg/g 3 times/week) as previously described (44). The combination regimen administered to VAF DR-BB rats induces IDDM in ~90% of animals (44). Treatment with both mAb and poly I:C was stopped when diabetes was diagnosed or after 30 days of treatment. All experimental animals were screened three times weekly for the presence of glycosuria (Tes-Tape; Eli Lilly, Indianapolis, IN). The presence of diabetes in glycosuric rats was established on the basis of a plasma glucose concentration >250 mg/dl (Glucose Analyzer 2; Beckman Instruments, Fullerton, CA).

Histological evaluation of insulinitis. After the diagnosis of diabetes or at the conclusion of the experiment, rats were killed in an atmosphere of 100% CO₂, and pancreases were removed and fixed in Bouin's solution. Paraffin-embedded sections of pancreas were prepared for histologic analysis and stained with hematoxylin and eosin. Tissues were scored by an evaluator who was not informed of the donor's glycemic status. Based on histological appearance, pancreatic insulinitis was scored as both a qualitative (present or absent) phenotype and a quantitative trait locus (QTL). Pancreases were graded on a scale of 0 to 4+ as follows: 0, no inflammatory mononuclear cell infiltration of any islets; 1+, islet mononuclear cell infiltration only at the periphery of the islet (peri-insulinitis); 2+, small numbers of mononuclear cells infiltrating into islets with preservation of islet architecture; 3+, large numbers of mononuclear cells within most islets affected and with some distortion of islet architecture; and 4+, florid infiltration of mononuclear cells or classical end-stage islets. Islets in at least three sections of pancreatic tissue were examined.

DNA samples. Genomic DNA was prepared using two protocols. First, snap-frozen livers were ground on dry ice, and the dispersed tissue was treated with Proteinase K in the presence of 10% sarkosyl and 0.5 mol/l EDTA (pH 8.0). DNA was purified from these digests by phenol-chloroform extraction and dialysis against Tris EDTA (0.01 mol/l Tris/0.001 mol/l EDTA, pH 7.4). Second, genomic DNA was extracted from rat tail snips using the QIAamp Tissue kit (Qiagen, Stanford, CA). DNA was extracted according to the manufacturer's instructions.

Microsatellite analysis. All microsatellite primers used in this study are available from Research Genetics (Huntsville, AL). The general map location of these microsatellites was taken from maps published by the Whitehead Institute (<http://www.genome.wi.mit.edu/rat/public/>) and by Drs. R. Wilder and E. Remmers (<http://www.nih.gov/niams/scientific/ratgbase/index.htm>). Primers were tested against a panel of parental genomic DNA samples to determine polymorphisms between DP-BB and WF rat strains. Those primers found to be polymorphic between parents were then used in a genome-wide screen of the backcross progeny. Primers were handled in one of two ways. In some cases, they were end-labeled using [³²P]ATP, used in a polymerase chain reaction (PCR), and resolved by PAGE. Alternatively, they were added directly to a PCR, and the product was resolved using agarose gel electrophoresis.

For radiolabeled primers, a standard kinase reaction was performed as previously described (45,46). The PCR mixture was prepared using a modification of a published protocol (46). DNA templates were added to a 96-well plate (TKR Biotech Products, Huntington Valley, PA) at a concentration of 120 ng/well. A 7-µl aliquot of the combined PCR and kinase mixture was added to each well containing the DNA template, followed by a drop of mineral oil per well. The 96-well plate was sealed with a lid, and the DNA templates were amplified in an Omnigene Thermocycler (Hybaid; TKR Biotech Products). The thermocycler was programmed for 35 cycles of 94°C for 1 min, 55°C for 2 min, and 72°C for 2 min, followed by one cycle of 72°C for 5 min. Reaction products were resolved by PAGE.

When unlabeled primers were to be used, a standard PCR reaction was performed; 120 ng of DNA was amplified in a 20- μ l PCR reaction as above. The final primer concentration was 30 nmol \cdot l⁻¹ \cdot μ l⁻¹, and the thermocycler was programmed for 50 cycles of the program above. PCR products were resolved on 3% agarose gels (Metaphor; FMC BioProducts, Rockland, ME). Sizes of the amplified products were estimated by comparison to a 100-base pair ladder (Pharmacia Biotech, Piscataway, NJ). Gels were stained using ethidium bromide and viewed by ultraviolet light.

Data analysis. The probability of linkage between marker loci and the presence of diabetes or insulinitis was detected using Map Manager QT software (Version b21; K. Manly, Roswell Park Memorial Cancer Institute, Buffalo, NY). Probabilities are expressed as logarithm of odds (LOD) ratio scores for qualitative traits and likelihood ratio statistics (LRSs) for quantitative traits. Probabilities were independently confirmed using analysis of variance (ANOVA) for QTL markers linked to insulinitis and using the nonparametric χ^2 statistic for the qualitative trait of diabetes. Thresholds for declaration of significance of linkage were calculated using a permutation program based on regression models developed by Churchill and Doerge (47). For $\alpha = 0.1$, the experiment-wise cut-off for the insulinitis trait is LR = 12.9, and for $\alpha = 0.05$, LR = 14.3 (48).

RESULTS

Study population and disease incidence. A total of 140 (DP \times WF)F1 \times WF animals were generated for this study. As expected, ~50% were RT6.1⁺. These animals were entered into the disease induction protocol and treated with anti-RT6.1 mAb and poly I:C. Of the 68 treated animals, 20 (29%) developed diabetes, and all 20 had histological evidence of insulinitis with a mean grade of 3.75. Pancreases from the 48 nondiabetic animals were collected, but 15 samples were technically unsatisfactory. Among the remaining 33 samples from nondiabetic animals, 18 (55%) exhibited insulinitis of grades 2, 3, or 4. The other 15 were either completely normal or evidenced only peri-insulinitis (grade 1). Both sexes were equally represented in each of these phenotypic categories (data not shown).

Genome mapping. Approximately 500 microsatellite primers were screened for their ability to detect polymorphisms between DP-BB and WF rats, and 164 polymorphic primers were chosen to cover the genome at an intermarker

distance of ~20 cM. In areas of interest, denser mapping was performed. The final mean distance between markers for all primers was 3.01 cM. The largest interval was 43 cM on chromosome 17. The rate of polymorphism was observed to be ~35%. Because only those animals that expressed the RT6.1 alloantigen could be entered into the induction protocol, allelic markers linked to the RT6 locus on chromosome 1 did not segregate and could not be analyzed. A map was constructed using these primers to determine or verify the position of each primer in the rat genome and to provide map locations of diabetes-modifying loci in the backcross cohort. In most cases, the map position of the primers in our analysis correlated closely with positions published by Jacob et al. (46) (<http://www.genome.wi.mit.edu/rat/public/>), Wilder and Remmers (<http://www.nih.gov/niams/scientific/ratgbase/index.htm>), and Bihoreau et al. (49) (<http://www.well.ox.ac.uk/pub/genetics/ratmap>).

Genes linked to diabetes expression. The subset of primers found to show linkage with diabetes expression and presence of insulinitis is shown in Table 1. Expression of diabetes in backcross rats was linked to three regions of the genome. The strongest linkage mapped to an ~5 cM interval on chromosome 4 ($\chi^2 = 10.4$, df = 1, $P = 0.0012$, Table 1). This region is ~45 cM from the top of chromosome 4, in an interval bordered by D4Wox10 and D4Wox22 (*NPY*). We have tentatively designated the diabetes-modifying locus on chromosome 4 as *iddm4*. It can be seen that the LOD scores for markers in this region are relatively low. This is because LOD scores are based on the total number of recombinants, whereas 2 \times 2 contingency scores consider the two individual recombinant classes (*iddm4*^{d/w}-nondiabetic and *iddm4*^{d/w}-diabetic) separately. The lower LOD scores reflect the fact that not all *iddm4*^{d/w} rats became diabetic, because *iddm4*^{d/w} is not fully penetrant for diabetes in the absence of a second *iddm* locus in this cross. Consistent with this interpretation, a second strong linkage mapped to an ~5 cM inter-

TABLE 1
Loci on chromosomes 4, 13, and 3 linked to diabetes and insulinitis in (WF \times DP-BB) \times WF rats

Chromosome	QTL in interval	cM*	Marker	Diabetes LOD score	Diabetes χ^2	<i>P</i> value	Insulinitis LOD score	Insulinitis χ^2	<i>P</i> value	Origin†	
4	<i>iddm4</i>	32	D4Wox10	NS	5.2	0.022	2.2	9.9	0.0016	DP	
4			D4Arb11	NS	7.2	0.0072	3.1	11.3	0.0008	DP	
4			D4Mgh16	NS	7.2	0.0072	3.1	11.3	0.0008	DP	
4		39	D4Mgh24	1.0	10.4	0.0012	3.1	11.3	0.0008	DP	
4			D4Arb29	1.0	10.4	0.0012	3.6	12.8	0.0003	DP	
4			42	D4Mit5	1.0	10.4	0.0012	3.6	12.8	0.0003	DP
4			42	D4Arb9	1.0	10.4	0.0012	3.6	12.8	0.0003	DP
4			42	D4Mit3	1.0	10.4	0.0012	3.6	12.8	0.0003	DP
4			44	D4Arb30	1.0	10.4	0.0012	3.6	12.8	0.0003	DP
4			46	D4Wox22 (<i>NPY</i>)	NS	8.0	0.0046	2.2	7.5	0.0063	DP
13	<i>iddm5</i>	5	D13Mit1	2.2	8.7	0.0032	NS	0.16	0.898	DP	
13			D13Mgh1	2.2	8.7	0.0032	NS	0.16	0.898	DP	
13			9	D13Mgh2	1.7	7.1	0.010	NS	0.4	0.816	DP
13		10	D13Mgh3	1.6	6.8	0.0335	NS	0.5	0.797	DP	
3	<i>iddm6</i>	80	D3Mit4	NS	1.7	0.1975	NS	5.5	0.02	WF	
3			96	D3Mgh10	1.3	3.4	0.663	NS	0.1	0.8104	WF
3			98	D3Mgh1	1.3	3.4	0.663	NS	0.1	0.8104	WF

LOD scores were calculated by Map Manager QT. χ^2 was calculated from 2 \times 2 contingency tables. *P* values of linkage are associated with χ^2 . Origin is the strain of origin of susceptible allele. *cM from top of the chromosome map; †strain of origin of susceptible allele.

TABLE 2
Quantitative trait loci linked to severity of insulinitis in (WF × DP-BB) × WF rats

Chromosome	QTL in interval	cM*	Marker	Insulinitis LRS	% Variance	ANOVA <i>P</i> value	Origit
4	<i>iddm4</i>	32	D4Wox10	7.7	12	0.0066	DP
4		39	D4Mgh24	14.9	23	0.0002	DP
4		42	D4Mit5	14.9	23	0.0002	DP
4		42	D4Arb9	14.9	23	0.0002	DP
4		42	D4Mit3	14.9	23	0.0002	DP
4		44	D4Arb30	14.9	23	0.0002	DP
4		46	D4Wox22 (<i>NPY</i>)	6.4	10	0.014	DP
13	<i>iddm5</i>	5	D13Mit1	NS		0.1673	DP
13		5	D13Mgh1	NS		0.1673	DP
13		9	D13Mgh2	NS		0.3142	DP
13		10	D13Mgh3	NS		0.3757	DP
3	<i>iddm6</i>	80	D3Mit4	9.1	14	0.0033	WF
3		85	D3D1Mgh18(Cst3)	5.0	7	0.0236	WF
3		96	D3Mgh10	NS		0.1753	WF
3		98	D3Mgh1	NS		0.1753	WF

LRS comes from from Map Manager QT analysis ($4.6 \times \text{LOD}$). The percent variance is that due to susceptible alleles at this marker as calculated by Map Manager QT. *P* values are of linkage as assessed by independently calculated ANOVA. *cM from the top of the chromosome map; †strain of origin of susceptible allele.

val on proximal chromosome 13. Statistical significance associated with the linkages in this interval varied slightly ($P = 0.0018$ to $P = 0.0032$; LOD scores 1.9–2.4, Table 1). We have tentatively designated the diabetes-modifying locus on chromosome 13 as *iddm5*. The diabetes-associated haplotypes of *iddm4* and *iddm5* are derived from the DP parent and are fully expressed in heterozygotes.

The third locus was less robust, mapping to a broad 18 cM interval on chromosome 3. The strongest linkage was associated with markers D3Mgh10 and D3Mgh1 (LOD score = 1.3, Table 1) and has been given the tentative designation *iddm6*. The diabetogenic allele of *iddm6* is derived from the WF parent.

Genetic control of the insulinitis phenotype. Pancreatic insulinitis was analyzed as both a qualitative (present or absent) phenotype and as a QTL. Genetic linkage of insulinitis as a qualitative trait was observed for markers on chromosome 4 in the interval bordered by D4Mgh24 and D4Arb30. All markers in this interval showed significant linkage to insulinitis (LOD score = 3.1–3.6, $P < 0.0008$, Table 1). The 95% CI for these diabetes-modifying loci extends from D4Wox10 ($P = 0.0016$) to neuropeptide Y (D4Wox22, $P = 0.0063$). This interval includes the trypsinogen gene (D4Arb9) and the carboxypeptidase A gene (D4Mit3) and entirely overlaps the interval that defines *iddm4*. Among rats with histological evidence of insulinitis (grade 2+ or greater), 30 of 38 (79%) had an allele of D4Mit3 from the DP parent ($P = 0.0003$ when compared with allele frequency in the whole cross). The average insulinitis score for *iddm4*^{di/w} heterozygotes was 2.9 and that for *iddm4*^{di/w} homozygotes was 1.4 ($P = 0.0002$). When analyzed as a qualitative trait, it is noteworthy that insulinitis showed no statistically significant linkage to *iddm5*, and its linkage to *iddm6* was marginal.

The insulinitis phenotype was also evaluated as a quantitative trait, and, as expected from the qualitative analysis, severity of insulinitis segregated with markers defining *iddm4* (Table 2). This association achieved statistically significant linkage (LRS = 14.9) in the region proximal to the *NPY*

genetic marker. In addition, linkage of the severity of insulinitis was found with markers on chromosome 3 (e.g., D3Mit4, $P = 0.0033$), but this linkage did not achieve an LRS above the $\alpha = 0.1$ cutoff. Because the interval containing *iddm6* is broad, it is difficult to determine whether the effect is due to the same locus that had some association with diabetes (Table 1) or to a different locus. Again, *iddm5* appears to play no role in determining the severity of insulinitis as evaluated by QTL analysis.

Genetic control of the progression of insulinitis to frank diabetes. The observation that the *iddm5* locus was associated with diabetes, but not with either measure of insulinitis, led us to hypothesize that it might act by facilitating the progression of disease. To test this hypothesis, the subset of all animals with documented insulinitis was analyzed separately. Results, which are shown in Table 3, indicate that the presence of *iddm5*^{di/w} is highly correlated with the eventual expression of diabetes among rats with insulinitis (LOD scores of 1.9–2.4). Only 3 of 17 rats with insulinitis and *iddm5*^{di/w} failed to progress to hyperglycemia. The *iddm6*-linked markers D3Mgh1 and D3Mgh10 demonstrated weaker linkage to the expression of diabetes among rats with insulinitis (LOD score = 1.1, $\chi^2 P$ value = 0.014). *Iddm4* displayed no linkage to diabetes in this subset.

When combinations of alleles at *iddm4* and *iddm5* are examined in backcross progeny, a pronounced skewing is seen in the insulinitis-positive rats. *Iddm4* and *iddm5* alleles from the DP parent are seen together more frequently in animals with evidence of insulinitis than predicted from their respective allele frequencies in the whole population ($\chi^2 P$ value = 0.009). All 20 diabetic animals had inherited either D4Mit3 or D13Mgh1 from the DP parent, and 12 rats had inherited both ($P < 0.0001$).

DISCUSSION

The goal of this study was to identify new susceptibility and resistance alleles that control the predisposition to, and progression of, autoimmune diabetes in the BB rat. We

TABLE 3
Linkage of *iddm4*, *iddm5*, and *iddm6* to diabetes in (WF × DP-BB) × WF rats with insulinitis

Chromosome	QTL in interval	cM*	Marker	Diabetes LOD score	Diabetes χ^2	P value	Origit
4	<i>iddm4</i>	32	D4Wox10	NS			
4		39	D4Mgh24	NS			
4		42	D4Mit5	NS			
4		42	D4Arb9	NS			
4		42	D4Mit3	NS			
4		44	D4Arb30	NS			
4		46	D4Wox22 (<i>NPY</i>)	NS			
13	<i>iddm5</i>	5	D13Mit1	2.4	10.9	0.0010	DP
13		5	D13Mgh1	2.4	10.9	0.0010	DP
13		9	D13Mgh2	2.0	9.0	0.0027	DP
13		10	D13Mgh3	1.9	8.7	0.0032	DP
3	<i>iddm6</i>	80	D3Mit4	NS		NS	WF
3		96	D3Mgh10	1.1	6.0	0.014	WF
3		98	D3Mgh1	1.1	6.0	0.014	WF

LOD scores were calculated by Map Manager QT. χ^2 was calculated from 2×2 contingency tables. P values are of linkage associated with χ^2 . *cM from the top of the chromosome map; †strain of origin of susceptible allele.

analyzed a backcross of the DP-BB rat to the histocompatible immunologically normal WF strain and used an environmental perturbant to unmask susceptibility in the absence of congenital lymphopenia. Using simple sequence length polymorphism analysis, we mapped loci on chromosomes 4 (*iddm4*) and 13 (*iddm5*) that show significant or suggestive linkage to IDDM in animals treated with poly I:C and depleted of RT6.1⁺ regulatory T-cells. We further observed that the *iddm5* locus is associated with diabetes, but not with insulinitis. In contrast, *iddm4*^{d/w} is significantly associated with insulinitis. These observations led us to test the hypothesis that *iddm5*^{d/w} facilitates the progression of disease, and consistent with that hypothesis, we found that *iddm5*^{d/w} is strongly correlated with the eventual expression of diabetes among rats with insulinitis.

Based on these findings and previous observations, we propose a disease model for the BB rat. As is also true of IDDM in humans and NOD mice, the first genetic component maps to the MHC, which, in the case of the BB rat, is the RT1^u haplotype. In addition, we now propose that a new locus on chromosome 4 (*iddm4*) is responsible for disease initiation and insulinitis, the pathological substrate that precedes and then accompanies the development of hyperglycemia. We also propose that a new locus on chromosome 13 (*iddm5*) participates in disease progression. In the backcross system used in these studies, *iddm5* facilitates progression in response to environmental perturbation (poly I:C and anti-RT6.1 mAb). In the special case of the DP-BB rat, we propose that the *lyp* gene causes lymphopenia, unmasking the latent predisposition to autoreactivity and disease progression associated with *iddm4* and *iddm5*. We would predict that rats with the RT1^u class II MHC haplotype will spontaneously develop autoimmune diabetes in the presence of *lyp*^{d/d}, *iddm4*^{d/-}, and *iddm5*^{d/-}.

***Iddm4* and *lyp* are different.** Our data prove that homozygosity for *lyp* is not required for the predisposition to autoimmune diabetes. No animals in our backcross could be homozygous for *lyp*. The present data do leave open the question as to whether heterozygosity for *lyp* could account for our results, that is to say, that *iddm4* is *lyp*. We believe

this is unlikely for several reasons. First, BB rats heterozygous for *lyp* never exhibit insulinitis (29,35), whereas in our analysis, *iddm4*^{d/w} is strongly associated with the presence of insulinitis. It is difficult to ascribe a diabetogenic role to heterozygous *lyp* in the absence of this phenotype in rats that have a full complement of diabetogenic loci, i.e., (DP-BB × DR-BB)F1 hybrids. It remains likely, however, that the spontaneous occurrence of insulinitis requires homozygosity for the *lyp* gene (29).

Second, the most likely position for *iddm4* is proximal to *lyp*. Because *lyp* is recessive, the distinction between *lyp*^{d/w} and *lyp*^{w/w} can be inferred only from linked markers, such as *NPY*. There is strong evidence that the *NPY* locus is a good marker for *lyp*. Among 370 (DP-BB × non-BB) backcross animals reported by Awata et al. (35), for example, all lymphopenic rats were homozygous for a BB rat-derived allele at the *NPY* marker; in contrast, all nonlymphopenic rats were heterozygous for this allele. Both Awata et al. (35) and Jacob et al. (31) have observed that D4Mit5, statistically the strongest surrogate for *iddm4* in our study, maps ~2 cM proximal to *lyp*/*NPY*, which is in agreement with our results. In addition, using additional crosses, we have mapped an intervening marker (D4Arb30) between D4Mit5 and *NPY*, and its linkage to the insulinitis phenotype was intermediate between those associated with D4Mit5 and *NPY* (data not shown). These observations suggest to us that *iddm4* and *lyp* are unlikely to be identical. Very recent mapping data (32) also support the inference that *lyp* maps to a location telomeric to *iddm4*.

Finally, there are in vivo observations that argue against any role for *lyp* in predisposing to autoimmunity. It is known, for example, that the *lyp* gene is completely normal in the DR-BB rat. In the Worcester colony, lymphopenia has not been detected in over 60 generations of inbreeding, and spontaneous autoimmunity has never been observed among animals housed under VAF conditions, confirming that the *lyp* mutation does not exist in this subline of the BB rat. In addition, as noted above, (DP-BB × DR-BB)F1 rats, which are obligate *lyp* heterozygotes, are free of both insulinitis and diabetes. However, the DR-BB rat does harbor autoreactive cells that can be detected either by treatment of animals with anti-RT6.1 mAb

and poly I:C or by transfer of spleen cells to histocompatible nude recipients (18). We suggest that DR-BB rats have a diabetogenic allele of *iddm4*, which, together with the RT1^u MHC haplotype, is responsible for the predisposition to diabetes in this rat strain. We suggest further that the action of *iddm4* is revealed when the rats undergo depletion of regulatory T-cells. This hypothesis can be tested directly, and we have, in fact, induced diabetes in both DR-BB (18) and (DR-BB × WF)F1 hybrid rats (unpublished observations).

In their aggregate, these observations suggest 1) that *lyp* homozygosity generates a specific lymphopenic environment permissive to the expression of spontaneous autoimmune disease, but 2) that *lyp* is not responsible in either the homozygous or heterozygous state for the fundamental latent predisposition to autoimmunity of the BB rat.

Spontaneous and induced IDDM. It could be argued that the genetic loci that we have mapped are not relevant to the expression of autoimmunity because of their dependence on perturbation with poly I:C and depletion of regulatory cells. We would point out, however, that the perturbants do not generate autoreactivity, but rather facilitate its detection. In addition, IDDM is generally believed to reflect the interaction of a genetic predisposition with environmental determinants (50–53). There are many examples of the generation of autoimmunity in nonautoimmune backgrounds. Both Penhale et al. (54,55) and Mason et al. (56,57) have reported the induction of autoimmune diabetes in the normal PVG rat after radiation and thymectomy. Our laboratory has also reported the adoptive transfer of diabetes using spleen cells from RT6-depleted (but nondiabetic) YOS and PVG rats (18) and the induction of diabetes in PVG rats treated with both poly I:C and anti-RT6.1 mAb (58). Interestingly, in all of these reports, the induction of autoimmune diabetes was facilitated by the use of RT1^u congenic strains of the PVG rat. In their aggregate, these observations suggest that the predisposition to autoimmune disease may be common, but that its expression depends on an appropriate concatenation of MHC haplotype, additional susceptibility genes (e.g., *lyp*), and environmental perturbation.

The model used in the present study does not depend on lymphopenia, as in the DP-BB rat, or on a unique class II haplotype, as in the NOD mouse. In many ways, the perturbation we used mimics the effects of viral infection. The first component of our treatment protocol, poly I:C, is a synthetic double-stranded polyribonucleotide (59). Presumably because of structural resemblance to double-stranded viral RNA, poly I:C elicits immune responses that mimic viral infection. These include stimulation of type I interferon production by various cells (60), interleukin (IL)-1 production by monocytes (61), and activation of NK cells (62), B-cells (63), and endothelial cells (64). Treatment of DR-BB rats with low doses of poly I:C (5 µg/g) induces diabetes in ~20% of animals (44), a rate similar to that observed when DR-BB rats are infected with Kilham rat virus (KRV) (65). Higher doses of poly I:C (10 µg/g) induce diabetes in nearly all DR-BB rats (66).

The second element of our protocol, depletion of RT6.1⁺ T-cells, does not induce IDDM in VAF DR-BB rats, but it does induce disease in seropositive animals that have experienced infection with KRV (44). For studies of IDDM induction in VAF animals, we substituted low-dose poly I:C for KRV infection; this form of combined immune system perturbation induces

diabetes in >95% of DR-BB rats (44). Note that neither KRV infection nor treatment with anti-RT6.1 mAb causes pan T-cell lymphopenia or immunoincompetence (9,44,67). We hypothesize that treatment with anti-RT6.1 mAb depletes a population of regulatory cells that normally play a role in preventing the expression of autoimmune diabetes (10).

Syntenic genes. Two of the loci detected in our cross, *iddm5* and *iddm6*, have syntenic homologs identified in previous studies of mouse and human diabetes susceptibility (Fig. 1). Rat *iddm5*, located on proximal chromosome 13, is syntenic to distal mouse chromosome 1 and human chromosome 18q. In all three species, the Bcl-2 gene is found in this region. Bcl-2 is an intriguing candidate gene (68), as it could act to extend the life span of inflammatory T-cells already in the pancreas (69). Importantly, this syntenic group also contains known diabetes-modifying loci: both human IDDM6 (70) and mouse *Idd5* (71) map here (Fig. 1). *Idd5* is located in a broad interval on mouse chromosome 1 (72–75). The distribution of IDDM QTLs from both human and rat studies to two different syntenic groups in these species indicates that the mouse *idd5* interval may actually contain two separate *Idd* loci. On mouse chromosome 2, the *Idd13* locus is syntenic to the rat *iddm6* locus found on distal rat chromosome 3. The rat *iddm6* locus is associated with both insulinitis and progression to diabetes and is located within a broadly defined interval. It is therefore possible that two rat diabetes-modifying loci exist within this region. If so, it is interesting that a recent report proposes that a mouse diabetes-progression locus resides in or near *Idd13* (76). It maps to an interval containing the β₂-microglobulin, IL-1α, and IL-1β genes, syntenic to *iddm6* in our rat cross. Variants of the β₂-microglobulin locus have recently been associated with diabetes in the NOD mouse (77).

The insulinitis-associated QTL that we have designated *iddm4* is in an interval that is syntenic with human chromosome 7 and mouse chromosome 6. This is a strongly conserved region that contains the T-cell receptor β-chain locus, trypsinogen, *NPY*, and carboxypeptidase A. No linkage with diabetes in these regions has been reported in human studies, although an *Idd* locus was noted to segregate with *D6Nds1* on mouse chromosome 6 (75). However, the locus on rat chromosome 4 maps to the same general area as the predisposition to collagen-induced arthritis (78), and it is associated with severity of experimental allergic encephalomyelitis in LEW rats (79) (A.-M.M., E.P.B., unpublished observations).

Limitations of this analysis. We recognize that the analysis we have performed has depended on a relatively small number of backcross animals. We have identified two very strong diabetes-associated loci. The fact that we observed 4 of the 20 cases of diabetes in the absence of *iddm5* suggests that other progression loci remain to be found. The weaker associations, like *iddm6*, that we have reported remain to be confirmed, and others may have been missed. This is particularly true for proximal regions of chromosomes 17, 18, 19, and X, for which few polymorphic primers were available at the time of these studies, and for chromosome 1, which was fixed by the design requirement for RT6.1⁺ rats. In future studies, it will be necessary to determine whether or not treatment of susceptible backcross animals homozygous for RT6.2 with a nondepleting anti-RT6.2 reagent will lead to the expression of autoimmunity.

In addition, we cannot confirm the identity of *iddm3* on chromosome 18 (80) because it is likely to be a dominant

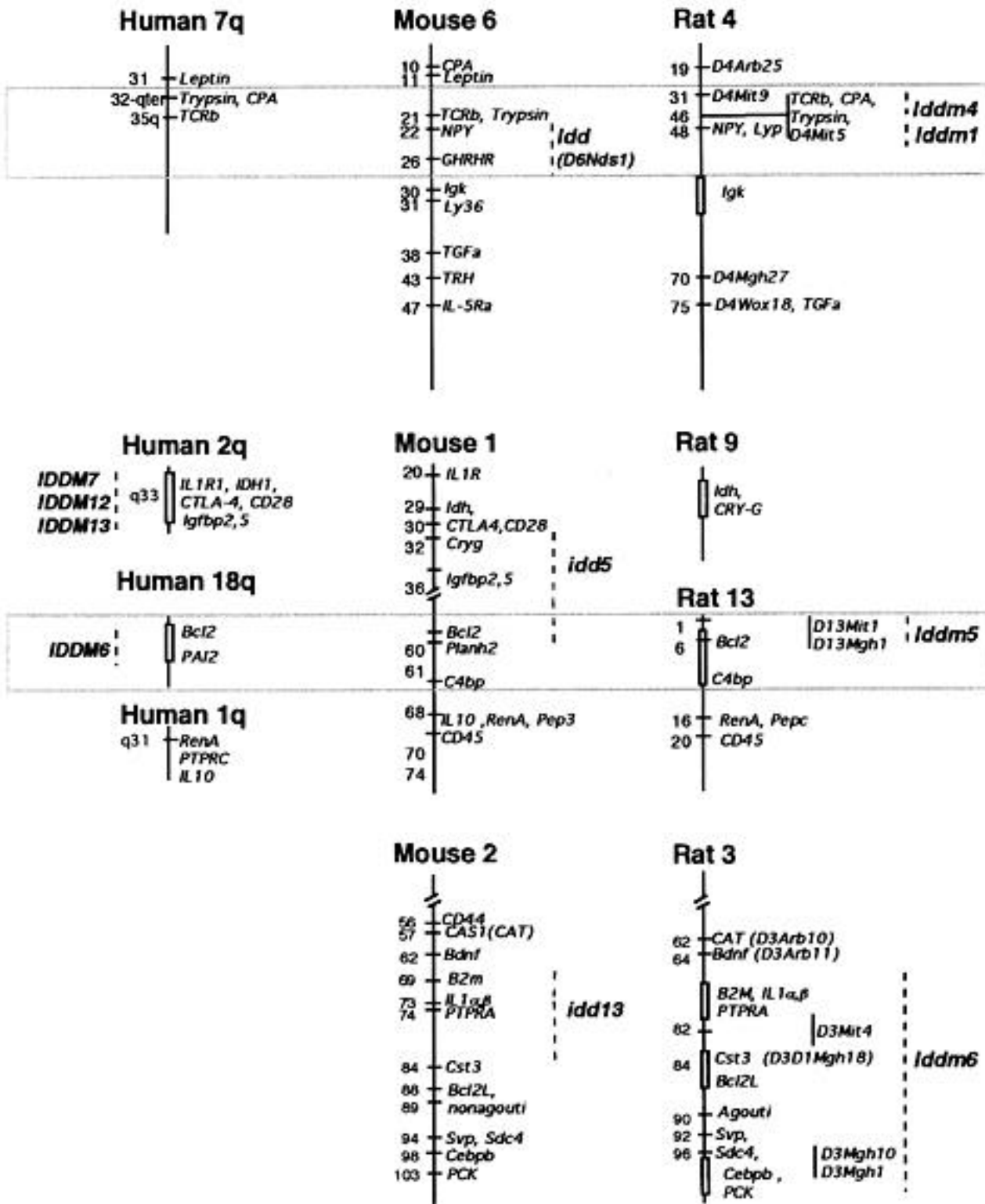


FIG. 1. Mouse and, where known, human syntenic groups are shown for each of the rat diabetes-modifying loci identified in this paper (73). For example, *iddm4* (top panel) on rat chromosome 4 is syntenic to conserved regions containing *NPY*, *trypsin*, and the T-cell receptor β -chain (*Tcrb*) genes in both human and mouse chromosomes. In addition, an *Idd* locus was noted in this region on mouse chromosome 6, near *D6Nds1* (75). In the case of rat *iddm5*, both human chromosome 18 and mouse chromosome 1 contain diabetes-modifying loci in conserved syntenic regions (middle panel). For *iddm6*, the conserved syntenic region in mice contains another diabetes-modifying locus, *Idd13* (bottom panel); there is no similar corresponding region in the human genome. Locations of typed microsatellite and genetic markers in the rat are shown with their approximate map position by solid vertical lines within the interval (dashed lines) of the *iddm* locus. Where the precise location of genes is not known, shaded boxes show their approximate orientation on the chromosome.

resistance gene donated by a resistant parent. However, an advantage to our approach is that we could map and study dominant or codominant diabetes-promoting loci from the DP-BB rat. In addition, we found it very useful to measure insulinitis as both a quantitative and qualitative parameter; this approach allows both the identification of new genes and the appreciation of the stages in disease pathogenesis during which they play an important role.

Summary and future directions. We propose a disease initiation (insulinitis) model for the BB rat that requires the RT1^u MHC haplotype and a newly identified locus on chromosome 4. We further propose that a newly identified locus on chromosome 13 is required for disease progression in response to environmental perturbation, whereas, in the DP-BB rat, *Iyp* is required for spontaneous expression of disease. The available data suggest that, in addition to the MHC, a relatively small number of major genes may account for the predisposition to, and progression of, IDDM in the BB rat. Creation of speed congenic animals may enable us to identify these genes. Finally, the availability of an inducible model of autoimmune diabetes suggests that it may be possible to perform genetic analyses of environmental perturbants, like viral infection, that are relevant to human IDDM.

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Author Queries (please see Q in margin and underlined text)

Q1a: Please check address. Last part of address is inconsistent on pgs. 2 & 3.

Q1: Please provide location of Roswell Park?

Q2: Tables 2 and 3 have lowercase i for iddm4, iddm5, iddm6; Table 1 has uppercase I for Iddm4, Iddm5, Iddm6. Should all be lowercase?

Q3: Table 3. First row, under LOD score. What is the superscript 6 for?

Q4: Edit of sentence beginning "As is also true..." OK?

Q5: Please list all authors of unpublished observations.

Q6: *idd5* or *Idd5*?

Q7: What is EAE?

Q8: Please list all authors of unpublished observations.

Q9: Text has lowercase i for iddm4, iddm5, iddm6; Figure legend has uppercase I for Iddm4, Iddm5, Iddm6. Should all be lowercase?