Brief Genetics Report

The Rat Diabetes Susceptibility Locus *Iddm4* and at Least One Additional Gene Are Required for Autoimmune Diabetes Induced by Viral Infection

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BBDR rats develop autoimmune diabetes only after challenge with environmental perturbants. These perturbants include polyinosinic:polycytidylic acid (poly I:C, a ligand of toll-like receptor 3), agents that deplete regulatory T-cell (Treg) populations, and a non–β-cell cytopathic parvovirus (Kilham rat virus [KRV]). The dominant diabetes susceptibility locus *Iddm4* is required for diabetes induced by treatment with poly I:C plus Treg depletion. *Iddm4* is penetrant in congenic heterozygous rats on the resistant WF background and is 79% sensitive and 80% specific as a predictor of induced diabetes. Surprisingly, an analysis of 190 (BBDR × WF)F2 rats treated with KRV after brief exposure to poly I:C revealed that the BBDR-origin allele of *Iddm4* is necessary but not entirely sufficient for diabetes expression. A genome scan identified a locus on chromosome 17, designated *Iddm20*, that is also required for susceptibility to diabetes after exposure to KRV and poly I:C (logarithm of odds score 3.7). These data suggest that the expression of autoimmune diabetes is a complex process that requires both major histocompatibility complex genes that confer susceptibility and additional genes such as *Iddm4* and *Iddm20* that operate only in the context of specific environmental perturbants, amplifying the immune response and the rate of disease progression. *Diabetes* 54:1233–1237, 2005

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KRV, Kilham rat virus; MHC, major histocompatibility complex; poly I:C, polyinosinic:polycytidylic acid; QTL, quantitative trait loci; Treg, regulatory T-cell.

Type 1 diabetes results from inflammatory infiltration of pancreatic islets and selective β-cell destruction. It is thought to be caused by environmental factors operating in a genetically susceptible host (1,2). Susceptibility loci include the major histocompatibility complex (MHC), a promoter polymorphism of the insulin gene, and an allelic variant of CTLA4 (3). Among candidate environmental perturbants, viral infection is one of the most likely (4). How genes interact with the environment to transform diabetes susceptibility into overt disease is unknown.

BBDR rats model virus-induced autoimmune diabetes remarkably well (5). They are phenotypically normal and, in clean housing, never develop diabetes. They do, however, become diabetic when challenged with environmental perturbants, including polyinosinic:polycytidylic acid (poly I:C) in combination with depletion of regulatory T-cells (Tregs) (6). Diabetes can also be induced in BBDR rats with Kilham rat virus (KRV), a non–β-cell cytopathic parvovirus (7). Naturally occurring KRV infection induces diabetes in ~1% of animals; intentional infection with 10^7 plaque forming units (PFU) induces diabetes in ~30% of BBDR rats (7). Infection with KRV following brief pretreatment with a low, subdiabetogenic dose of poly I:C (1 μg/g daily for 3 days) leads to diabetes in 100% of animals (8). The effect is virus specific; H-1, which is 98% sequence identical, uniformly fails to induce diabetes (8).

In analyses of (BBDP × WF) × WF rats, we used poly I:C plus Treg depletion to map a locus on chromosome 4 (*Iddm4*) with significant linkage to diabetes (6,9), and we recently positioned *Iddm4* in a 2.8-cM region (10). The BB-origin allele of *Iddm4* is dominant and 79% sensitive and 80% specific as a predictor of diabetes induced by Treg depletion and poly I:C. A radiation hybrid map has assigned *Iddm4* to a 6.3-Mb segment between *PTN* and *ZYX* at 7q32 in the human genome and to a 5.7-Mb segment between *Ptn* and *Zyx* in the mouse genome (11).

We now report a linkage analysis of 190 (BBDR × WF)F2 rats. It reveals that the BBDR-origin allele of *Iddm4* is necessary but not entirely sufficient for diabetes expression in response to KRV infection. An additional gene or genes on chromosome 17 are necessary.

TABLE 1
Frequency of diabetes in rats treated with KRV, poly I:C, and Treg depletion

Rat strains	Protocols			
	KRV (group 1)	KRV + poly I:C (group 2)	Anti-ART2.1 mAb + poly I:C (group 3)	Poly I:C alone (group 4)
BBDR	11/27	27/27	12/12	0/6
WF	0/3	0/8	3/53	—
WF.*Iddm4*^{d} (N6)	0/4	0/18	7/12	—
(BBDR × WF)F1	0/6	5/13	11/11	—
(WF × BBDR)F2	—	59/190	—	—

Frequency of induced diabetes. Male and female rats were entered into the indicated treatment protocols at 21–28 days old. In groups 1 and 2, KRV-UMass was given intraperitoneally at a dose of 10^7 PFU. In group 2, poly I:C was given at a dose of 1 μg/g body wt i.p. on days −3, −2, and −1 relative to KRV. In group 3, poly I:C (1 μg/g) was given 3 times/week for 40 days and anti-ART2.1 mAb (25 μg) was given 5 times/week for 40 days as described (9). Rats in group 4 received only poly I:C (1 μg/g) for 3 consecutive days. Diabetes was defined as a plasma glucose concentration >11.1 mmol/l (250 mg/dl). The WF.*Iddm4*^{d} congenic rats were from the N6 generation and bear ~2.8 cM of the genetically dominant BBDR rat-derived *Iddm4* region on chromosome 4 and at least one BBDR-origin "a" allele of the ART2 T-cell alloantigen on chromosome 1 (10). Diabetic rats in all groups had severe insulitis or "end-stage" islets.

RESEARCH DESIGN AND METHODS

BBDR/Wor, WF.*Iddm4*, and WF.*ART2a* rats (all *RT1*$^{u/u}$, *ART2*^{a}) were obtained from colonies maintained by us. WF.*Iddm4* congenic rats were generated by repetitive (BBDR/Wor × WF) × WF backcrosses using a marker-assisted selection protocol as described (10). They were studied at the N6 generation. WF.*ART2a* congenic rats were also developed by us and differ from ordinary WF animals in that they express the "a" rather than the "b" allotype of the ART2 T-cell alloantigen on chromosome 1 (10). For simplicity, we refer to them here as WF rats. An F2 intercross was bred from (BBDR × WF)F1 hybrids. Animals were housed in viral antibody–free conditions, confirmed monthly to be serologically free of rat pathogens (10), and maintained in accordance with recommendations in the Guide for the Care and Use of Laboratory Animals (National Academy of Sciences, 1996).

Microsatellite and mapping analyses. Genomic DNA was prepared as described (10) (online appendix Fig. 3 [available from http://diabetes.diabetesjournals.org]). Microsatellite markers were placed evenly throughout the 20 autosomes. The source of primers and positions of markers on the genetic map are given in online appendix Fig. 3. Primers were end labeled using ^{32}Pγ-ATP, used in a PCR reaction, and resolved by polyacrylamide gel electrophoresis as described (6). 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 (cM) or megabases (Mb) according to the rat genome sequence, June 2003 build (available at http://genome.ucsc.edu).

Linkage of diabetes with segregation of BBDR-origin alleles was evaluated by composite interval mapping (CIM) using model 6 of the Zmapqtl program in Windows QTL Cartographer v1.30 (available at statgen.ncsu.edu/qtlcart/cartographer.html). CIM combines classical interval mapping with multiple regression analysis, allowing for more precise quantitative trait loci (QTL) localization than classical interval mapping (12).

Treatment protocols. KRV-UMass was propagated in normal rat kidney cells grown in Dulbecco's minimal essential medium. Poly I:C (Sigma, St. Louis, MO) was dissolved in Dulbecco's PBS, sterile filtered, and stored at −20°C until used. Contaminating endotoxin concentration was <50 units/mg (Charles River Endosafe, Charleston, SC). In studies of KRV alone, rats of either sex 22–28 days old were injected intraperitoneally with 10^7 PFU in a volume of 1 ml. In other experiments, rats 21–25 days of age of either sex were injected intraperitoneally with poly I:C (1 μg/g body wt on 3 consecutive days) and either not treated further or injected on the following day with KRV. Pretreatment with poly I:C was used because it increases the frequency of diabetes in KRV-treated BBDR rats from ~30 to 100% (8). Animals were screened three times weekly for glycosuria (Tes-Tape; Eli Lilly, Indianapolis, IN). Diabetes was diagnosed on the basis of a plasma glucose concentration >11.1 mmol/l (OneTouch Ultra Glucometer; LifeScan, Milpitas, CA). For study of insulitis, pancreata were removed, fixed in formalin, and stained with hematoxylin and eosin. Insulitis was graded by a qualified pathologist on a scale of increasing intensity from 0 to 4+ as described (10).

RESULTS

Autoimmune diabetes induced by KRV and TLR3 ligation. We first confirmed (8) that a significant fraction (41%) of parental BBDR rats become diabetic after infection with KRV alone, that none become diabetic in response to a 3-day course of poly I:C alone, and that 100% become diabetic in response to KRV after poly I:C (Table 1). We also confirmed (13) that WF rats resist diabetes induction in response to either KRV or KRV plus poly I:C (Table 1).

We next tested N6 generation WF.*Iddm4* congenic rats (10) for disease susceptibility. To our surprise, we observed WF.*Iddm4*^{d} rats to be uniformly resistant to diabetes in response to KRV infection either alone or after poly I:C, despite maintaining the expected degree of susceptibility to diabetes after treatment with poly I:C and Treg depletion (Table 1).

Autoimmune diabetes induced by KRV and TLR ligation segregates with *Iddm4* and a second locus in (BBDR × WF)F2 rats. To determine whether *Iddm4* acts only in the presence of additional BBDR-origin genes, we generated (BBDR × WF)F1 progeny. We observed that all F1 rats were resistant to KRV alone, but 38% were susceptible to treatment with KRV plus poly I:C. To identify susceptibility genes, we then generated (BBDR × WF)F2 progeny and treated them with KRV plus poly I:C. Diabetes occurred in 59 of 190 animals (31%) in this segregating population and affected both males and females (Table 1). Looking first at the *Iddm4* interval, we observed that diabetes occurred almost exclusively in animals with at least one BBDR-origin allele of *Iddm4* (58 of 59 diabetic animals, Table 2). The presence of the BBDR allele of *Iddm4* was 98% sensitive but only 31% specific in predicting susceptibility to diabetes, implying that at least one gene of BBDR origin is required for the expression of diabetes in response to infection. We therefore performed

TABLE 2
Frequency of diabetes in (BBDR × WF)F2 rats as a function of *Iddm4* genotype

Iddm4 genotype	*N*	*n* (%) diabetic
BBDR/BBDR	49	23 (47%)
BBDR/WF	100	35 (35%)
WF/WF	41	1 (2%)

All (BBDR × WF)F2 rats in Table 1 were genotyped as described in RESEARCH DESIGN AND METHODS. They are grouped according to the presence of the WF and BBDR alleles of the microsatellite marker *D4Arb9* used as the genotype for *Iddm4* as described (10). Overall $\chi^2 = 22.2$, df = 2, $P < 0.001$.

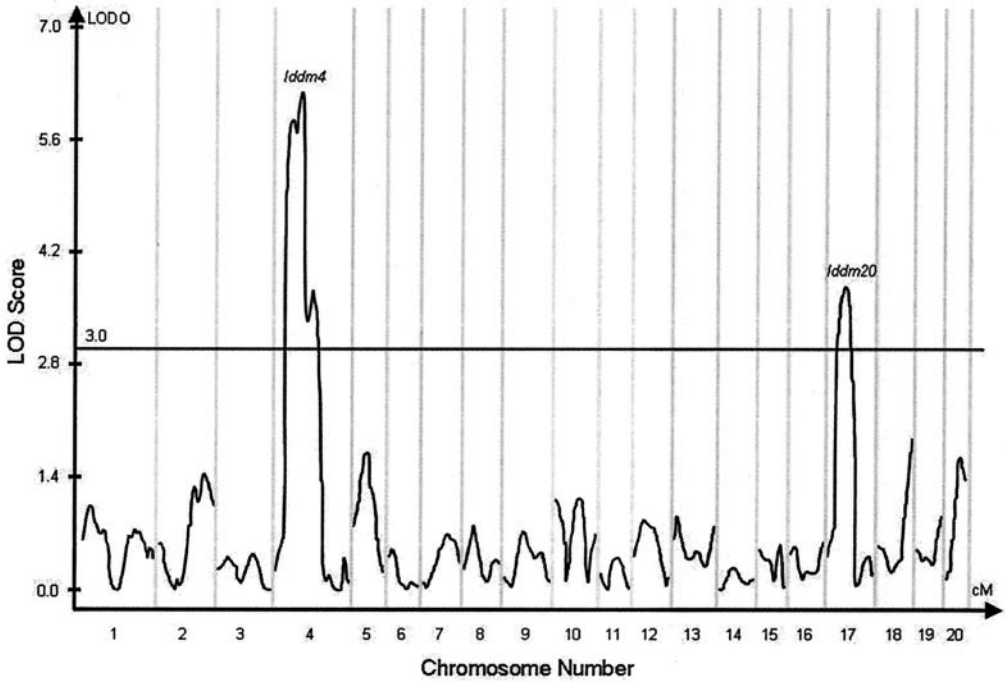

FIG. 1. Composite interval analysis of linkage to the diabetes phenotype in 190 (WF × BBDR)F2 rats. Significant markers were first chosen using a linear regression model with a forward/backward selection procedure in the SRmapqtl module of QTL Cartographer. Markers flanking the test interval were added to the regression model to control for the presence of linked QTL. LOD scores are displayed as peaks over the entire genome, with chromosomes 1–20 displayed on the *x*-axis. The curves are discontinuous, and the vertical gray lines delimit each individual chromosome and its associated curve. The horizontal reference line identifies the standard LOD = 3 cutoff. The only peaks that significantly exceed this standard are on chromosome 4 (*Iddm4*) and on chromosome 17 (*Iddm20*).

a genome-wide scan on this F2 cohort. The remaining genome was assessed for linkage using 144 markers on the 20 autosomes (online appendix Fig. 3).

Composite interval analysis of the linkage data is shown in Fig. 1. *Iddm4*, as expected, showed strong linkage to diabetes (logarithm of odds [LOD] >6.0 at ~36 cM on chromosome 4). A second locus on chromosome 17 was linked to diabetes in the F2 population with an LOD score of 3.7. This locus has been designated *Iddm20* by the curators of the Rat Genome Database (available at www.rgd.mcw.edu).

To determine the mode of inheritance and the interaction between these two loci, we analyzed the dataset using a life-table analysis. As shown in Fig. 2, the highest likelihood of diabetes onset occurs in animals in which the BBDR-origin allele of *Iddm4* is homozygous or heterozygous and *Iddm20* is homozygous.

Candidate gene analysis. The genome-wide scan positioned the *Iddm20* locus in a 1-LOD interval bounded by *D17Rat61* (at 19.7 Mb) and *D17Rat115* (at 27.7 Mb) (available at http://genome.ucsc.edu). To identify candidate genes within this interval, we constructed a preliminary map using the databases at UCSC and Ensembl (available at http://genome.ucsc.edu and http://www.ensembl.org). The genes in the *Iddm20* region have their human orthologs on human chromosomes 5, 6, and 9 and on mouse chromosome 13. Of interest is a confirmed mouse diabetes QTL (*Idd14*) in this homologous region (14,15). Candidate genes in the *Iddm20* interval are listed in online appendix Table 4.

Histology. Histologic analysis of islets revealed nearly complete concordance of insulitis scores with diabetes phenotype. Among the 59 diabetic F2 animals, the mean insulitis score was 3.7 with 49 scored 4+ or end-stage insulitis. In contrast, among 127 nondiabetic rats with technically satisfactory specimens, the mean insulitis score was 0.3 with 111 (87%) being entirely normal and 8 of the remaining 16 exhibiting only 1+ insulitis. Exocrine pancreatitis was absent.

DISCUSSION

These data establish linkage of rat genotype to a form of environmental perturbation—infection—that is potentially important in the pathogenesis of autoimmunity. They confirm that *Iddm4* is an exceptionally strong non-MHC determinant of susceptibility to autoimmune diabetes in the rat (6,9–11). In previous studies of *Iddm4*, diabetes was induced by chronic treatment with poly I:C plus Treg depletion. The present data now extend the role of *Iddm4* in diabetes pathogenesis to virus-induced disease expression. They also illuminate the complexity of environmental interaction with genetic susceptibility. The diabetogenic potential of *Iddm4* is readily discernable in congenic rats treated with poly I:C and Treg depletion but is far less apparent in animals treated with KRV plus poly I:C unless additional BBDR genes are present. We have discovered at least one of these genes, designated *Iddm20*, on chromosome 17.

The *Iddm20* interval (online appendix Table 3) contains at least one gene of particular interest: *Syk*. This gene is involved in T-cell receptor–dependent signaling pathways and interacts with *Cblb* (16). This could be important because *Cblb* is a known rat diabetes susceptibility gene (17). Loss of function mutations in *Cblb* lead to activation

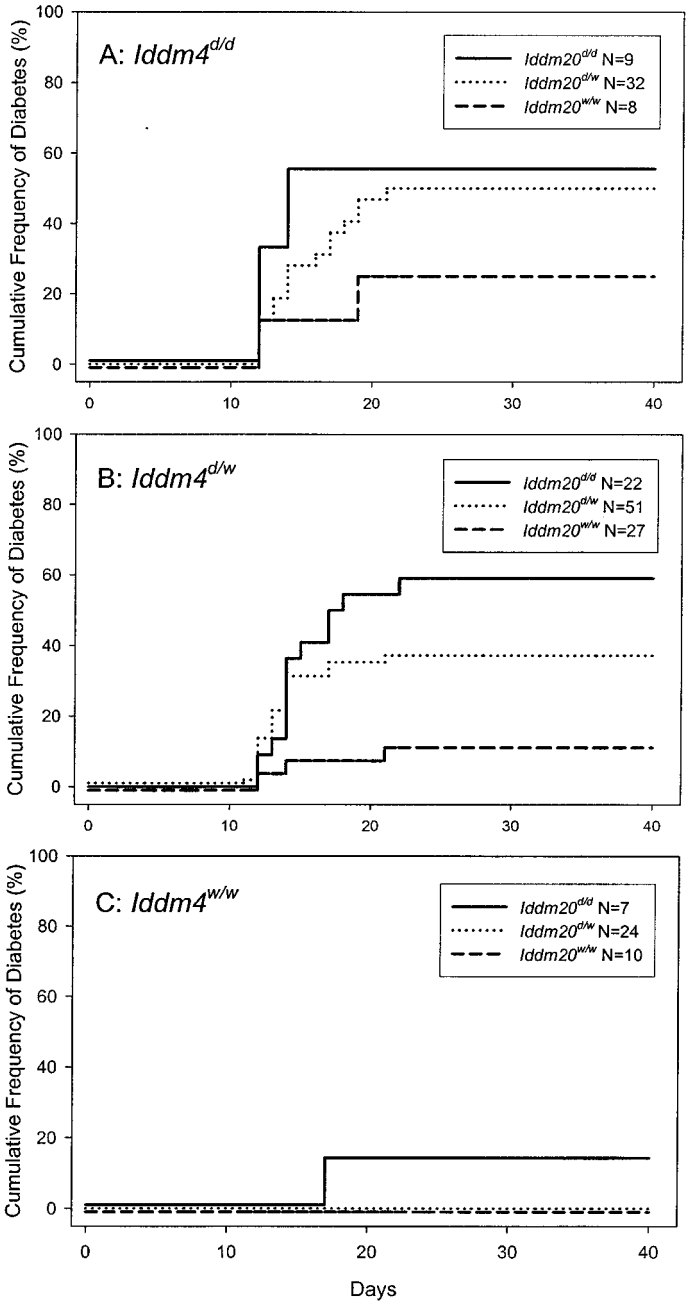

FIG. 2. Kaplan-Meier analysis of the cumulative frequency of autoimmune diabetes by inheritance of BBDR-origin diabetes susceptibility loci on chromosomes 4 and 17 in 190 (WF × BBDR)F2 rats. Time on the horizontal axis is in days after infection with KRV. *A*: Data for F2 rats homozygous for the BBDR-origin allele of *Iddm4*. *B*: Data for the heterozygotes. *C*: Data for F2 rats homozygous for the WF-origin allele of *Iddm4*. Within each panel, individual curves show data for subgroups expressing the indicated BBDR- and WF-origin alleles of *Iddm20*. The number of rats in each subgroup is indicated in the figure. Overall, 59 of 190 F2 animals (31%) became diabetic. Among the diabetic rats, 98% (58 of 59) expressed at least one BBDR-origin allele of *Iddm4*, and of these, 91% (53 of 58) expressed at least one BBDR-origin allele of *Iddm20*. Among all rats homozygous for the WF-origin allele of *Iddm20*, 11% (5 of 45) became diabetic and all 5 expressed at least one BBDR-origin allele of *Iddm4*. Overall statistical analysis of the entire dataset for the effect of *Iddm4* genotype stratified by *Iddm20* genotype was statistically significant (log rank = 20.22, df = 2, $P < 0.0001$). Similarly, analysis for the effect of *Iddm20* genotype stratified by *Iddm4* genotype was statistically significant (14.03, 2, $P < 0.001$). These data are shown in tabular form in online appendix Table 3.

of autoreactive diabetogenic T-cells in the absence of full costimulation (17).

Iddm20, like *Iddm4*, appears to act as a genetic dominant with incomplete penetrance. There is clear disease-promoting activity in the *Iddm20* heterozygote. In the poly I:C plus Treg system, ~70% of both WF.*Iddm4*$^{d/w}$ heterozygotes and WF.*Iddm4*$^{d/d}$ homozygotes become diabetic (10). In the KRV plus poly I:C model, homozygosity for diabetogenic alleles at the *Iddm20* locus increases the penetrance of diabetes in *Iddm4*$^{d/d}$ rats from 25% (in *Iddm20*$^{w/w}$ animals) to 56%, and it increases penetrance in *Iddm4*$^{d/w}$ rats from 11 to 59%. We therefore regard *Iddm20* as a modifier of the *Iddm4* locus.

The mechanisms by which poly I:C and KRV infection act to induce diabetes in genetically susceptible rats are not yet known. We speculate that *Iddm4*, *Iddm20*, or both define a strain-specific response of BBDR rats to KRV infection. KRV is known to infect lymphocytes in the pancreatic lymph nodes (but not the islets) of BBDR rats (18). More recent studies have revealed that KRV also causes a decrease in splenic $CD4^+CD25^+$ Treg cells in both BBDR and normal WF rats (8). In adult LEW rats, KRV-UMass infection is associated with several potentially important effects on both $CD4^+$ and $CD8^+$ T-cell populations (19) but whether allelic variations in *Iddm4* or *Iddm20* regulate those responses is not yet known. By itself, KRV infection typically induces diabetes in ~30–40% of BBDR/Wor rats (8). Pretreatment poly I:C, at a dose that is itself incapable of inducing disease, dramatically increases the penetrance of diabetes (8). The mechanism of this synergy is not clear but is likely to relate to innate immunity because poly I:C is a ligand of toll-like receptor 3 and a potent inducer of type I interferon production by various cells (20) and interleukin-1 production by monocytes (21). It also activates natural killer cells (22) and B-cells (23). In rats, it has been shown that interferon production in response to poly I:C varies substantially in different inbred strains (24), an effect that is presumably genetically determined. It will be of interest to determine whether *Iddm4* and/or *Iddm20* is a determinant of the magnitude of the immune response to poly I:C.

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