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The *iddm4* Locus Segregates With Diabetes Susceptibility in Congenic WF.*iddm4* Rats

John P. Mordes[1], **Jean Leif**[1], **Stephen Novak**[2], **Cheryl DeScipio**[2], **Dale L. Greiner**[1], and **Elizabeth P. Blankenhorn**[2]

[1]Department of Medicine, University of Massachusetts Medical School, Worcester, Massachusetts

[2]Department of Microbiology and Immunology, MCP Hahnemann University, Philadelphia, Pennsylvania.

Abstract

Viral antibody–free BBDR and WF rats never develop spontaneous diabetes. BBDR rats, however, develop autoimmune diabetes after perturbation of the immune system, e.g., by viral infection. We previously identified a disease-susceptibility locus in the BBDR rat, *iddm4*, which is associated with the development of autoimmune diabetes after treatment with polyinosinic:polycytidylic acid and an antibody that depletes $ART2^+$ regulatory cells. We have now developed lines of congenic WF.*iddm4* rats and report that in an intercross of N5 generation WF.*iddm4* rats, ~70% of animals either homozygous or heterozygous for the BBDR origin allele of *iddm4* became hyperglycemic after treatment to induce diabetes. Fewer than 20% of rats expressing the WF origin allele of *iddm4* became diabetic. Testing the progeny of various recombinant N5 WF.*iddm4* congenic rats for susceptibility to diabetes suggests that *iddm4* is centered on a small segment of chromosome 4 bounded by the proximal marker *D4Rat135* and the distal marker *D4Got51*, an interval of <2.8 cM. The allele at *iddm4* has 79% sensitivity and 80% specificity in prediction of diabetes in rats that are segregating for this locus. These characteristics suggest that *iddm4* is one of the most powerful non–major histocompatibility complex determinants of susceptibility to autoimmune diabetes described to date.

Type 1A autoimmune diabetes is heritable, but the mode of inheritance is complex and non-Mendelian (1). Intensive analysis has identified several high-risk class II major histocompatibility complex (MHC) haplotypes associated with the disease, but none are necessary or sufficient for diabetes expression. More than 16 additional non-MHC loci have also been linked to the disease, but the analysis of such a complex trait in outbred populations is extremely difficult (2). The relevant genes and their mechanism of action have not been identified.

The inbred NOD mouse also develops a heritable type 1A–like diabetic syndrome that affects up to 90% of female mice. As in humans, diabetes in NOD mice is associated with a

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

high-risk class II MHC haplotype (I-A^{g7}) and at least 17 non-MHC loci, but only one gene (β 2 microglobulin) has been identified with absolute certainty (3).

The genetic control of type 1A diabetes in the BB rat model has been studied less extensively, but new data suggest that it may now provide important advantages for the analysis of this disorder (4). Approximately 90% of inbred viral antibody–free (VAF) diabetes-prone BBDP rats develop spontaneous autoimmune hyperglycemia (5,6). Unlike humans with type 1 diabetes, however, BBDP rats are lymphopenic. In contrast, the inbred diabetes-resistant BBDR rat was developed from diabetes-prone forebears selected for normoglycemia (4) and is nonlymphopenic and immunocompetent (5). Under VAF conditions, no BBDR rats develop autoimmunity spontaneously (7), but type 1A–like diabetes can be induced by several interventions. These include low-dose irradiation, cyclophosphamide, high-dose polyinosinic:polycytidylic acid (poly I:C), viral infection, and in vivo depletion of $ART2^+$ regulatory cells in combination with low-dose poly I:C (5). Arguably, the BBDR rat models type 1A diabetes in humans with considerable fidelity; the predisposition to disease is clearly heritable but requires the kind of interaction with the environment that is often believed to be critical for disease expression in humans (8,9).

As is the case for human type 1A diabetes, inheritance of disease in BB rats is associated with a permissive MHC locus, designated *iddm2* in the rat. The BB rat expresses the class I $RT1A^u/RT1C^u$, class II $RT1B/D^u$ MHC haplotype (10). Expression of diabetes is independent of class I haplotype but requires the presence of at least one class II $RT1B/D^u$ allele (11-15). The $RT1^u$ allele of the BB rat does not appear to be a unique diabetogenic variant (11), and diabetes can be induced by immunological perturbation in several class II $RT1^u$ rat strains (16).

The autosomal recessive locus *lyp (*or *iddm1)* causes T-cell lymphopenia in BBDP rats (17-19). The *lyp/iddm1* locus has been mapped to chromosome 4 (RNO4) (19,20). Multiple genetic crosses have shown that deficiency in peripheral T-cells is necessary, but not sufficient, for the expression of spontaneous type 1 diabetes in BB rats (12,17-19,21). Studies of (BBDR × WF) × WF backcross animals, all of which are $lyp^{+/+}$, have demonstrated that *lyp* is not required for the latent predisposition to autoimmune diabetes in BBDR rats that develop disease in response to appropriate immunomodulatory and environmental perturbants (22). The *lyp/iddm1* gene has recently been identified as *Ian4*, a mitochondrial membrane protein (23).

Additional susceptibility loci have been mapped in backcrosses of (BBDP × WF)F1 and (BBDR × WF)F1 rats to the WF rat. Neither F1 rat develops spontaneous diabetes, but the disease was inducible in >95% of F1 animals after combined treatment with low-dose poly I:C and depletion of the regulatory $ART2^+$ cell population (24). In both backcrosses, we mapped a locus on chromosome 4 (*iddm4*) with significant linkage to insulitis and type 1A–like diabetes expression. The *iddm4* locus is linked to, but different from, *lyp/iddm1/Ian4*. It has been mapped in two separate (DP × WF)F1 × WF backcrosses, and identity-by-descent analysis suggested that the *iddm4* locus is likely to play a role in disease initiation in both BBDP and BBDR rats (22).

Using a marker-assisted breeding strategy, we have now created WF.*iddm4* congenic rat lines as a first step toward identifying the *iddm4* gene and to confirm our hypothesis that *iddm4* participates in generating the latent autoreactivity that is revealed when animals are exposed to appropriate environmental perturbation. We also generated a control WF.*ART2*^{a} congenic rat, which, unlike the standard *ART2*^{b} WF rat, can be depleted of ART2^{+} regulatory cells using available reagents. We now report that diabetes segregates in a genetically dominant manner with *iddm4* in an N5 intercross of WF.*iddm4* congenic rats and that thymocytes from WF.*iddm4*$^{d/-}$ rats transfer diabetes to adoptive recipients with high efficiency. Finally, in a progeny testing analysis of N6 WF.*iddm4* congenic rats, we have established likely boundaries for the *iddm4* interval on chromosome 4.

RESEARCH DESIGN AND METHODS

1. Animals

BBDR/Wor rats (*RT1*$^{u/u}$, *ART2*^{a}) were obtained from a VAF colony originally maintained at the University of Massachusetts Medical School and now at BRM (Worcester, MA). Wistar Furth (WF) rats (*RT1*$^{u/u}$, *ART2*^{b}) were purchased from Harlan Sprague Dawley (Indianapolis, IN). (BBDR/Wor × WF) × WF animals were bred in our facilities, as described previously (22), and backcrossed repetitively to generate WF.*iddm4* congenic lines, as described below. (BBDR/Wor × WF) × WF animals were also backcrossed repetitively to generate a WF.*ART2*^{a} congenic line, as described below. Athymic WAG *rnu/rnu* (*RT1*$^{u/u}$) rats were obtained from BRM. Animals in these studies were maintained under VAF conditions, tested monthly, and consistently confirmed to be serologically free of Sendai virus, pneumonia virus of mice, sialodacryoadenitis virus, rat corona virus, Kilham's rat virus, H-1 (Toolan's virus), GD7, Reo-3, *Mycoplasma pulmonis*, lymphocytic choriomeningitis virus, mouse adenovirus, Hantaan virus, and *encephalitozöon cuniculi*. All animals were maintained 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 School.

2. Marker-assisted congenic rat production

Congenic rats were bred using a marker-assisted selection protocol (25). Rats from each backcross generation were treated as follows. DNA samples from all progeny were prepared from tail snips and screened using microsatellite markers that define the *iddm4* and *ART2* intervals plus a selected panel of 194 additional microsatellite loci chosen to be distributed as evenly as possible over all the autosomes. Typed rats found suitable for breeding were mated with diabetes-resistant rats to create the next generation. Initially, the diabetes-resistant rats were standard *ART2*^{b} WF rats, and later in the breeding program they were WF.*ART2*^{a} congenic rats (see below). Rats for breeding were selected according to the following criteria: *1*) they were *ART2*$^{a/b}$ (so their progeny could be depleted with anti-ART2.1 antibody); *2*) they had DR-derived alleles for the *iddm4* interval, as determined conservatively by heterozygosity for markers between *D4Rat16* and *D4Rat44*; and *3*) they had the fewest DR-derived alleles at all other typed loci. At the next round, the same strategy was used for *iddm4* and *ART2*, and so on for several generations. Congenic parental

rats though the N5 generation were males. Thereafter, due to periodic unavailability of males, female congenic rats were used as parents when needed. Induced diabetes in BBDR rats is observed equally in both sexes (4), and at no time in the breeding program was an effect of sex on susceptibility to diabetes noted.

A total of seven rats with smaller recombinant *iddm4* intervals were identified at generation N5 and used to generate multiple litters of subcongenic progeny, which are hereafter designated as "types" 5–11, Rats studied for susceptibility to diabetes induction in the present report were either these seven types of N6 progeny (n = 124) or the progeny (n = 58) of an intercross between N5 congenic siblings bearing the full *iddm4* interval. The breeding schemes were as follows.

iddm4$^{d/d}$ homozygote production

By the N5 generation, much of the WF.*iddm4* congenic rat genome was fixed for WF alleles (~98–99%, see below), and we intercrossed selected *iddm4*$^{d/w}$ N5 animals to create N5F1 rats with *iddm4*$^{d/d}$, *iddm4*$^{d/w}$, and *iddm4*$^{w/w}$ on the WF background.

WF.ART2^{a} congenic production

Using similar procedures, we selected an N3 (BBDR × WF) × WF *iddm4*$^{w/w}$ male having >95% WF origin genome to create a congenic WF rat that expresses the ART2.1 allotype. The male was analyzed using a genome-wide scan of the available polymorphic microsatellite markers and flow cytometry to document the presence of ART2.1^{+} T lymphocytes. Screening for ART2 allotype in subsequent generations was done by direct typing for ART2 using conformation-sensitive gel electrophoresis (CSGE) as described below. This line is now at the N8 generation and is >99% WF, The BBDR origin ART2 interval on chromosome 1, as determined by typing for flanking markers, is <5.6 cM.

Residual DR origin chromosomal segments and ART2 genotype

In the rats studied, there were eight segregating DR origin chromosomal segments outside the *iddm4* and *ART2* intervals. These were located on chromosome 10 at 0–34 cM (N5F1; types 6 and 7), 12 at 27–54 cM (N5F1; types 5, 6, 7, 9, and 11), 16 at 34–45 cM (N5F1; types 5, 6, 7, and 9), 19 at 20–43 cM (N5F1; types 5 and 6), 20 at 0–23 cM (N5F1; types 5, 6,7,10, and 11) 5 at 76–105 cM (types 7, 8, and 10), 6 at 76–85 cM (types 6–10), and 13 at 23–38 cM (N5F1; types 5, 6, 7, 9, and 10). It should be noted that none of the WF.*iddm4* animals used in these studies, including the N5F1 series and the seven congenic subtypes, carried all eight of the residual DR origin intervals.

The protocol for induction of diabetes in these studies requires the depletion of ART2^{+} regulatory T-cells. Until the creation of the animals described here, all analyses of *iddm4* in the rat (22,26) were carried out using animals heterozygous for *ART2*^{a} (which encodes ART2.1) and *ART2*^{b} (which encodes ART2.2) (27). These genes are codominant (28,29) but expressed somewhat asymmetrically, with the *ART2*^{b} gene product being favored (30). The generation of the N5F1 animals and the mating scheme used to generate the N6 congenic types led to the generation of rats homozygous for *ART2*^{a}. All statistical analyses included an adjustment for *ART2* genotype. Because there are no available reagents that deplete

ART2.2$^+$ T-cells, no *ART2*^{b} homozygotes have been studied. The flanking markers of the *ART2* region in the WF.*iddm4* rats in this study were *D1Rat29* and *D1Rat287*.

3. DNA samples

Genomic DNA was prepared using one of two protocols. 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/1 EDTA (pH 8.0). DNA was purified from these digests by phenol-chloroform extraction and dialysis against Tris EDTA (0.01 mol/1 Tris/0.001 mol/l EDTA, pH 7.4). Alternatively, 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.

4. Microsatellite and mapping 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 our own segregating backcrosses and from maps published by the Rat Genome Database (http://www.rgd.mcw.edu) and by Dr. R. Wilder and Dr. E. Remmers (www.nih.gov/niams/scientific/ratgbase/index.htm). Those primers found to be polymorphic between parental strains were then used in a genome-wide screen of the progeny. Primers were end-labeled using [γ-^{32}P]ATP, used in a PCR, and resolved by polyacrylamide gel electrophoresis as described (26). For detection of polymorphisms within expressed sequence tags (ESTs), appropriate primers were chosen from the EST database (www.ratest.uiowa.edu) and were added unlabeled to a PCR. The products were resolved using CSGE as described (31). Autoradiographic films of CSGE gels were also used to resolve the two alleles of the ART2 gene, amplified using radiolabeled primers that flank the polymorphic nucleotides (forward: 5′-CCAATGCATTTGATGACCAG-3′; reverse: 5′-TCCCAGTGTAGGCAACTAAAGC-3′). The position of markers on the genetic map was established by inspection of the dataset and conventional calculation methods to establish meiotic map distances, which are expressed in centiMorgans.

5. Induction of diabetes

For the induction of autoimmune diabetes, all congenic animals were screened for expression of the ART2.1 allotype as described above. It was necessary to do so because, as noted earlier, there is no depleting anti-ART2.2 monoclonal antibody (mAb) comparable to the T-cell–depleting DS4.23 anti-ART2.1 mAb used in all prior analyses of diabetes induction in the BBDR rat. When 28–32 days of age, ART2.1$^+$ rats were treated with the cytotoxic DS4.23 anti-ART2.1 mAb (1 ml of 2× hybridoma supernatant five times per week) and poly I:C, a nonspecific immune system activator (2.5 μg/g, three times per week), as described (32). The combination regimen administered to VAF BBDR/Wor rats induces type 1 diabetes in >90% of animals (32). Treatment with both mAb and poly I:C was stopped when diabetes was diagnosed or after 40 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).

6. Histological evaluation of insulitis

After the diagnosis of diabetes or at the conclusion of the experiment, rats were killed and pancreata were removed and fixed in 10% buffered formalin. Paraffin-embedded sections of pancreas were prepared for histologic analysis and stained with hematoxylin and eosin. An evaluator who was not informed of the donor's glycemic status scored the tissues. On the basis of histological appearance, pancreatic insulitis was scored as both a qualitative (present or absent) phenotype and a quantitative trait locus. Pancreata were graded on a scale of 0–4+ as follows: 0, no inflammatory mononuclear cell infiltration of any islets; 1+, islet mononuclear cell infiltration only at the periphery of the islet ("peri-insulitis"); 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 some distortion of islet architecture; and 4+, florid infiltration of mononuclear cells or classical end-stage islets.

7. Thymocyte adoptive transfer

Thymocytes for use in adoptive transfer studies were obtained from the N5F1 WF.*iddm4* donor animals described above. These rats had all been treated with poly I:C and anti-ART2.1 mAb, and thymi were recovered from diabetic donors at the time of disease onset and from nondiabetic donors immediately after the 40th day of treatment. Recovered thymi from each individual donor were processed as described (33) and adoptively transferred at a dose 200×10^6 cells i.v. into a single athymic WAG recipient. The recipients were treated with anti-ART2.1 mAb plus poly I:C as described (33). Neither treatment of the thymocyte donor with anti-ART2 mAb plus poly I:C nor the presence of diabetes in the donor influences the ability of BBDR rat thymocytes to adoptively transfer disease (33,34).

8. Data analysis

Differences among groups of rats with respect to diabetes-free survival were analyzed by the method of Kaplan and Meier using the log-rank statistic (35); when necessary, analyses were stratified by *ART2* genotype (36). Parametric data are shown as arithmetic means ± SD. Insulitis scores were analyzed nonparametrically using Mann Whitney *U* tests (37). Analyses of proportions used χ^2 analysis or the Fisher's exact statistic (37), and *P* values <0.05 (two tailed) were considered statistically significant.

The relative positions of markers on chromosome 4 were determined by examining the rate of recombination between them in both the N5F1 intercross and the progeny of the N4 and N5 congenic strains. In most cases, these positions were found to be very similar to the positions of the same markers, as determined by radiation hybrid panel analysis (rgd.mcw.edu).

RESULTS

1. Diabetes segregates with *iddm4* in an N5 intercross of WF.*iddm4* congenic rats

A panel of informative microsatellite markers on RNO4 was used to verify the retention of the *iddm4* interval in each generation of marker-assisted congenics. From among the N5 WF.*iddm4* progeny scored for markers linked to *iddm4* (22,26), two males and two females, each bearing a full-length nonrecombinant *iddm4* interval, were selected to generate intercross progeny. This interval was flanked by *D4Rat16* and *D4Rat44* (22). A total of 58 offspring were generated and treated with poly I:C and anti-ART2 mAb to induce diabetes beginning at 28–32 days of age and continuing for 40 days or until the onset of diabetes.

Confirming our original results (22,26), diabetes segregated significantly with markers in the *iddm4* interval; 29/32 diabetic rats carried BBDR-derived alleles at these markers. As before, linkage analysis revealed that the diabetic phenotype correlated best with inheritance of BBDR-derived alleles at *D4Arb9/D4Arb38/D4Mgh32* ($P = 0.0011$). We therefore used these markers to distinguish $iddm4^{d/d}$, $iddm4^{d/w}$, and $iddm4^{w/w}$ animals. Among $iddm4^{d/d}$ rats ($n = 12$), 67% became diabetic, with a mean latency of 22 days. Among $iddm4^{d/w}$ animals ($n = 30$), 70% became diabetic, with a mean latency of 25 days. In contrast, the cumulative frequency of diabetes in $iddm4^{w/w}$ progeny ($n = 16$) was significantly less (18%, $P = 0.007$ vs. $iddm4^{d/d}$ and $P = 0.003$ vs. $iddm4^{d/w}$), with a mean latency of 28 days in these three rats (Fig. 1). There was no statistically significant difference in the cumulative frequency or latency to onset of diabetes in the homozygous $iddm4^{d/d}$ rats when compared with the heterozygous $iddm4^{d/w}$ animals (Fig. 1). Each of these analyses was adjusted for *ART2* genotype because this experimental cohort of N5F1 rats included animals homozygous for $ART2^{a}$. The overall life table analysis, considering all three *iddm4* groups and adjusting for *ART2* genotype, was significant at the $P = 0.0027$ level.

The analysis also revealed that the *ART2* genotype was a determinant of the degree of penetrance of *iddm4*. Overall, 94% of N5F1 WF.$iddm4^{d/-}$ rats homozygous for $ART2^{a}$ became diabetic ($n = 16$ of 17), compared with 52% of rats that were WF.$iddm4^{d/-}ART2^{a/b}$ heterozygotes ($n = 13$ of 25, $P < 0.004$, Fisher's exact test). Among animals that were WF.$iddm4^{w/w}$, two of four rats homozygous for $ART2^{a}$ became diabetic, compared with 1 of 12 that were $ART2^{a/b}$ heterozygotes ($P = 0.047$).

2. Progeny analysis

Analysis of candidate parents for the N5 generation of WF.*iddm4* rats revealed several recombinants carrying various segments of the interval bounded by *D4Rat16* and *D4Rat44*, which we had used to define the original *iddm4* locus (22). To narrow the boundaries of the *iddm4* interval, we analyzed the offspring of recombinant progeny bearing BBDR origin markers in seven selected subcongenic intervals, each of a different length, on chromosome 4. It was necessary to adopt this strategy because the diabetes phenotype is incompletely penetrant and the resistant phenotype is incompletely resistant (22,26). The approximate map locations of these intervals, together with intermarker distances in centiMorgans, are shown in Fig. 2. Mapping analysis of the progeny in this study (based on >500 meioses)

places the typed markers at positions that were generally consistent with the radiation hybrid and intercross maps (rgd.mcw.edu).

The susceptibility to diabetes induction of rats bearing at least one DR-derived allele within the interval was compared with that of their WF.*iddm4*$^{w/w}$ littermates. As shown in Table 1 (bottom row), the susceptibility of N6 WF.*iddm4*$^{w/w}$ animals to diabetes induction was low; only 3 of 53 animals became diabetic. Among the seven subcongenic types tested, the frequency of diabetes in chromosome 4 heterozygotes ranged from 0 to 58% (Table 1).

The linkage of diabetes in the congenic progeny to markers in the *iddm4* interval is shown in Table 2. As was true for the N5F1 analysis, susceptibility to diabetes was best predicted by inheritance of a set of markers in the middle of the *iddm4* interval, including *D4Arb38*, *D4Arb9*, and *D4Mgh32*. Inheritance of DR-derived alleles at these loci provided a surrogate for the *iddm4* locus itself. There were no recombinants detected in the congenic population studied between *D4Rat96* and *D4Arb9* (Fig. 2), We therefore classified all of the N6 progeny as either *D4Arb9*$^{d/-}$ or *D4Arb9*$^{w/w}$. Using this peak marker, we calculated the cumulative frequency and kinetics of induced diabetes in WF.*iddm4*$^{w/w}$ and WF.*iddm4*$^{d/W}$ rats, and the result demonstrates a striking difference in diabetes susceptibility between the two groups (Fig. 3). As was done for the N5F1 analysis, the analysis was stratified by *ART2* genotype. After adjusting for *ART2*, the effect of *D4Arb9* was highly statistically significant ($P < 0.0001$). The analysis confirmed that the *ART2* genotype was an important determinant of the penetrance of *iddm4*. Overall, 78% (14 of 18) of WF.*iddm4*$^{d/w}$ rats that were homozygous for *ART2*^{a} became diabetic, compared with 24% (5 of 21) of rats that were WF.*iddm4*$^{d/w}$ *ART2*$^{a/b}$ heterozygotes ($P < 0.002$, Fisher's exact test). Among WF.*iddm4*$^{w/w}$ rats, very few developed diabetes, and there was no statistically significant difference between ART2$^{a/a}$ homozygotes (3 of 25) and *ART2*$^{a/b}$ heterozygotes (2 of 28, $P = 0.15$, Fisher's exact test).

The linkage of diabetes in the various types of congenic progeny to markers in the *iddm4* interval, as shown in Table 2, establishes the current working boundaries for *iddm4*. The key observations in Table 2 are *1)* type 7 congenics (58% frequency of diabetes) had a WF-derived allele at *D4Rat135* but a DR-derived allele at *D4Rat96*, and *2*) type 6 congenics (0% frequency of diabetes) had WF-derived alleles at *D4Arb9*, *D4Arb38*, and *D4Mgh32* and a DR-derived allele of *D4Got51*. Our mapping of the two flanking markers (*D4Got51* and *D4Rat135*) places them in the central region of rat chromosome 4, ~40–45 cM distal to the centromere. These observations, together with the map locations shown in Fig. 2, allowed us to narrow the *iddm4* interval to a <2.8-cM segment of chromosome 4 in the vicinity of the trypsin and T-cell receptor β-chain loci.

We next analyzed the frequency and intensity of insulitis, the pathological substrate of autoimmune diabetes, in a sample of the congenic rats shown in Table 1 that were still nondiabetic at the conclusion of the period of observation on day 40. In previous reports (22,26), we noted an association between the presence of the BBDR origin allele of *iddm4* and higher intensity insulitis scores. As shown in Table 3, the intensity of insulitis in our congenic rats with the WF origin allele of *D4Arb9* was low. In contrast, insulitis was both more common and more intense in the nondiabetic rats with the DR origin allele. A

complete assessment of the effect of *ART2* genotype on insulitis was not possible because insulitis scores were available for only two rats that were both *iddm4*^{d} and *ART2*$^{a/b}$. The overall mean insulitis score in nondiabetic *ART2*$^{a/a}$ rats (2.3 ± 1.5, n = 18) was slightly higher than in *ART2*$^{a/b}$ animals (1.2 ± 1.5, n = 49, $P < 0.025$).

We also analyzed insulitis in a sample of diabetic rats of all types to determine whether diabetes was induced at varying degree of insulitis in different groups. We observed, however, that for both the N5F1 intercross and the congenics, the mean insulitis scores were almost uniformly 4+. (Thirty-five of 36 scored pancreata from diabetic rats showed 4+ insulitis and the one exception was scored 2+.)

The linkage of insulitis scores in the nondiabetic congenic progeny to markers in the interval was assessed by ANOVA and a 2 × 2 χ^2 analysis (Table 2). Once again, the most highly significant linkage occured between insulitis and the *iddm4*-associated markers within the larger interval. These include *D4Arb9*, *D4Arb38*, and the EST UI-R-C3-tv-a-09-0-UI (hereafter abbreviated *tva*) (http://www.ratest.uiowa.edu). Each of these genetic markers identifies one of the set of pancreatic trypsin genes on chromosome 4. Table 2 also demonstrates that the linkage maps for insulitis and diabetes do not coincide for the region of chromosome 4 distal to *tva*.

Analysis by ANOVA documented that there was no independent segregating contribution of those chromosomal segments outside the *iddm4* and *ART* regions that still carry BBDR-derived alleles (see RESEARCH DESIGN AND METHODS) to susceptibility to diabetes or insulitis.

3. Thymocyte transfer

It is known that thymocytes from BBDR rats can adoptively transfer diabetes to athymic recipients (33,38), and we next sought to determine whether the ability to transfer diabetes was related to the presence of DR origin alleles at the *iddm4* locus. Thymocytes for adoptive transfer were obtained from a sample of the WF.*iddm4*$^{d/w}$, WF.*iddm4*$^{d/d}$, and WF.*iddm4*$^{w/w}$ N5F1 animals described in Fig. 1. As shown in Fig. 4, the majority of adoptive recipients of thymocytes became diabetic within 90 days, irrespective of the genotype of the donor. However, diabetes appeared earlier when thymocytes were obtained from either WF.*iddm4*$^{d/d}$ N5F1 (n = 11, 10 diabetic, median latency 30 days) or WF.*iddm4*$^{d/w}$ N5F1 donors (n = 24, 3 diabetic, median latency 34 days) rather than WF.*iddm4*$^{w/w}$ donors (n = 14, 11 diabetic, median latency 56 days). Life table analysis, after adjustment for the *ART2* genotype of the thymocyte donor, confirmed a statistically significant difference in the behavior of the three groups at the $P < 0.005$ level. Between-group analyses revealed that thymocytes from WF.*iddm4*$^{d/d}$ and WF.*iddm4*$^{d/w}$ were equally effective in transferring diabetes ($P = 0.5$), and both of these groups differed significantly from the WF.*iddm4*$^{w/w}$ thymocyte donors ($P < 0.01$). Overall, there was no difference in latency to onset of diabetes when the thymocyte donor was *ART2*$^{a/a}$ versus *ART2*$^{a/b}$ ($P = 0.36$).

DISCUSSION

These data confirm and extend our hypothesis that *iddm4*, now mapped to a <2.8-cM interval on chromosome 4, is an exceptionally strong non-MHC determinant of

susceptibility to autoimmune diabetes in the rat. This hypothesis was developed in previous studies of (WF × BBDP) × WF and (WF × BBDR) × WF backcrosses. Those studies clearly established *iddm4*, localized initially to a 40-cM interval, as a major determinant of diabetes susceptibility that is common to both the DP and DR sublines of the BB rat (22,26). Our observation here that nearly 75% of N5 generation WF.*iddm4*^{d} intercross rats developed autoimmune diabetes after immunological perturbation confirms the importance of *iddm4* and the fidelity of our phenotype within the congenic breeding scheme. The intercross also confirmed that the diabetogenic BBDR origin allele of *iddm4* is genetically dominant.

Testing the N6 progeny of various recombinant N5 WF.*iddm4* congenic parents revealed that susceptibility to diabetes is controlled by a segment of RNO4 containing *tva* and bounded by the proximal marker *D4Rat135* and the distal marker *D4Got51*. Analysis of the entire dataset generated in the progeny study suggests that the presence of the BB origin allele of *iddm4* is 79% sensitive and 80% specific in the prediction of diabetes in rats that segregate for this locus. In the N5F1 cohort, the relative risk of developing diabetes in treated animals bearing the BBDR origin allele of *iddm4* was 3.31 (95% CI 1.00–10.8). In the N6 congenic cohort, the relative risk was 8.60 (95% CI 2.90–25.6).

The experimental cohort of N5F1 and congenic rats presented here is the first to include animals homozygous for ART2^{a}. In previous analyses of the *iddm4* locus, all rats were *ART2*$^{a/b}$ heterozygotes. The present data reveal that the ART2 genotype is an important determinant of the penetrance of *iddm4*. There are several possible mechanisms by which homozygosity for ART2.1 could affect the penetrance of *iddm4*. *1*) There may be differential efficiency of depletion of ART2^{+} regulatory T-cells in the peripheral lymphoid compartment of homozygotes versus heterozygotes. In at least one strain combination (BBDP × LEW.B6), ART2.1 and ART2.2 are expressed differentially on T-cells (30). In the heterozygous rats, not only was there more ART2.2 on cells expressing both it and ART2.1, but there also was noted a small population of T-cells that expressed only ART2.2 and would therefore not be depleted by the DS4.23 mAb used in these studies (30). We have confirmed this phenotype in a small sample of (BBDR × WF)F1 rats (J.P.M., unpublished observations). *2*) A second possibility is that ART2.1^{+} and ART2.1^{+}/ART2.2^{+} cells in the intestinal lymphoid compartment might be differentially affected by antibody treatment (39). *ART2*$^{+}$ cells in the gut include a population of NK cells that could be important modulators of autoimmunity (39). *3*) It is also possible that the effect on penetrance is due to genes within the *ART2* interval on chromosome 1. The present study, designed solely to map *iddm4*, did not generate sufficient data to distinguish among these alternatives, the analysis of which will become part of future studies.

We also analyzed insulitis, the pathological precursor and substrate of autoimmune diabetes. In animals that were treated but not diabetic at the conclusion of the protocol, the frequency of insulitis was greater in WF.*iddm4*^{d} rats than in controls. In addition, the quantitative trait locus controlling the intensity of insulitis was found within the same segment of RNO4 as susceptibility to diabetes. These data suggest that the *iddm4* gene plays a role during the early stages of autoimmune diabetes pathogenesis in the rat.

We did observe that a small fraction of WF.*iddm4* congenic rats homozygous for the WF origin allele of *iddm4* became hyperglycemic after treatment to induce diabetes (8 of 101, considering both the N5F1 and congenic progeny testing analyses). This observation is consistent with previous reports that some degree of susceptibility to autoimmune diabetes is common among rats that express the class II *RT1B/D*^{u} MHC haplotype (16). With respect specifically to the WF rat, Ellerman and Like (16) reported that 7% of these animals treated for 3 weeks with poly I:C alone could be induced to become diabetic. They also reported that spleen cells from treated WF rats were capable of the adoptive transfer of autoimmune diabetes. The simplest interpretation of these observations, given the common ancestry of the WF and BBDR rats, is that a mutation in the *iddm4* gene has amplified the susceptibility to autoimmune diabetes that is inherent in the ancestral Wistar rat. These data can also be interpreted to suggest that *iddm4*^{w} is a recessive non-MHC resistance gene for autoimmunity. Because diabetes in all of these animals was induced using poly I:C, it is interesting to speculate that differences in the metabolism of poly I:C could determine diabetes susceptibility. A corollary possibility is that the differences in the response to poly I:C (e.g., IL-18, IL-12, and interferons) could influence diabetes susceptibility. We have previously reported that rat strains differ in the amount of interferon produced in response to poly I:C (40), but that report did not measure responses in either BBDR or WF rats. There is no independently measured response to poly I:C in the present genetic analysis, but it remains our intention to test this and other immunophenotypes when the WF.*iddm4* rat is fully congenic.

The region of rat chromosome 4 containing the *iddm4* interval contains a number of interesting potential candidate genes. Among these are the T-cell receptor β-chain genes, the trypsin multigene family, ephrin B6, caspase 2, and others. None of these candidates has been formally excluded. As the sequence of the rat genome becomes available and bioinformatics tools become more sophisticated, the prospects for identifying *iddm4* would appear to be good.

Our initial analyses of the *iddm4* locus suggest that it is not syntenic with any of the known human *iddm* or mouse *idd* diabetes susceptibility loci. It is important to point out, however, that this does not imply that *iddm4* is rat specific. To identify a locus as conferring susceptibility, it must be associated with a difference that segregates, and such unrecognized nonsegregating loci could be present in either humans or NOD mice.

Although our data clearly demonstrate that *iddm4* is a major determinant of autoimmune diabetes in the *RT1*^{u} rat, the data are also indicative of the action of additional disease-modifying genes. We show here that *iddm4* is incompletely penetrant and that its penetrance is modified by *ART2* genotype. In several reports, the presence of a diabetes resistance gene has been inferred from crosses between diabetic BBDP and resistant, nonlymphopenic non-BB rats. In two studies, the *RT1*^{u} and *lyp* genes were placed on either the ACI (41) or the F344 (42) strain background, and these animals were found to be resistant to the development of diabetes. A third study reported results from F2 offspring of (DP × F344)F1 hybrids. The authors found six F2 rats homozygous for both *RT1*^{u} and *lyp* (19). Because none of these became diabetic, they 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. (43) who, using the BB/OK variant of the BB rat, discovered a candidate location for *iddm3* on chromosome 18. Using the BB/OK rat and crosses to several strains, including DA and SHR, Klöting and colleagues have also reported several other disease-modifying loci in the BB/OK rat on chromosomes 1, 6, 18, 20, and X (44-48). BB/OK rats exhibit several features not observed in the more common BB rats of Worcester origin (4), and the relationship of these loci to those observed in the BBDR rat of Worcester origin is uncertain.

With respect to the likelihood that multiple genes are responsible for diabetes susceptibility in the BB rat, it is important to point out that the frequency of diabetes in our various "susceptible" subcongenic lines expressing $D4Arb9^{d}$ varied from 30 to 70%. These data are consistent with the possibility that one or more additional genes important for the expression of autoimmunity may be present in the original 40-cM *iddm4* interval. As noted previously (22), a number of genes important for autoimmunity in the rat map to this region of chromosome 4. These include loci associated with susceptibility to experimental autoimmune uveitis, adjuvant arthritis, and collagen-induced arthritis. Although our data are consistent with the possibility of a second gene, the sample size lacks the power to formally prove or disprove the hypothesis.

Many studies suggest that autoimmune diabetes in the BB rat is T-cell–dependent and that cells capable of diabetes induction are present in the thymus. This was most clearly demonstrated in studies showing that both unmanipulated and cultured BB rat thymocytes can transfer disease to adoptive recipients (33) and that transfer can be prevented by coculture in the presence of islet tissue (38). We hypothesized, accordingly, that the diabetogenic action of $iddm4^{d}$ could be detected in populations of thymocytes. To test this hypothesis, we used an adoptive transfer protocol and showed that thymocytes from susceptible WF.$iddm4^{d}$ animals were capable of the adoptive transfer of diabetes at high efficiency to athymic histocompatible WAG nude recipients. Surprisingly, however, thymocytes from WF.$iddm4^{w}$ animals also transferred the disease, and the effect of *iddm4* appeared to be related to the kinetics of diabetes onset. Given the multigenic nature of diabetes in the rat and the latent susceptibility of the WF and other $RT1^{u}$ rat strains to diabetes, we interpret our transfer data to suggest that $iddm4^{d}$ is expressed at the level of the thymus and in some way facilitates the action of other susceptibility genes that are otherwise masked in the context of a normal *regulatory* immune environment in vivo.

In conclusion, we propose that *iddm4* on chromosome 4 is a critical determinant of susceptibility to the induction of diabetes in the rat after environmental perturbation of the immune system. With respect to its ability to predict disease, *iddm4* is 79% sensitive and 80% specific. These characteristics suggest that *iddm4* is a major determinant of susceptibility to autoimmune diabetes in the rat. As additional markers become available, it should be possible to narrow further the *iddm4* interval. Identification of *iddm4* and elucidation of the disease-determining pathways that it affects may eventually provide useful information for the understanding of human type 1A diabetes.

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Glossary

CSGE	conformation-sensitive gel electrophoresis
EST	expressed sequence tag
mAb	monoclonal antibody
MHC	major histocompatibility complex
poly I:C	polyinosinic:polycytidylic acid
VAF	viral antibody free

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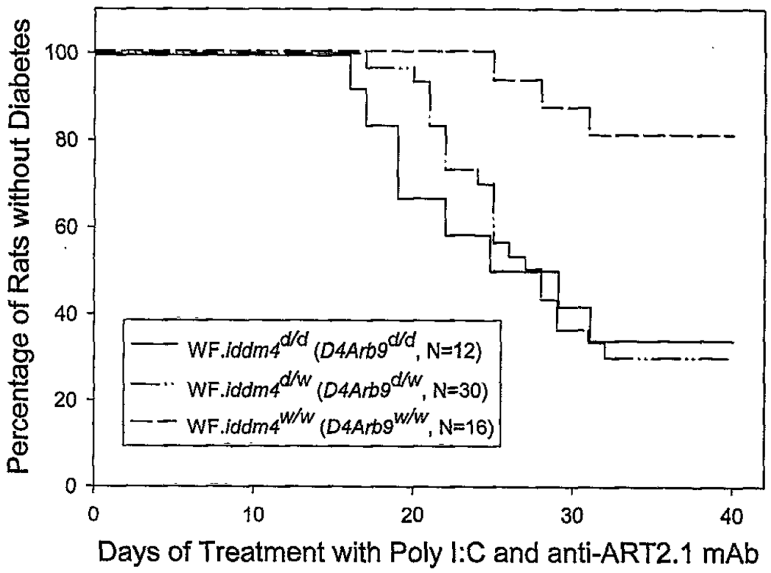

FIG. 1.

Kaplan Meier analysis of diabetes in WF.*iddm4* N5F1 rats. From among N5 WF.*iddm4* progeny scored for markers linked to *iddm4*, suitable rats were selected to generate intercross progeny as described in RESEARCH DESIGN AND METHODS. In this figure, progeny were typed as homozygous or heterozygous for the presence WF or BBDR origin alleles of the microsatellite marker *D4Arb9*. A total of 58 of the progeny expressed the ART2.1 regulatory T-cell alloantigen and could therefore be tested for susceptibility to the induction of diabetes by administration of poly I:C and anti-ART2.1 mAb. Among WF.*iddm4*$^{d/d}$ rats, 67% became diabetic, with a median latency of 25 days. Among WF.*iddm4*$^{d/w}$ animals, 70% became diabetic, with a median latency of 27 days. The cumulative frequency of diabetes in *iddm4*$^{w/w}$ progeny was statistically significantly less (18%, $P = 0.007$ vs. iddm4$^{d/d}$ and $P = 0.003$ vs. iddm4$^{d/w}$). The cumulative frequency of diabetes in homozygous *iddm4*$^{d/d}$ rats and heterozygous *iddm4*$^{d/w}$ rats was statistically similar ($P = 0.57$). Significance levels were determined by log-rank statistic with adjustment for *ART2* genotype (see RESULTS).

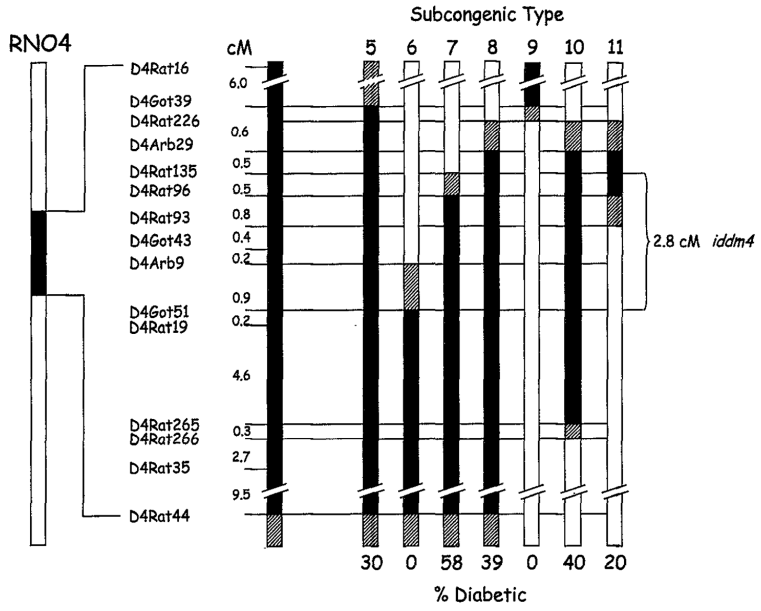

FIG. 2.

Map of diabetes susceptibility in heterozygous WF.*iddm4* congenic rats. The left-most bar represents the entire length of rat chromosome 4 (RN04). The solid region of the bar indicates the *iddm4* interval present in the N5 parents of the N5F1 rats shown in Fig. 1 and the N6 congenic progeny analyzed in Table 1. A magnified view of the *iddm4* interval with the relevant microsatellite markers is shown in the adjacent bar, with the intermarker distances indicated in centiMorgans. The remaining bars represent the segment of this interval present in the parent of each of the seven subcongenic types, with the type number indicated on the top of each bar. At the bottom of each bar is the percentage of progeny of these animals that developed diabetes after treatment, as explained in Table 1 and RESULTS. Areas of the bars with no fill indicate WF origin alleles, and areas with solid fill indicate BBDR-derived alleles. Diagonal fill indicates transition intervals for which the strain of origin could not be determined with available markers. Note that these chromosomal segments are those of the parents and do not take into account recombinations that may be present in their progeny. Considering only the definitively typed regions (solid fill) and omitting from consideration type 11, which is indeterminate in its susceptibility to diabetes, the data from progeny testing define a 2.8-cM interval for *iddm4* bounded by *D4Rat135* and *D4Got51*.

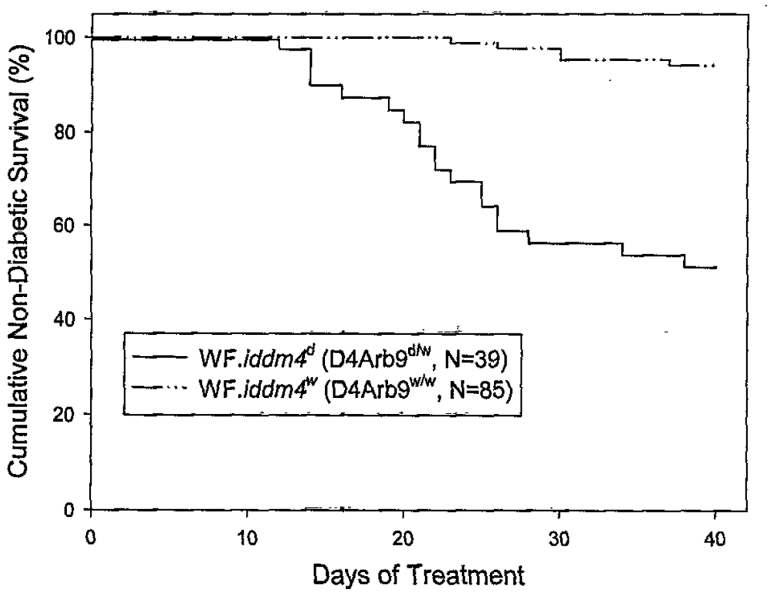

FIG. 3.

Kaplan Meier analysis of diabetes in WF.*iddm4*^{d} (*D4Arb9*$^{d/w}$) and WF.*iddm4*^{w} (*D4Arb9*$^{w/w}$) N6 congenic rats. Congenic N6 generation WF.*idddm4* rats were bred and treated with poly I:C and anti-ART2.1 mAb as described in RESEARCH DESIGN AND METHODS. All animals were genotyped and grouped according to the presence of the WF and BBDR alleles of the microsatellite marker *D4Arb9*, as described in RESULTS and in the legend to Table 1. One WF.*iddm4*^{d} rat that died on day 17 with unknown glycemic status has been omitted. The cumulative frequency of diabetes was 49% in the WF.*iddm4*^{d} rats and 6% in the WF.*iddm4*^{w} group ($P < 0.0001$, log-rank statistic after adjustment for *ART2* genotype).

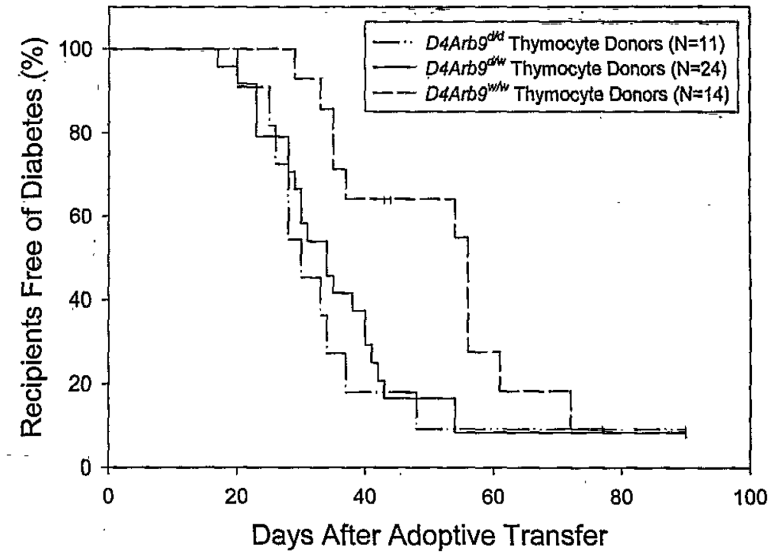

FIG. 4.

Kaplan Meier analysis of diabetes in adoptive recipients of thymocytes. Thymocytes were obtained from the N5F1 WF.*iddm4* rats described in Fig. 1 and prepared for adoptive transfer to WAG *rnu/rnu* rats, as described in RESEARCH DESIGN AND METHODS. Thymi were obtained from diabetic donors within 1 day of diagnosis; additional thymi were obtained from animals still nondiabetic at the conclusion of the protocol on day 40. Recipients were monitored for the development of diabetes for 90 days after transfer. Overall life table analysis, after adjustment for the *ART2* genotype of the thymocyte donor, confirmed a statistically significant difference in the behavior of the three groups at the $P < 0.005$ level. Thymocytes from WF.*iddm4*$^{d/d}$ and WF.*iddm4*$^{d/w}$ were equally effective in transferring diabetes ($P = 0.5$), and both of these groups differed significantly from the WF.*iddm4*$^{w/w}$ thymocyte donors ($P < 0.01$). Recipients of thymocytes from donors bearing the WF allele of *D4Arb9* became diabetic, with a median latency to onset of 56 days, whereas recipients of thymocytes from donors bearing a BBDR origin allele of *D4Arb9* became diabetic, with a median latency of 33 days. There was no statistically significant independent effect attributable to the presence or absence of diabetes in the thymocyte donor. *N* indicates the number of rats entered into the study, and the small vertical bars indicate censored data, i.e., rats that either died during the study ($n = 4$) or were still normoglycemic at the end of the period of observation.

TABLE 1

Analysis of WF.*iddm4* N6 progeny

Progeny type	*n*	*n* (%) diabetic	Mean latency to diabetes (days)	*P* *	Number of diabetic rats with *D4Arb9*^{d} allele
Congenic rats with BBDR-derived chromosome 4 alleles					
5	10	3 (30)	17	<0.01	3/3
6	12	0 (0)	—	NS	0/0
7	12	7 (58)	24	<0.001	7/7
8	18	7 (39)	20	<0.001	7/7
9	4	0 (0)	—	NS	0/0
10	5	2 (40)	30	<0.01	2/2
11	10	2 (20)	30	NS	0/2
Congenic rats homozygous for WF-derived chromosome 4 alleles					
Pooled from progeny types 5–11	53	3 (5)	29	—	0/3

Data are the results of WF.*iddm4* congenic progeny testing. N5 generation WF.*iddm4* congenic rats with recombinant *iddm4* intervals were identified and bred to WF.*Art2*^{a} rats. Their N6 progeny were tested for susceptibility to the induction of diabetes as described in RESEARCH DESIGN AND METHODS. Seven subcongenic types were generated in this way. The approximate location and size of the BBDR rat-origin interval carried by each line is shown in Fig. 2.

* *P* values were calculated by 2 × 2 χ^2 analysis. Indicated *P* values are with respect to the pooled group having all WF-derived chromosome 4 markers. Progeny of types 6, 9, and 11 could not inherit *D4Arb9*^{d} alleles, which were not present in their N5 congenic parents. A fraction of the progeny from types 5, 7, 8, and 10 were recombinants that did not inherit *D4Arb9*^{d}. The number of diabetic rats in each type of congenic that carried *D4Arb9*^{d} is shown in the right-most column. NS, not significant.

TABLE 2

Linkage of markers near *D4Arb9* to insulitis and diabetes

Trait	Insulitis score *P* value	Insulitis χ^2	Insulitis *P* value	Diabetes χ^2	Diabetes *P* value	Genomic exclusions determined by subcongenic types*
D4Rat16	NS	0.1	NS	0.46	NS	7, 10
D4Rat134	NS	0.1	NS	0.00	NS	7, 10
D4Mit9	0.03	4.0	NS	0.19	NS	7, 10
D4Wox10	0.03	1.9	NS	0.19	NS	7, 10
D4Arb11	0.03	4.6	0.03	0.06	NS	7
D4Rat226	0.02	7.1	0.008	1.22	NS	7
D4Arb29	0.01	7.1	0.008	9.26	0.0023	7
D4Rat122	0.01	5.7	0.02	9.98	0.0016	7
D4Rat135	0.01	5.7	0.02	9.98	0.0016	7
D4Got48	<0.003	8.6	0.003	28.70	<0.0001	None
D4Rat96	<0.003	8.6	0.003	28.70	<0.0001	None
D4Rat93	<0.003	8.6	0.003	31.43	<0.0001	None
EST tva	<0.003	8.6	0.003	31.43	<0.0001	None
D4Arb9	<0.003	8.6	0.003	31.43	<0.0001	None
D4Got51	NS	3.6	NS	24.12	<.0001	6
D4Got52	NS	3.6	NS	24.12	<.0001	6
D4Rat19	NS	3.6	NS	24.12	<.0001	6
D4Mit5	NS	1.7	NS	20.53	<.0001	6
D4Rat228	NS	1.1	NS	19.58	<.0001	6
D4Arb30	NS	1.1	NS	19.58	<.0001	6
D4Rat265	NS	0.9	NS	9.18	0.002	6
D4Rat266	NS	1.7	NS	6.79	0.009	6
D4Rat35	NS	1.9	NS	3.33	NS	6
D4Rat38	NS	1.0	NS	4.15	NS	6
D4Rat44	NS	1.6	NS	3.18	NS	6

Data are linkage of diabetes and insulitis to markers near *D4Arb9*^{d} in congenic rats. Microsatellite markers in the left-most column were linked to insulitis severity or the presence of insulitis in the 67 progeny that did not develop overt diabetes, as shown in Table 3. ANOVA was used to determine significance of linkage to the insulitis score, and the 2 × 2 χ^2 test was used to determine the significance of the frequency of insulitis and diabetes, scored as binary traits. The frequency of diabetes with respect to inheritance of the DR-derived allele at each of the markers was calculated for the entire congenic cohort shown in Table 1 (N = 124 rats).

* The right-most column identifies congenic subtypes with either a high (types 7 and 10) or a zero (type 6) frequency of diabetes (see Table 1) that were used to exclude regions of RN04 from the *iddm4* interval. At the indicated markers, types 7 and 10 are “w” but are diabetes-susceptible and type 6 has the “d” allele but is diabetes-resistant. This information was used to construct the map shown in Fig. 2. NS, not significant (P > 0.05).

TABLE 3

Insulitis in nondiapetic WF.*iddm4* congenic rats

Group	*n*	Mean insulitis score*	*n* (%) with 0 or 1 + insulitis	*n* (%) with 2+, 3+, or 4+ insulitis
Congenic WF.*iddm4* rats that inherited D4Arb9$^{d/w}$	16	2.50 ± 1.41	4 (25)†	12 (75)†
Congenic WF.*iddm4* rats homozygous for D4Arb9$^{w/w}$	51	1.22 ± 1.46	34 (67)	17 (33)

A sample of pancreata from animals described in Table 1 that were nondiabetic at the conclusion of the experiment was scored for the presence of insulitis on a scale of 0–4 as described in RESEARCH DESIGN AND METHODS. Insulitis scores are shown as the arithmetic means ± SD.

*$P < 0.005$ (Mann-Whitney *U* test).

†$P = 0.004$ (Fisher's exact statistic). Comparisons used nonparametric statistics to avoid making assumptions about the distribution of scores. Not all pancreata from the rats shown in Table 1 were analyzed because some of the nondiabetic animals were needed for subsequent breeding ($N = 23$), one rat died before harvest of the pancreas, and some histology specimens were unsatisfactory and could not be interpreted ($N = 9$).