Two Genetic Loci Regulate T Cell–Dependent Islet Inflammation and Drive Autoimmune Diabetes Pathogenesis

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Insulin-dependent diabetes mellitus (IDDM) is a polygenic disease caused by progressive autoimmune infiltration (insulitis) of the pancreatic islets of Langerhan, culminating in the destruction of insulin-producing β cells. Genome scans of families with diabetes suggest that multiple loci make incremental contributions to disease susceptibility. However, only the *IDDM1* locus is well characterized, at a molecular and functional level, as alleleic variants of the major histocompatibility complex (MHC) class II HLA-DQB1, DRB1, and DPB1 genes that mediate antigen presentation to T cells. In the nonobese diabetic (NOD) mouse model, the *Idd1* locus was shown to be the orthologous MHC gene I-Ab. Inheritance of susceptibility alleles at *IDDM1/Idd1* is insufficient for disease development in humans and NOD mice. However, the identities and functions of the remaining diabetes loci (*Idd2–Idd19* in NOD mice) are largely undefined. A crucial limitation in previous genetic linkage studies of this disease has been reliance on a single complex phenotype—diabetes that displays low penetrance and is of limited utility for high-resolution genetic mapping. Using the NOD model, we have identified an early step in diabetes pathogenesis that behaves as a highly penetrant trait. We report that NOD-derived alleles at both the *Idd5* and *Idd13* loci regulate a T lymphocyte–dependent progression from a benign to a destructive stage of insulitis. Human chromosomal regions orthologous to the *Idd5* and *-13* intervals are also linked to diabetes risk, suggesting that conserved genes encoded at these loci are central regulators of disease pathogenesis. These data are the first to reveal a role for individual non-MHC *Idd* loci in a specific, critical step in diabetes pathogenesis—T cell recruitment to islet lesions driving destructive inflammation. Importantly, identification of intermediate phenotypes in complex disease pathogenesis provides the tools required to progress toward gene identification at these loci.

Introduction

Type 1 or insulin-dependent diabetes mellitus (IDDM) is a complex trait that is conferred by allelic variation at multiple loci. However, efforts to define the genetics of common polygenic disorders have been hampered by the low penetrance of individual loci, and IDDM is an intensively studied example. Genetic analyses of families with IDDM have generated discrepancies likely reflecting variability among study populations, which limits consensus on disease linkage to only a few loci (Concannon et al. 1998; Mein et al. 1998). Genetic dissection of IDDM has been enabled by the nonobese diabetic (NOD) mouse model (Makino et al. 1980), which closely recapitulates the human disease. Nineteen *Idd* susceptibility loci have been identified in the NOD mouse, and

human-genome scans reveal similarly complex inheritance (Davies et al. 1994; Concannon et al. 1998) in mice (Makino et al. 1980; Ikegami et al. 1995; McAleer et al. 1995; Wicker et al. 1995; Vyse and Todd 1996; Podolin et al. 1997, 1998). The major diabetes-susceptibility locus, *IDDM1* in humans (MIM 222100) and *Idd1* in mice, maps to variation in the major histocompatibility complex (MHC) class II genes. The remarkable sequence similarity between human HLA-DQB1 and mouse H-2 I-AB susceptibility alleles lies in residues crucial for antigen presentation to T cells (Hattori et al. 1986; Acha-Orbea and McDevitt 1987; Todd et al. 1987). However, the molecular basis of MHC-linked diabetes susceptibility is more complex than was originally appreciated. For example, different HLA-DQB alleles are associated with diabetes risk in distinct populations, and evidence for association of both DRB1 (Sheehy et al. 1989) and DPB1 alleles has been reported elsewhere (Erlich et al. 1996; Noble et al. 1996*a*, 1996*b*, 2000). Beyond the critical role of class II alleles, evidence for linkage to extended MHC haplotypes includes variants in the peptide transporter genes in some human populations (Caillat-Zucman et al. 1995; Deng et al. 1995). In the NOD mouse, recent data support at least

two additional diabetes-associated genes in the MHC region near the transporter gene *Lmp2* (Hattori et al. 1999). Thus, MHC markers are unique in displaying strong linkage to the diabetes phenotype in humans and mice, but a susceptible MHC haplotype alone is insufficient to confer disease.

In marked contrast to the strong effects of MHC haplotype, whole-genome scans in NOD mice (Cordell and Todd 1995) and in humans (Davies et al. 1994; Concannon et al. 1998; Mein et al. 1998) suggest that many non-MHC *Idd* loci make incremental contributions to disease susceptibility. Only two human loci outside the MHC have been clearly established in independent studies. *IDDM2* on chromosome 11p15 maps to a VNTR sequence upstream of the insulin gene (Bennett et al. 1995) and has been suggested to affect insulin expression and T cell tolerance to the protein (Bennett and Todd 1996; Vafiadis et al. 1997). A region on human chromosome 2q has been linked to *IDDM,* by several groups (Davies et al. 1994; Copeman et al. 1995; Owerbach and Gabbay 1995; Morahan et al. 1996; Esposito et al. 1998; Mein et al. 1998) and is syntenic to the *Idd5* locus on mouse chromosome 1 (Todd et al. 1991; Ghosh et al. 1993; Garchon et al. 1994; Cordell and Todd 1995; Cordell et al. 1998). However, identification of non-MHC susceptibility genes in humans and mice has been difficult, and neither the identities nor the pathogenic contributions of most of the remaining loci are known.

Previous efforts to map diabetes-susceptibility genes in humans and rodents share a crucial limitation—the reliance on a single complex phenotype, diabetes. Since this clinical endpoint reflects the actions of many genes acting in complex pathways, it is not surprising that the influence of single loci is hard to resolve. We reasoned that the identification of intermediate (preclinical) diabetes phenotypes in islet inflammation might better reflect the action of one or a subset of essential genes common to IDDM-susceptible humans and rodents. One such phenotype was provided by identification of a pivotal early step in the islet inflammatory process.

Infiltration of the pancreatic islets of Langerhans by T cells and antigen-presenting cells (APCs) is a protracted process that precedes overt diabetes by months to years (Hanninen et al. 1992; Itoh et al. 1993). Infiltration begins as a leukocyte accumulation around the islet perimeter (peri-insulitis), which then invades the islet interior (invasive insulitis), ultimately resulting in diabetes in 70%–90% of female NOD mice by age 6 mo (Signore et al. 1989; Castano and Eisenbarth 1990; Jarpe et al. 1991; Waters et al. 1992; Jansen et al. 1994; Signore et al. 1994; Fox and Danska 1997). T cells are critical for invasive insulitis, since protracted peri-insulitis occurs in NOD mice depleted of T cells by administration of antibodies directed against T cell surface

markers (Charlton et al. 1988; Sempe et al. 1991; Chatenoud et al. 1994, 1997). Considerable evidence indicates that destruction of islet β cells is also T cell–dependent (reviewed in Toyoda and Formby 1998). Although T cell function is clearly central to destructive islet inflammation, the *Idd* loci that control this aspect of autoimmune pathogenesis remain undefined.

A clue to the genetic control of T cell pathogenesis was provided by our previous study, in which we compared insulitis progression in both NOD and the nonobese, diabetes-resistant (NOR) strain of mice (Fox and Danska 1998). NOR is a recombinant inbred strain that inherits 88% of the genome from NOD and the remaining 12% from the C57BLKS/J strain (Prochazka et al. 1992). The NOR and NOD strains share the diabetogenic MHC haplotype H-2^{g7} (*Idd1*) but differ at 5 of the remaining 18 *Idd* loci (Prochazka et al. 1992; Serreze et al. 1994). Thus, the inheritance of C57BLKS/J-derived alleles at *Idd4, -5, -9, -11,* and *-13* is sufficient to protect NOR from IDDM. We found that NOR diabetes resistance correlated with a protracted peri-insulitis involving minimal T cell contribution (Fox and Danska 1998). Thus, transition from peri- to invasive insulitis appeared to be dependent on T cell infiltration and the *Idd* loci that are allelically variable between NOR and NOD mice. Importantly, these results implicated distinct genetic control of different immune-cell types in early islet inflammation. Here, we have investigated the genetic control of this step in diabetes development by performing a linkage analysis in (NOD × NOR) F2 progeny. Progression from peri- to invasive insulitis behaved as a highly penetrant trait controlled by an interaction between 2 of the 19 *Idd* loci, *Idd5* and *-13*. Remarkably, chromosomal regions syntenic to *Idd5* and *-13* are the only known non-MHC diabetes-susceptibility loci that are shared by humans and mice. Our data reveal for the first time that these two loci regulate a critical early step in diabetes pathogenesis, orchestrated through T cell recruitment to nascent autoimmune islet lesions.

Material and Methods

Mice

All mice used in these studies were maintained in a barrier facility at the Hospital for Sick Children (Toronto). In our colony, diabetes incidence at age 6 mo in NOD animals is 83% in females, 9% in males, and 0 in NOR and (NOD × NOR) F1 mice.

Immunohistochemistry

Pancreata were removed from 80-d-old NOD and NOR (NOD × NOR) F$_1$ female mice and from (NOD × NOR) F$_2$ intercross male and female mice, were

immersed in TissueTec (Bayer Labs), snap-frozen in liquid nitrogen, and stored at $-70°$ C. Preparation of serial frozen sections was performed with a Leica CM 3050 Cryostat (Leica Canada). To maximize analysis of independent islet infiltrates, serial 5-μm sections were prepared from each of three depths of the 5-mm-thick tissue block, in which each series of sections examined was sampled at intervals of $\geqslant 300$ μm apart, as described elsewhere (Fox and Danska 1998). Previous morphometric studies demonstrate that 2–3-mo-old mice have islets averaging 100 μm in diameter and that each pancreas contains ~1,000 islets (Bonnevie-Nielsen et al. 1983). Pancreatic sections were stained with Mayer's hematoxylin and eosin Y (H+E; Sigma), to visualize leukocyte infiltration. For immunohistochemical analysis, serial sections were then incubated with biotin–anti-CD3 (145-2C11; [Leo et al. 1987]) or biotin–anti-MHC class II (10.2-16 [Oi et al. 1978]) antibodies and ExtrAvidin-Horseradish peroxidase (Sigma). Antibody staining was visualized with AEC substrate (Sigma). Antibodies were prepared by protein G purification from tissue-culture supernatants and were biotin conjugated (Cedarlane Labs), as described elsewhere (Fox and Danska 1997). Insulitis severity was scored in H+E–stained pancreatic sections. Approximately 250 islets from three different tissue depths >300 μm apart were examined in each animal and were graded by assignment of the following scores: 0, no visible infiltrates; 1, peri-insulitis, indicated by perivascular and peri-islet infiltrates; 2, <50% of the islets displaying invasive infiltrates (defined as islet interior occluded by leukocytes); 3, >50% of the islets displaying invasive insulitis when <50% of the islet is occluded by leukocytes; or 4, complete infiltration, virtually all islets displaying invasive insulitis of 50%–100% of the islet. This approach and scoring system has been used extensively for analysis of autoimmune insulitis, by many laboratories (Wicker et al. 1987, 1992; Stein et al. 1992; Yui et al. 1996).

Genomic DNA Preparation

Splenic DNA from C57BLKS/J mice was purchased from the Jackson Labs DNA Resource (Jackson Laboratory). Genomic DNA was also prepared from tail snips of 30 d-old DBA/2J, C57BL/6J, NOD, NOR (NOD × NOR) F_1, and (NOD × NOR) F_2 intercross mice, by standard methods. Each preparation was diluted 1:100 for use in PCR amplification.

Genotyping

Genotyping of NOR and (NOD × NOR) F_2 intercross progeny was performed using microsatellite markers (Whitehead Institute for Biomedical Research/ MIT Center for Genome Research). PCR primer pairs were obtained from Research Genetics. (NOD × NOR)

F_2 intercross animals were typed for microsatellite alleles at *Idd4* (D11Mit30 and D11Mit320), *Idd5* (D1Mit3, D1Mit8, D1Mit24, D1Mit46, D1Mit77, and D1Mit122), *Idd9/11* (D4Mit13, D4Mit16, and D4Mit72), and *Idd13* (D2Mit17, D2Mit135, D2Mit338, D2Mit395, D2Mit411, D2Mit423, and D2Mit490). These mice were also typed at C57BLBKS-derived regions in the NOR genome that do not contain known *Idd* loci on chromosomes 7 (D7Mit105), 12 (D12Mit230), and 18 (D18Mit4 and D18Mit197). NOR mice were typed for NOD-, DBA/2J- or C57BL/ 6J-derived alleles at multiple markers on chromosome 1 (D1Mit3, D1Mit8, D1Mit11, D1Mit18, D1Mit22, D1Mit24, D1Mit46, D1Mit48, D1Mit65, D1Mit66, D1Mit72, D1Mit77, D1Mit122, D1Mit178, D1Mit212, D1Mit213, D1Mit231, D1Mit245, D1Mit279, D1Mit305, D1Mit306, D1Mit322, D1Mit383, D1Mit411, D1Mit414, and D1Mit430) and chromosome 2 (D2Mit17, D2Mit22, D2Mit62, D2Mit135, D2Mit144, D2Mit147, D2Mit206, D2Mit229, D2Mit256, D2Mit333, D2Mit338, D2Mit343, D2Mit393, D2Mit395, D2Mit411, D2Mit423, D2Mit447, D2Mit452, D2Mit480, D2Mit490, and D2Mit493). The order of these markers derives from the Whitehead Institute for Biomedical Research/MIT Center for Genome Research. For most primer pairs, 30 ng of genomic DNA was amplified for 35–40 cycles, with the following conditions: 30 s at 94°C, 30 s at 55°C, and 1 min at 72°C. For D11Mit30, D4Mit11, D12Mit51, and D18Mit21, PCR amplification was performed with 30 ng of genomic DNA for 40 cycles (30 s at 94°C, 40 s at 50°C, and 1 min 10 s at 72°C in 2.5 mM $MgCl_2$). The PCR amplification products were electrophoresed through 2% NuSieve (American Bioanalytical) and 2% agarose (Gibco BRL) gels and were visualized with ethidium bromide.

Statistical Analysis

Quantitative-trait linkage analysis was performed as follows: a genetic map was built on the basis of the Whitehead Institute for Biomedical Research/MIT Center for Genome Research and EUCIB (UK Human Genome Project Mapping Center) marker orders, and markers were analyzed for trait linkage, by the MAP-MAKER EXP v3.0 and QTL v1.1 suite of programs (Lander et al. 1987; Genome Center Software Information and Documentation). Multipoint analysis was conducted using recessive, dominant, and additive models. Linkage to chromosome 1 (*Idd5*) was maximal under an additive model, and linkage to chromosome 2 (*Idd13*) was maximal under a dominant model (see the Results section). The LOD scores, proportion of the trait variance explained, and 1-LOD confidence intervals of the position of the peaks were determined for all animals

and separately for females and males. Haplotype construction was performed, to detect close double recombinants, and microsatellite typing was reexamined, to correct any errors. For chromosome 2 markers, several mice still typed as close double recombinants and were excluded from the study. Furthermore, since the marker order on chromosome 2 differs markedly between different reference maps, we also analyzed chromosome 2 by using pairwise linkage.

Results

T Cell Dependence of Transition from Peri- to Invasive Insulitis

To determine whether progression from peri- to invasive insulitis was invariably associated with T cell infiltration, we analyzed T cell and APCs presence in serial pancreatic sections of 80-d-old NOD, NOR, (NOD × NOR) F_1, and (NOD × NOR) F_2 intercross mice. Invasive insulitis in NOD and (NOD × NOR) F_1 animals (fig. 1*A*, panels *a* and *b*) was characterized by coincident infiltration of MHC class II$^+$ APCs (fig. 1*A*, panels *d* and *e*) and CD3ε^+ T cells (fig. 1*A*, panels *g* and *h*). In contrast, only mild peri-insulitis was seen in NOR mice (fig. 1*A,* panel *c*). Although robust MHC class II$^+$ staining was evident in NOR peri-islet infiltrates (fig. 1*A,* panel *f*), few or no CD3ε-expressing T cells were observed in these lesions (fig. 1*A,* panel *i*), a result concordant with our previous study (Fox and Danska 1998). In (NOD × NOR) F_2 intercross progeny (*n* = 19), both the NOD and NOR parental phenotypes were observed. Progression to invasive insulitis (fig. 1*B,* panel *a*) was associated with islet infiltration by APCs and T cells (fig. 1*B,* panels *c* and *e,* respectively). In contrast, other (NOD × NOR) F_2 mice displayed peri-insulitis (fig. 1*B,* panel *b*) characterized by MHC class II$^+$ APCs in the absence of CD3ε^+ T cells (fig. 1*B,* panels *d* and *f,* respectively). These results suggested that the transition from peri- to invasive insulitis depends on T cell recruitment and is regulated by loci that are allelically variable between NOD and NOR mice.

Next, we examined whether the progression from peri- to invasive insulitis behaved as a preclinical trait suitable for genetic linkage analysis. Insulitis severity was measured in 82 male and female (NOD × NOR) F_2 progeny at age 80 d. Two distinct phenotypes were evident in the F_2 animals: 39% had invasive insulitis (insulitis score ≥ 1.5) and 61% had mild peri-insulitis (insulitis score ≤ 0.3), a result characteristic of a bimodal trait (fig. 2). Sex dimorphism is well established in NOD diabetes, in which disease incidence is higher in female, compared with male, mice (Makino et al. 1980; Pozzilli et al. 1993). In 80-d-old (NOD × NOR) F_2 animals, only a marginal increase in insulitis severity was observed in

female, compared with age-matched male, mice (fig. 2; Mann-Whitney test for significance, $U = 590$, $P = .03$). We previously used quantitative reverse transcriptase–coupled PCR to examine inflammatory progression in purified male and female NOD islets. At age 30 d, T cell cytokine profiles in islet lesions were already biased toward T helper 1 in females and T helper 2 in males. Sex differences in the abundance of T cells and APCs were not evident until age 70 d (Fox and Danska 1997). Examination of the insulitis phenotype at age 80 d in the current study focused on an early stage in autoimmune disease progression, a stage prior to the appearance of substantial differences in severity of male and female islet inflammation.

NOD-Derived Alleles at Idd5 and Idd13 Control Invasive Insulitis

All (NOD × NOR) F_2 intercross progeny were genotyped for NOD- or BKS-derived alleles at microsatellite markers spanning the *Idd4, -5, -9/11,* and *-13* loci and at markers in C57BLKS/J-derived regions of the NOR genome that lacked previously identified *Idd* loci. Pairwise (table 1) and multipoint (fig. 3) statistical analyses of the insulitis-severity scores and genotypes revealed significant linkage (Lander and Kruglyak 1995) of invasive insulitis to markers of *Idd5* on chromosome 1 and *Idd13* on chromosome 2. LOD scores were calculated separately for males and females, as well as for all the (NOD × NOR) F_2 animals. A significant LOD score for all animals was observed at the marker D1Mit305 (LOD, 5.7; fig. 3*A*) when an additive model was used, with the females displaying strong linkage (LOD, 5.3) whereas the males produced virtually no linkage across this region when the same model was used (LOD, <1.0). We also observed significant linkage to a second locus with maximal linkage at the marker D2Mit395, with LOD scores of 5.7 for all animals, 4.0 for females, and 1.7 for males, under the assumption of a dominant model (fig. 3*B* and table 1). In contrast, all other markers examined in regions of allelic variation between the NOD and NOR strains did not show significant linkage (LOD, ≤2.6; data not shown). D1Mit305 and D2Mit395 accounted for 31% and 27%, respectively, of the genetic variation in insulitis phenotype and generated scores 2 log greater than the benchmark defined for significant linkage (Lander and Kruglyak 1995).

These findings suggest that NOD-derived alleles at both *Idd5* and *Idd13* drive invasive insulitis, providing evidence of a genetic interaction controlling this phenotype (Frankel and Schork 1996). To analyze this interaction, we compared the degree of insulitis in (NOD × NOR) F_2 progeny with all possible combinations of NOD and NOR alleles at the two loci (fig. 4). Clearly, homozygosity for BKS-derived alleles at either
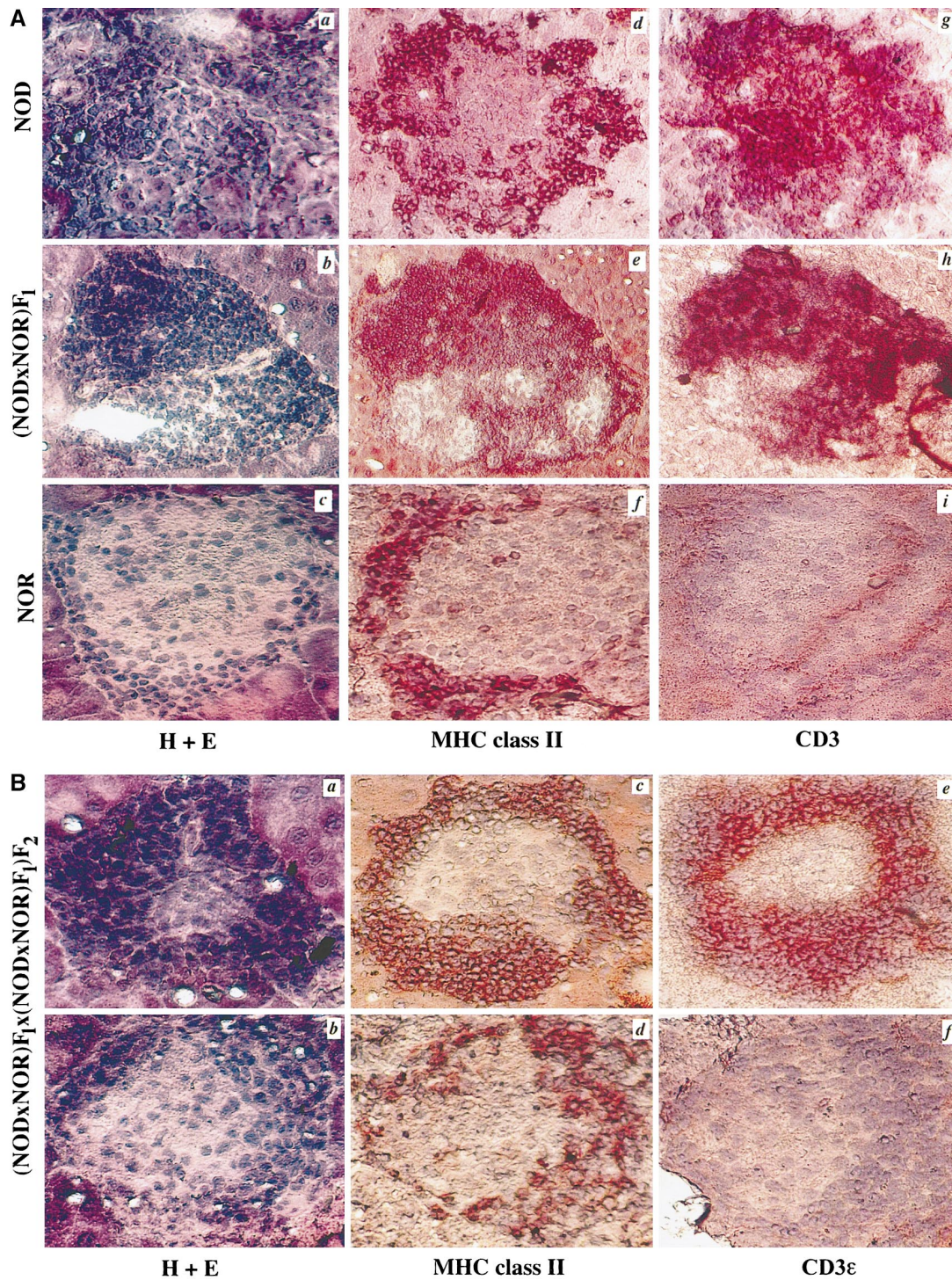
Figure 1   Invasive insulitis is T cell dependent. *A,* Serial pancreatic cross-sections from 80-d-old female NOD, (NOD × NOR) F$_1$, and NOR mice were stained with H+E, to visualize infiltrating cells (panels *a–c*); with the anti-MHC class II antibody 10.2-16, to visualize APC (panels *d–f*); and with the anti-CD3e antibody 145-2C11, to visualize T cells (panels *g–i*). Panels *d–i* were counterstained in Mayer's hematoxylin. *B,* Serial sections from two representative (NOD × NOR) F$_2$ intercross progeny were stained with H+E (panels *a* and *b*), anti-MHC class II (panels *c* and *d*), and anti-CD3 (panels *e* and *f*). Positive protein expression is indicated by dark brown coloration. Original magnification, × 250.
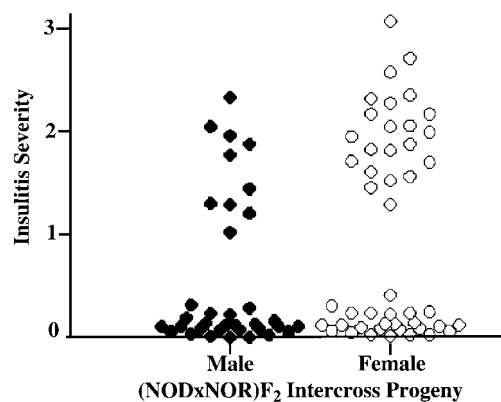
Figure 2 Insulitis severity in (NOD × NOR) F$_2$ intercross progeny. The average insulitis severity was calculated in 82 80-d-old (NOD × NOR) F$_2$ intercross mice by the scoring of H+E–stained pancreatic cross-sections. As shown, a scatter plot comparing individual insulitis severity in male (*blackened circles*) and female (*unblackened circles*) animals clearly indicates that insulitis severity behaves as a bimodal trait.

Idd5 (D1Mit305) or *Idd13* (D2Mit395) conferred potent resistance to invasive insulitis; all animals of these genotypes displayed insulitis scores <0.3 (fig. 4). The powerful role of NOD alleles at these two loci was also evident in this study. Insulitis progression (severity score >1.0) was observed only in the (NOD × NOR) F$_2$ animals that carried at least one NOD-derived allele at both *Idd5* and *Idd13* (fig. 4). (NOD × NOR) F$_2$ mice carrying NOD alleles at Idd5 and Idd13 provided evidence for the penetrance of the insulitis-progression phenotype. Seventy-four percent (32/43) of F$_2$ animals carrying at least one NOD-derived allele at both *Idd5* and *Idd13* displayed insulitis scores >1.0. Evidence for genetic interaction between *Idd5* and *Idd13* is provided by comparison of insulitis values of mice with at least one NOD allele at each locus (n = 38) to the other animals (n = 44). Using a t test for nonequal variances in the two groups yielded t = 9.3, 44 df; two-tailed P < .0001. Thus, a genetic interaction between NOD-derived alleles at the *Idd5* and *Idd13* loci promotes the transition from peri- to invasive insulitis, an obligate step in early islet inflammation. The effect could be blocked by homozygosity for BKS-derived resistance alleles at either locus. Interestingly, although the phenotype distributions were very similar between male and female (NOD × NOR) F2 progeny (fig. 2), males contributed little to the *Idd5* linkage (fig. 3). This observation likely reflects the relatively small numbers of animals with each of the nine possible *Idd5* and *Idd13* genotype combinations when they are subdivided by sex (fig. 4).

Idd5 and Idd13 Regulate T Cell Influx into Islet Lesions

Progression to invasive insulitis was associated with T cell invasion of islet lesions (fig. 1*A* and *B*), suggesting

that the two features are controlled by the same loci. We used immunohistochemistry to examine T cells and APCs in 19 randomly selected (NOD × NOR) F$_2$ intercross mice. Eight of these animals (3 male and 5 female) had inherited at least one NOD-derived allele at *Idd5* and *Idd13;* all displayed invasive insulitis (severity scores 1.2–2.0), and 98% of their affected islets showed T cell infiltration (table 2, *top*). In contrast, 11 animals (2 male and 9 female) were homozygous for BKS-derived alleles at either or both of the *Idd5* and *Idd13* loci; all of these mice displayed mild peri-insulitis (severity scores 0.08–0.23), and T cells were observed in only 4.8% of the islet lesions scored in these animals (table 2, *bottom*). Strikingly, these results suggest that the mechanism by which *Idd5* and *Idd13* drive insulitis progression is T cell recruitment to, or retention in, islet lesions.

Genetic Maps of NOR Chromosomes 1 and 2

Progress toward the identification of the genes at *Idd5* and *Idd13* depends on high-resolution genetic mapping. NOR is a recombinant inbred strain providing an opportunity to define the C57BLKS/J-derived segments, of chromosomes 1 and 2, in which the genes regulating insulitis progression reside. The C57BLKS/J strain was derived from two parental strains, C57BL/6J (B6) and DBA/2J (Naggert et al. 1995). Consequently, NOR mice harbor chromosomal segments of NOD, DBA/2J, and B6 origin. To refine the boundaries of the NOR chromosome 1 and 2 segments that were of NOD, DBA/2J or B6 origin, NOR mice were typed with microsatellite markers, and these markers were ordered according to current genetic maps (see the Materials and Methods section).

Table 1

Pairwise Linkage Analysis of Chromosome 2

	%	LOD	POSITION	
MARKER	VARIANCE	SCORE	MIT	JAX
D2Mit395	26.9	5.3	55.7	66.9
D2Mit17	16.4	3.1	56.8	69.0
D2Mit338	23.3	4.5	59.0	57.5
D2Mit135	20.2	3.9	61.2	73.0
D2Mit490	19.7	3.8	64.5	64.5
D2Mit423	14.3	2.7	68.9	68.9
D2Mit411	7.9	1.4	77.6	77.6

NOTE.—The (NOD × NOR) F$_2$ data for markers of chromosome 2 were also analyzed by pairwise linkage analysis. The marker name, percentage of variance, and LOD score are shown. As is also shown, the relative order of these markers differs according to information available at the Whitehead Institute for Biomedical Research/MIT Center for Genome Research (MIT) and the Jackson Laboratory (JAX). For both databases, the position of each marker from the centromere of chromosome 2 is shown in centimorgans.
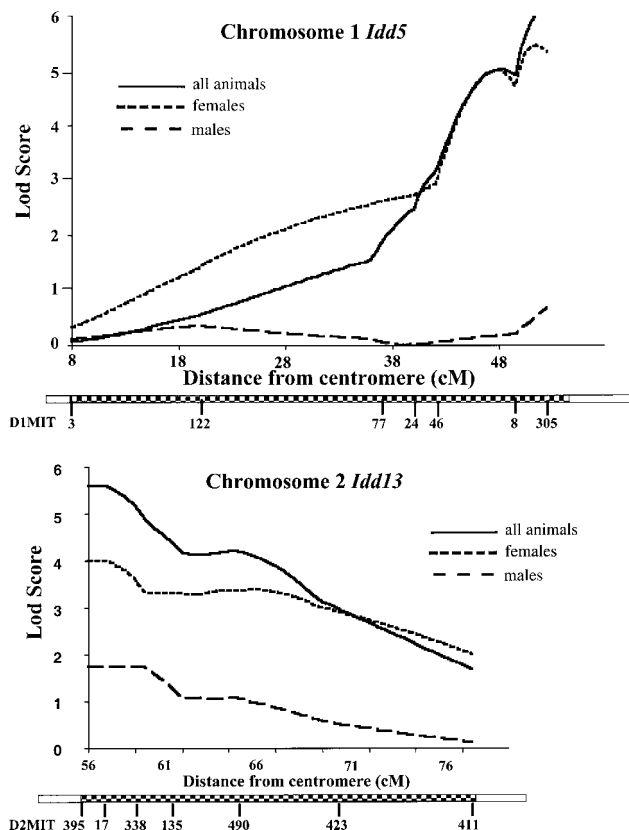
3 and 5B). As mentioned in the context of multipoint linkage analysis, there is not yet a clear consensus on the order of some chromosome 2 markers. On the basis of these marker positions and the linkage analysis, we computed a 5-cM 1-LOD confidence interval for *Idd5* on chromosome 1 (fig. 5A) and a 4-cM 1-LOD confidence interval for *Idd13* on chromosome 2 (fig. 5B).

Discussion

Studies in humans, NOD mice, and BioBreeding (BB) rats have demonstrated that the generation and function of islet-reactive T cells depends on MHC haplotype (Colle et al. 1981; Hattori et al. 1986; Todd et al. 1987; Wicker et al. 1987). Clearly, β islet–cell destruction requires the coinheritance of multiple susceptibility loci (reviewed in Cordell and Todd 1995). Unraveling how and when non-MHC *Idd* genes regulate IDDM pathogenesis is key to the development of therapies to modulate disease progression in individuals at high-risk. The success of identification of MHC class II as a major diabetes gene, as well as the remarkable sequence similarity between human DQβ and mouse I-Aβ susceptibility alleles, established a precedent that will not be easily fulfilled by other *Idd* genes. Unlike the high diabetes risk conferred by MHC class II alleles, it has been suggested that non-MHC genes contribute a small, incremental influence on disease susceptibility. Whole-genome scans in humans and in mice (Cordell and Todd 1995) have supported this idea, although the former have yielded disparate results in different study popu-
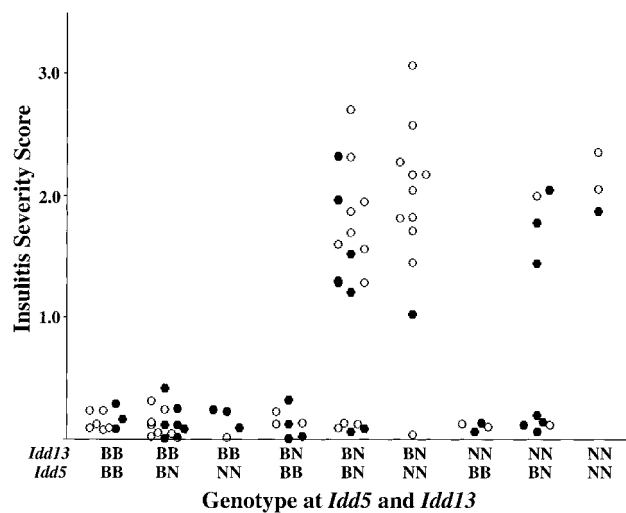
Figure 3 NOD-derived alleles at *Idd5* and *Idd13* confer invasive insulitis. The LOD scores for the linkage of invasive insulitis in females, males, and all animals are shown vs. the distance in centimorgans from the centromeres of *A,* chromosome 1 and *B,* chromosome 2. The relative order of the microsatellite markers along each chromosome (according to Whitehead Institute for Biomedical Research/MIT Center for Genome Research) is shown beneath the *x* axis of each graph. C57BL/6J-derived regions (*checkered boxes*) are shown on NOR chromosomes 1 and 2. The remaining regions (*unblackened boxes*) are NOD-derived. *(NOD × NOR) F₂ intercross animals could not be scored for linkage telomeric to D1Mit305 or centromeric to D2Mit395 since these markers very close to the boundaries of these C57BL/6J-derived chromosome segments in NOR mice.

Using 25 microsatellite markers on chromosome 1, two B6-derived segments—one of 16 cM and another of 21 cM in length, located between the D1Mit66 and D1Mit322 loci and between the D1Mit178 and D1Mit84 loci, respectively—were resolved in NOR (fig. 5A). Control of invasive insulitis was most closely linked to D1Mit305, near the telomeric boundary of the latter segment (fig. 3). Analysis of 22 microsatellite markers on chromosome 2 showed that NOR mice contained a 25-cM B6-derived segment extending between D2Mit62 and D2Mit144 (fig. 5B). On chromosome 2, maximal linkage of T cell–dependent invasive insulitis was observed at D2Mit395, near the centromeric boundary of the B6-derived segment (fig.

Figure 4 Histogram scatter plots of the insulitis severity in male (*blackened circles*) and female (*unblackened circles*) (NOD × NOR) F₂ intercross progeny distributed by combination of NOD (N) and C57BKS/J (B) alleles at *Idd5* (D1Mit305) and *Idd13* (D2Mit395).

Figure 5   Chromosomes 1 and 2 in NOR mice. NOR genomic DNA was typed for NOD- (*unblackened boxes*) or C57BL/6J-derived (*checkered boxes*) alleles at each microsatellite marker shown on chromosomes 1 (*A*) and 2 (*B*). The distance in centimorgans between markers is shown to the far left. Several candidate genes that map to each region are also shown (in italics). * = 1-LOD confidence intervals were calculated with MAPMAKER QTL. The gene locations and distances in centimorgans are adapted from published studies (Seldin 1996, 1997; Siracusa et al. 1996, 1997), the Whitehead Institute for Biomedical Research/MIT Center for Genome Research, and the Jackson Laboratory. The original location of *Idd5* encompasses an interval 20–60 cM from the centromere of chromosome 1 (Cornall et al. 1991; Garchon et al. 1991). *Idd13* was originally mapped to a 4-cM interval of chromosome 2 that spans the $\beta_2 m$, *Il1*, and *Pcna* genes (Serreze et al. 1994), within which the 1-LOD confidence interval for insulitis progression resides.

number of genes without obvious links to diabetes pathogenesis map proximal to D1Mit305 (fig. 5A), including those for the nicotinic acetylcholine receptor, *Acrg* (Heidmann et al. 1986; Cohen-Haguenauer et al. 1989); collagen type VI, *Col6a3* (Schurr et al. 1990); retinal S-antigen, *Sag* (Danciger et al. 1989); and the high-density lipoprotein-binding protein, *Hdlbp* (Xia et al. 1993; LeBoeuf et al. 1994). At *Idd5*, as at all other *Idd* regions, the mapping of expressed sequence tags and defined coding regions is far from complete, so it remains likely that these regions contain genes that have yet to be identified and may include novel genes involved in diabetes susceptibility.

Idd13 in Diabetes Pathogenesis

The second locus to which we mapped T cell–dependent invasive insulitis is D2Mit395 in the *Idd13*

Table 3

Chromosomal Regions Orthologous to Murine *Idd5* and *Idd13* Contain Diabetes-Susceptibility Genes in the Rat and Human Genomes

MOUSE			RAT		HUMAN	
Name (Chromosome)	Locations	Phenotype (References)	Name (Chromosome)	Location (References)	Name	Location (References)
Idd5 (1)	Bcl2- D1Nds1	Peri-insulitis (Garchon et al. 1991)	*Iddm5* (13)	D13Mit1 diabetes progession (Martin et al. 1999)	IDDM7	Chromosome 2q (Coperman et al. 1995; Esposito et al. 1998)
Idd5 (1)	D1Nds4	Spontaneous and cyclophos-accelerated diabetes (Cornall et al. 1991)			IDDM12	Chromosome 2q31 (Owerbach and Gabby 1995; Esposito et al. 1998)
Idd5 (1)	D1Mit5	Insulitis kinetics and severity (Ghosh et al. 1993), insulitis susceptibility (Yui et al. 1996)			IDDM13	Chromosome 2q D2S164 (Morahan et al. 1996; Esposito et al. 1998)
Idd5 (1)	D1Mit4–Bcl2, D1Mit5–Mit15, D1Mit305	T cell recruitment and insulitis progression, interaction with *Idd13* (present study)			IDDM6	Chromosome 18q21 (Merriman et al. 1997, 1998)
Idd13 (2)	D2Mit490–Mit144, H3a-IL-1, IL-1-Pcna	IDDM (Serezze et al. 1998)	*Iddm6* (3)	D3Mgh10, diabetes; D3Mit4, insulitis severity, interaction with *Iddm5* (Martin et al. 1999)	IDDM3	Chromosome 15q26 D15S107 (Field et al. 1994; Zuberi et al. 1996)
Idd13 (2)	D2Mit395	T cell recruitment and insulitis progression, interaction with *Idd5* (present study)				

NOTE.—Mouse, human, and rat homology maps (NCBI Human/Mouse Homology Relationships) establish that regions of the human and rat genomes previously found to be linked to type 1 diabetes risk, are orthologous to the murine *Idd5* and *Idd13* intervals identified in this and prior studies of the NOD mouse. For *Idd5* on chromsome 1, multiple diabetes-susceptibility regions were identified over a broad region of human chromosome 2q and on human 18q. Interestingly, the region of the rat genome orthologous to murine *Idd13* on chromosome 2 (55–60 cM), is *Iddm6*, which demonstrates genetic interaction with *Iddm5* on rat chromosome 13. The latter region is orthologous to murine chromosome 1 (56–62 cM) which overlaps the 1-LOD confidence interval for the *Idd5* linkage to insulitis progression in this study.

locus. *Idd13* originally was mapped to a 4-cM region of chromosome 2, flanked by the genes for beta-2-microglobulin ($\beta_2 m$) and proliferating-cell nuclear antigen (*Pcna*; Serreze et al. 1994), although recent evidence suggests that it is ≥30 cM in length (Serreze et al. 1998). It is likely that the linkage reported here for the insulitis progression lies within the previously published *Idd13* interval (Serreze et al. 1998), although discrepancies between current chromosome 2 maps (Whitehead Institute for Biomedical Research/MIT Center for Genome Research, Jackson Laboratory [Siracusa et al. 1996, 1997]) remain to be clarified. Importantly, murine *Idd13* is orthologous to the *Iddm6* region of rat chromosome 3 (Martin et al. 1999) and may be orthologous to a human chromosome 15 segment that contains the *IDDM3* locus (Field et al. 1994; Zamani et al. 1996), but there is some disagreement on this point (Mein et al. 1998; table 3). Of particular interest, insulitis severity in the BB rat model was shown to be influenced by genetic interaction between the *Iddm6* locus and the *Iddm5* locus (Martin et al. 1999), a result consistent with our findings of strong interaction between the syntenic murine *Idd13* and *Idd5* loci, respectively. In all three species, this chromosome region includes $\beta_2 m$, a subunit of the MHC class I complex. In multiple studies, allelic variation between NOD and NOR mice has been reported to affect both the structure and the expression of MHC class I. For example, the interval containing the NOD-derived *Idd13* encodes a $\beta_2 m$ variant that affects an MHC class I serological determinant (Serreze et al. 1998). In addition, interferon-γ–mediated up-regulation of cell-surface MHC expression is reportedly absent in cultured NOD macrophages (Leiter and Serreze 1992), although the *Idd13* locus does not appear to control this effect (Serreze et al. 1998). Allelic variation in the $\beta_2 m$ sequence also exists between normal mouse strains (Michaelson 1983), so it remains unresolved how functional variants in this protein may contribute to autoimmune pathogenesis. Additional genes within the confidence interval computed from our analysis include a cluster encoding minor histocompatibility antigens (Graff et al. 1994), recombinase A (*Reca* [Takahasi et al. 1994; Roca and Cox 1997]), lymphocyte tyrosine kinase (*Ltk* [Snijders et al. 1997]), and receptor tyrosine kinase (*Tyro3* [Liao et al. 1996]) involved in bone resorption (Nakamura et al. 1998). The proinflammatory cytokine interleukin-1 (*Il1* [Siracusa et al. 1996, 1997]) also has been considered a candidate gene for mouse *Idd13* and for human *IDDM13* on chromosome 2q (Esposito et al. 1998). Considerable work remains to isolate and analyze the function of gene(s), at the *Idd13* and *Idd5* loci, that regulate the progression from benign to invasive insulitis.

Conclusions

Rodent models of polygenic disease provide a unique opportunity to coordinate genetic analyses with the study of cellular pathogenesis. By comparing NOD and NOR mice, we have identified a discrete step in insulitis progression that is conferred by two susceptibility loci and, by inference, two (sets of) genes that are common to IDDM-susceptible humans and rodents. NOR mice inherit genes from the NOD background that promote the peri-islet and perivascular infiltration by APCs, the earliest detectable stage in diabetes pathogenesis. However, this stage is apparently benign, since these lesions contain few T cells (Fox and Danska 1998), and NOR animals rarely progress to overt diabetes. Here we have shown that NOD alleles at *Idd5* and *Idd13* together regulate T cell influx into islet lesions, which is an obligate step in autoimmune pathogenesis and evident months before overt disease onset. Our identification of punctuated steps in the insulitis process suggested a "simpler" preclinical phenotype that enabled genetic linkage to two of many susceptibility loci and insight into the cellular biology that these loci control. Genetic analysis of other polygenic diseases underscores the utility of this strategy. In (NZB × NZW) F$_1$ mice, B cell phenotypes that are markers of the autoimmune events in systemic lupus erythematosus have greatly assisted in the identification of susceptibility loci (Vyse et al. 1996; Mohan et al. 1997). In type 2 diabetes, physiological and genetic studies in rat models has allowed linkage of multiple loci with specific facets of the disease, including impaired insulin secretion, increased body-mass index, and elevated blood-glucose levels (Galli et al. 1996; Gauguier et al. 1996). The genetic variants that confer diabetes risk in rats or mice may well differ from those in humans, and, similarly, different constellations of genes may mediate diabetes in genetically diverse human populations. Although these genes may differ between species and populations, they are likely to act on common biological pathways that control islet inflammation and β cell destruction. Importantly, the identification of rodent genes within these shared pathways has promise for resolving the molecular basis of diabetes pathogenesis, thereby illuminating targets for therapeutic intervention in high-risk or newly diagnosed individuals.

Acknowledgments

Electronic-Database Information

The accession number and URLs for data in this article are as follows:

Genome Center Software Information and Documentation, http://waldo.wi.mit.edu/genome_software
Jackson Laboratory, http://www.jax.org
NCBI Human/Mouse Homology Relationships, http://www.ncbi.nlm.gov/Homology
Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim (for *IDDM1* [MIM 222100]
Research Genetics, http://www.resgen.com
UK Human Genome Mapping Project Resource Centre, http://www.hgmp.mrc.ac.uk
Whitehead Institute for Biomedical Research/MIT Center for Genome Research, http://www-genome.wi.mit.edu

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