Brief Definitive Report

Use of Recombinant Congenic and Congenic Strains of NOD Mice To Identify a New Insulin-dependent Diabetes Resistance Gene

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Summary

Insulin-dependent diabetes mellitus (IDDM) in NOD/Lt mice represents a complex polygenic disease. NOR/Lt is a recombinant congenic strain (RCS) in which limited regions of the NOD/Lt genome have been replaced by genome from the C57BL/KsJ strain. NOR mice are insulitis resistant and diabetes free despite genetic identity with NOD at numerous chromosomal regions containing previously described insulin-dependent diabetes (*Idd*) genes, including the strongly diabetogenic $H2^{g7}$ major histocompatibility complex (MHC) haplotype. The present study revealed BKs-derived genome on segments of chromosomes (Chr) 1, 2, 4, 5, 7, 11, 12, and 18, approximating 11.6% of the total NOR genome analyzed. (NOD × NOR)F_2 segregation analysis was employed to identify chromosomal regions in NOR containing *Idd* resistance alleles. IDDM developed in 33% (10/30) of F_1 females, and 29.3% (36/123) of F_2 females aged to 1 yr. A previously unrecognized diabetes resistance locus (designated $Idd13^r$) strongly protective in homozygous state was identified on NOR Chr 2 in linkage with the *Il1α* structural gene. The existence of this locus was confirmed by construction of a NOD stock congenic for NOR-derived markers on Chr 2. Our analysis shows the utility of RCS and congenic stocks for the identification and isolation of non-MHC genes with strong antidiabetogenic functions.

NOR/Lt is an insulitis-resistant and diabetes-free recombinant congenic strain (RCS) produced from an isolated genetic contamination within a NOD/Lt pedigree line by the C57BL/KsJ (BKs) strain (1). Preliminary genetic screening of NOR/Lt for linkage markers delineating NOD-derived insulin-dependent diabetes (*Idd*), genes showed that both strains shared the diabetogenic $H2^{g7}$ MHC haplotype (*Idd1*, chromosome [Chr 17]) as well as NOD genome on regions of Chr 9, 3, 6, 7, and 14 previously shown to encompass *Idd2, 3* and *10, 6, 7,* and *8*, respectively (1, 2). The BKs strain, first identified as a MHC-incompatible substrain of C57BL/6J (B6), itself appears to be a RCS resulting from a genetic contamination of B6, most probably by the DBA/2J strain (3). Thus, the NOR/Lt stock apparently carried genetic input from three distinct inbred strains: NOD/Lt, B6, and possibly DBA/2J. The present study defines the tripartite origins of the NOR/Lt genome more completely, and uses segregation analysis to identify a new *Idd* linkage. Previous analyses of *Idd* genes utilized first backcross (BC1) progeny produced by breeding of diabetes-free F_1 hybrids to the NOD parental strain (4). Although allowing detection of recessive NOD susceptibility modifiers, BC1 is less well-suited for identification of strongly penetrant dominant susceptibility alleles. Given that the NOD and NOR parental strains share significant levels of genetic susceptibility to insulin-dependent diabetes mellitus (IDDM), an F_2 generation was analyzed since the effects of NOR-derived *Idd* resistance modifiers could be evaluated in both the homozygous and heterozygous states.

Materials and Methods

Mice. NOD/Lt mice have been maintained in our research colony by brother-sister mating. Currently, diabetes develops in 90% of female and 63% of male NOD/Lt mice by 1 yr of age. A single NOR/Lt male from the 12th generation of inbreeding was used as the progenitor for the (NOD × NOR)F_1 and F_2 segregants used in this study. All mice were maintained under specific pathogen-free conditions and allowed free access to food (diet 96W; Emory Morse Co., Guilford, CT) and acidified drinking water. The NOR/Lt strain is now inbred beyond the 20th generation of inbreeding and is available from The Animal Resources Unit of The Jackson Laboratory as a control strain of NOD/Lt.

Assessment of Diabetes and Insulitis Development. Female F_1 and F_2 progeny were monitored for the development of glycosuria with Tes-Tape™ (kindly supplied by Eli Lilly, Indianapolis, IN). Mice were confirmed as diabetic when glycosuria was 3^+ and plasma

glucose concentrations exceeded 16.7 mM. Upon diabetes onset, or upon reaching 1 yr of age, the segregants were necropsied and genomic DNA prepared from liver and kidney as previously described (5) for the genotypic analyses described below. Pancreases from 1-yr-old segregants remaining normoglycemic were fixed in Bouin's solution and sectioned at three nonoverlapping levels. Sections were stained with aldehyde fuchsin followed by hematoxylin and eosin counterstain to assess residual β cell mass and extent of leukocytic infiltration (insulitis). An insulitis index score for each mouse pancreas was obtained as previously described (6), with scores ranging between 0 (no lesions) and 4 (complete islet destruction).

Genotypic Analysis of NOR/Lt Mice and (NOD × NOR)F_2 Segregants. Initial genotyping of 53 markers in NOR mice at the F_8 and F_9 generation revealed recombinant regions of BKs genome on Chr 2, 4, 11 and 12 (1). At the 15th generation of inbreeding, the NOR strain was further genotyped at an additional 131 markers distinguishing NOD from BKs mice, either by our previously described Southern blot techniques (7, 8) to detect restriction fragment length variants (RFLV), or by PCR, using oligonucleotide primers for mapped simple sequence repeats known to distinguish the NOD genome from either B6 or DBA/2J genomes (9, 10). After an initial melting of genomic DNA for 3 min at 94°C, PCR products were generated over 30 cycles on a PTC-100 Thermal Cycler (MJ Research, Watertown, MA). Each cycle was programmed for a denaturation step at 94°C for 60 s, followed by primer annealing at 55°C for 90 s, and extension at 72°C for 120 s. Generally, PCR products were electrophoretically resolved in 4–6% Nusieve 3:1 agarose gels (FMC Corp., Rockland, ME), and then visualized by ethidium bromide staining. In cases where a locus-specific microsatellite sequence distinguishing NOD from BKs mice varied by <5 bp, α[^{32}P]dCTP-labeled PCR products were generated as previously described (9), electrophoretically separated in an 8% acrylamide gel, and then visualized by autoradiography.

BKs-derived genetic markers were analyzed in all F_2 segregants to establish which cosegregated with IDDM resistance. Linkage of a BKs-derived marker with IDDM resistance was assumed when a statistically significant segregation distortion favoring homozygosity for the NOD-derived allele in diabetic F_2 progeny was balanced by a concurrent skewing towards homozygosity for the BKs-derived allele in nondiabetic segregants. Segregation distortion for a given locus was statistically assessed by six-term contingency Chi-square (χ^2) analysis. In contingency χ^2 analysis, the expected number of the three possible F_2 genotypes within the diabetic and nondiabetic populations is based on the actual proportions of these genotypes in the total population, rather than on the theoretical 1:2:1 Mendelian ratio. Only markers showing a contingency χ^2 values $\geq$6.5 are listed.

Results and Discussion

Expanded Genotypic Analysis of NOR/Lt Mice. Of 184 nonrandomly selected polymorphic loci, 43 were BKs-derived (Fig. 1). In addition to previously reported recombinant regions on Chr 2, 4, 11, and 12, segments of BKs-derived genome on Chr 1, 5, 7, and 18 were found. BKs genome was present on proximal and medial Chr 1 (two discrete segments ~8 and 12.5 cM in size), distal Chr 2 (~32-cM segment), distal Chr 4 (~22-cM segment), proximal Chr 5 (~7-cM segment), distal Chr 7 (~16.5-cM segment), proximal and medial Chr 11 (three discrete segments ~4.5, 24, and 7.5 cM), and proximal and distal Chr 18 (~13 and 5 cM). A RCS should contain 12.5% of donor strain genome (11). Since the minimum estimated BKs genomic component in NOR totals 167.5 cM, and the mouse genome can be estimated as 1,450 cM, the BKs component of NOR is estimated at 11.6%, very close to the theoretical 12.5% for a RCS. Genotyping information provided by Dr. Jürgen Naggert (The Jackson Laboratory) allowed us to further delineate the BKs genomic contribution to NOR/Lt as being of B6 (29/183 loci) or DBA/2 origin (14/183 loci). As shown in Fig. 1, Chr 4 and 11 exhibit a complex mosaic of NOD, B6, and DBA/2-like genomes, whereas only B6 alleles were observed on the other recombinant chromosomes. As mentioned above, NOR contains nonrecombinant regions of NOD genome on five chromosomes where *Idd* susceptibility genes have been previously identified (2, 4, 5). These include Chr 17 (*Idd1* = *H2^{g7}*), Chr 9 (*Idd2*), Chr 3 (both *Idd3* and *Idd10*), Chr 7 (*Idd7*), and Chr 14 (*Idd8*).

Diabetes and Insulitis in NOR/Lt vs. (NOD × NOR)F_1 and F_2 Mice. Although distinguished from NOD/Lt mice in terms of IDDM resistance, aging NOR/Lt mice developed the heavy perivascular and periductular pancreatic leukocytic aggregates characteristic of NOD/Lt mice. Whereas these perivascular/periductular infiltrates in NOR were sometimes contiguous with one pole of an islet (peri-insulitis), islets exhibiting more severe insulitic stages were rare. Insulitis usually not more severe than grade 2 was observed in ~5% of the islets examined in 7-mo-old mice of either sex. Outcross of NOD to NOR markedly altered this resistance in F_1 female progeny. Of 30 F_1 females monitored for diabetes development through 1 yr of age, 10 (33%) developed IDDM. Widespread severe insulitis (index $\geq$3) characteristic of NOD parental mice was observed in 17 of the nondiabetic females, with only three exhibiting the insulitis resistance phenotype characteristic of the NOR parental females. This contrasts markedly with the complete absence of IDDM in (NOD × BKs)F_1 females which are heterozygous at all loci, including *H-2* (5). Of 123 (NOD × NOR)F_2 females produced, 36 (29.3%) became diabetic by 1 yr of age. As observed in nondiabetic F_1 progeny, all stages of insulitis were observed in the majority of pancreases of the nondiabetic F_2 segregants. Only 17/87 (20%) of these latter pancreases exhibited the insulitis-resistance profile characteristic of the NOR parental strain. The proportion of F_2 diabetics (29%), together with a comparable proportion of insulitis-resistant nondiabetic segregants (20%) might suggest segregation of a single, weakly dominant NOR-derived insulitis/diabetes resistance allele. However, the fact that 33% of female F_1 progeny developed diabetes, and 90% exhibited more widespread insulitis than NOR females, indicated a more complex genetic control.

Identification of a New Diabetes Locus on Chr 2. Genotypic analysis of all 123 F_2 progeny was performed to establish whether *Idd* resistance alleles were present on any of the eight recombinant chromosomes identified in NOR. NOD and NOR progenitors were homozygous for all alleles typed. Highly significant linkage with IDDM resistance was associated with homozygosity for NOR alleles on Chr 2. As shown in Table 1, the presence of an *Idd* resistance gene (provisionally designated *Idd13*) was established in an ~4-cM seg-

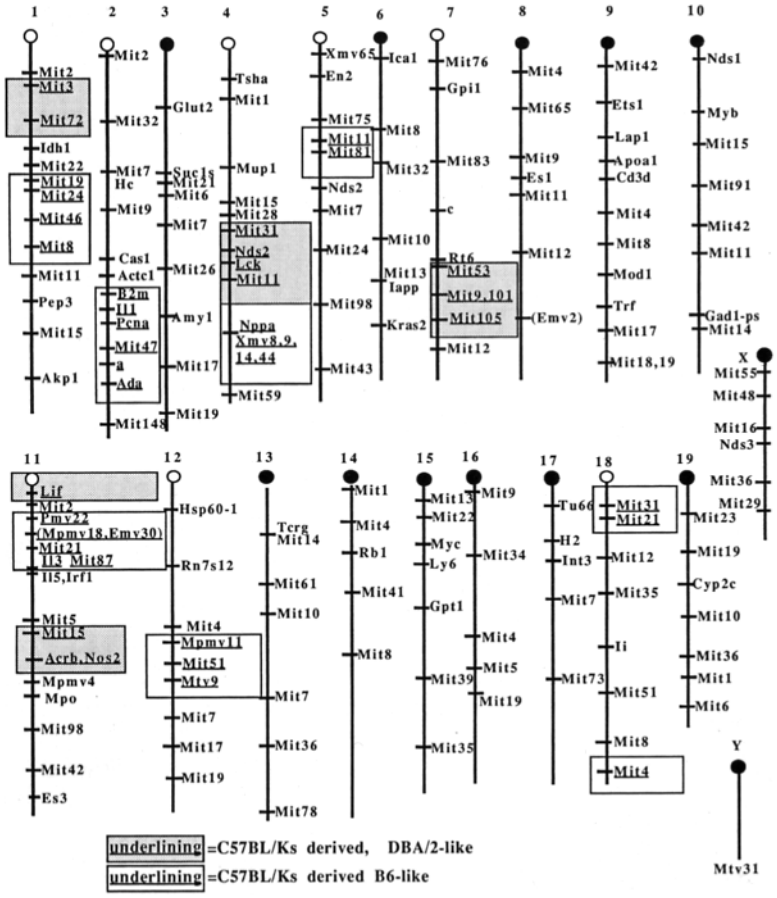

Figure 1. Genetic profile of NOR/Lt mice at the F15 generation of inbreeding. Markers of BKs origin are boxed, with loci originally derived from DBA/2J in shaded areas, and those of C57BL/6J origin in unshaded areas. All markers were typed by PCR analysis of locus-specific microsatellite sequences, except for the following loci. The IFN regulatory factor 1 gene (*Irf1*) was typed as a Southern blot RFLV with the previously described IRF-1 probe (24). The *Nos2* locus was typed by Southern blot analysis as a BglI RFLV with the previously described rat iNOS cDNA (20). *Xmv65* was typed as previously described (25) by Southern blot analysis as a HindIII RFLV with the JS-6, JS-10 oligonucleotide probes. The *Pcna* locus was typed as previously described (26) by HinfI/TaqI digestion of an intron 4 sequence amplified by PCR. Similarly, *B2m* was genotyped by BglI digestion of a PCR product spanning portions of exons 2 and 3 generated with the oligonucleotide primers 5′-CACGCCACCCAC-CGGAGAATG-3′ and 5′-GATGCTGATCACATGTCTCG-3′ (27).

Table 1. *Identification of Linkage Markers for BKs-derived Alleles Contributing to Diabetes Resistance in NOR/Lt Mice by Segregation Analysis of Female (NOD × NOR)F_2 Progeny*

		Diabetic genotypes	Control genotypes	
Chr	Marker	NOD:F_1:NOR	NOD:F_1:NOR	$\chi^2(p)$*
(*cM*)				
1 (37)	*D1Mit46*	9:24:3	19:41:27	7.4 (<0.0125)
(42)	*D1Mit8*	11:21:4	18:40:29	6.5 (<0.05)
2 (54)	*B2m*	18:17:1	17:41:29	18.2 (<0.0001)
(56)	*Il1*	19:16:1	16:42:29	20.5 (<0.0001)
(58)	*Pcna*	18:17:1	16:41:30	19.3 (<0.0001)
(69)	*D2Mit47*	16:18:2	18:38:30	13.5 (<0.001)
(73)	*D2Mit48*	15:19:2	18:38:28	11.7 (<0.0025)
4 (53)	*D4Mit11*	13:18:5	14:45:27	8.5 (<0.0125)
(66)	*Nppa*	14:16:6	15:45:26	7.2 (0.025)

* Markers within all recombinant regions depicted in Fig. 1 were typed in the F_2 segregants, and significant distortions in allele frequencies assessed by Contingency χ^2 analysis. Only those markers showing χ^2 values ≥6.5 are listed.

ment of NOR Chr 2 spanning BKs-derived linkage markers at β_2-microglobulin, (*B2m*), *Il1*, and proliferating cell nuclear antigen (*Pcna*). In the F_2 diabetic subpopulation, mice homozygous for the NOR allele at any of these three loci were rare, whereas they were present at higher than expected frequencies in the nondiabetic F_2 segregants. Since F_2 segregants either homozygous or heterozygous for the NOD alleles at these loci were equally IDDM susceptible, the susceptibility allele (*Idd13^s*) was assumed to be dominant over the NOR-derived resistance allele (*Idd13^r*). Homozygosity for NOR-derived alleles on the region of Chr 2 delineated by the *Il1* linkage marker did not prevent development of severe insulitis in females remaining diabetes free to 1 yr of age. Only 5/29 of nondiabetic F_2 females homozygous for NOR/Lt-derived alleles in this region had mean insulitis scores of $\leq$1.0, characteristic of the NOR/Lt parental strain. Similarly, of the 17/87 F_2 segregants that remained insulitis resistant through 1 yr of age (insulitis scores of $\leq$1.0), 12 of these mice were either homozygous or heterozygous for the NOD/Lt-derived *Il1* allele. Thus, resistance conferred by expression of the NOR-derived *Idd13^r* allele is not associated with the ability to prevent expression of the insulitis phenotype.

The strongest *Idd* linkage on Chr 2 currently is near the *Il1α* and *Il1β* structural loci. Since LPS-stimulated IL-1 secretion is defective in NOD/Lt peritoneal and bone marrow–derived macrophages (10, 11), and treatment of prediabetic NOD mice with IL-1α prevents IDDM (12), the *IL-1α* or *IL-1β* structural gene is a reasonable "candidate" gene to propose for *Idd13*. However, there is no compelling evidence to suggest that IDDM results from primary defects in the coding sequences of either IL-1 structural gene. Our unpublished data indicate that the IL-1 secretion defect is manifest post-transcriptionally. This might result from failure of NOD macrophages to differentiate fully from hematopoietic precursors (11). Further, in vivo treatment of NOD/Lt mice with IL-2 or poly [I:C] (13) restores normal patterns of macrophage IL-1 secretion. Most importantly, defective IL-1 secretion is observed in cultured peritoneal macrophages from NOR/Lt mice (1), which are characterized by B6-like *Il1* structural genes. Hence, the IL-1 secretion defect in NOD macrophages results from complex interactions between the *Il1* structural genes and *trans*-active factors encoded by other loci. The reduced ability of NOD T cells to acquire immunoregulatory activity in a syngeneic mixed lymphocyte reaction (SMLR) is partially a function of the IL-1 secretory defect (10). In contrast, T cells from NOR can acquire immunoregulatory function in a SMLR (1). However, a preliminary study using 8–12-wk-old F_2 males revealed that this immunodysfunction did not segregate with the *Il1* structural genes.

Other attractive candidate genes on Chr 2 are the two structural genes encoding the 67- and 65-kD isoforms of glutamic acid decarboxylase (*Gad1, Gad2*). These gene products are currently candidates for primary β cell autoantigens in NOD mice (14, 15). Although both *Gad* structural genes map to Chr 2 (16), they are positioned proximal to *Il1* in areas in which all markers typed to date have been NOD derived. Thus, NOD and NOR mice must be presumed to share the same *Gad* genes.

Other Potential Regions Conferring Weak Resistance in NOR. Presence of a weaker (and recessive or poorly penetrant) resistance gene on the more distal segment of Chr 4 was suggested by a marginally significant segregation distortion associated with the *D4Mit11* and the naturiuretic peptide precursor A (*Nppa*, formerly *Pnd*) markers. The possibility of an additional weak resistance gene in linkage with the *D1Mit46*marker on the more distal segment of NOR Chr 1 was also suggested. The *Idd9^r*, *Idd11^r*, and *Idd5^r* resistance alleles have been previously identified in these same chromosomal regions in outcross of NOD with other inbred strains (2, 4, 17, 18). Potential pathogenic interactions between *Idd13* and *Idd5* and/or *Idd9*, were assessed by comparing the genotypic frequencies of *Il1*, *D1Mit46*, and *Nppa* in the 40% (35/87) of nondiabetic F_2 segregants that clearly expressed widespread destructive insulitis (insulitis grade 3–4 present in most islets) vs. the 20% (17/87) exhibiting the lack of invasive insulitis (insulitis grades 0–1 in most islets). No such genetic interactions were discerned. Another relatively weak diabetes resistance allele (*Idd4^r*), cosegregating with the acetylcholine receptor β subunit (*Acrb*) linkage marker on Chr 11, has also been identified in outcross of NOD to the C57BL/10 strain (2, 19). As shown in Fig. 1, NOR is distinguished from NOD by polymorphisms in the *Acrb* gene, as well as in *Nos2*, the recently mapped gene for cytokine-inducible nitric oxide (20). However, neither *Acrb* nor any of the other markers in two more proximal recombinant regions on Chr 11 cosegregated with IDDM in the F_2 females (Table 1). It is interesting to note that NOR has acquired a BKs-derived murine mammary tumor virus (*Mtv9*) proviral gene on Chr 12. Since *Mtv* gene products modify the T cell repertoire, this new locus in NOR would be a logical candidate for the reduced insulitis and suppressed IDDM in NOR. However, no significant allelic distortion at either *Mtv9* or *D12Mit51* was observed in diabetic segregants.

Congenic Transfer of Diabetes Resistance Alleles on Chr 2 of NOR to the NOD Genetic Background. The strength of the NOR *Idd13* resistance allele on Chr 2 in the absence of other, more weakly penetrant resistance modifiers (putatively *Idd5^r* and *Idd9^r* or *Idd11^r*) was assessed by congenic transfer of the *Il1* and adenosine deaminase (*Ada*) linkage markers from NOR Chr 2 to the NOD background. At the N_7 generation of backcrossing to NOD/Lt, segregants heterozygous for both the *Il1* and *Ada* linkage markers on Chr 2 were intercrossed. Mice homozygous for the NOR/Lt-derived *Il1* allele were then selected from the resulting N_7F_1 progeny and assessed for diabetes development through 1 yr of age. Controls consisted of N_7F_1 progeny presumed homozygous or heterozygous for the NOD *Idd13* susceptibility allele based upon *Il1* genotyping. By this backcross generation, mice were homozygous for NOD alleles at *D1Mit46* and *D4Mit11*, respectively marking *Idd5* and *Idd9*. As expected from the results of the F_2 segregation analysis, 100% (12/12) of the *Idd13$^{s/s}$* segregants and 71% (24/34) of the *Idd13$^{s/r}$* segregants became diabetic by 1 yr of age. In contrast, only 36% (4/11) of the

Idd13$^{r/r}$ segregants became hyperglycemic over the same period of time. Of these four diabetics homozygous for the NOR *Il1* allele, two were also homozygous for NOR markers at *D2Mit47*, *D2Mit48*, and *Ada*, whereas the other two were heterozygous for these markers.

These results unequivocally establish the existence of *Idd13*^{r} on NOR Chr 2. However, in the absence of homozygosity for NOR resistance alleles at *Idd5*, *Idd9*, or *Idd11*, and perhaps more loci yet to be detected, homozygous expression of *Idd13*^{r} was incapable of completely suppressing diabetogenesis in N_7F_1 congenic females. Nevertheless, it should be noted that the first hyperglycemic *Idd13*$^{r/r}$ homozygote was not observed until 25 wk of age, when 41.2% (14/34) of the *Idd13*$^{s/r}$ and 66.7% (8/12) of the *Idd13*$^{s/s}$ segregants had developed IDDM (Fig. 2). The decrease in overall IDDM incidence, coupled with the delayed time of onset in *Idd13*$^{r/r}$ congenic mice becoming hyperglycemic, support the conclusion that *Idd13*^{r} is a major, but not the sole determinant of diabetes resistance in NOR. The presence of additional resistance loci in NOR was further supported by analysis of a reciprocal congenic stock in which the NOD susceptibility markers between *Il1* and *Ada* were transferred onto the NOR/Lt background in an effort to elicit IDDM. To date, after the same number of backcrosses (N_7) to NOR, IDDM developed in only 1/5 females heterozygous for NOD linkage markers at *Il1* and *Ada*. However, this single female did not develop IDDM until 51 wk of age.

Potential Relevance to IDDM Susceptibility in Humans. The importance of various MHC alleles as primary determinants of IDDM susceptibility in humans (21–23) is clearly established. However, it remains unresolved whether a fixed finite set of non-MHC susceptibility genes must interact with a susceptible MHC haplotype to effect IDDM pathogenesis, or whether different combinations of polygenes can deleteriously interact with IDDM-predisposing MHC alleles in different environments. Genetic analysis of IDDM susceptibility in NOD mice provides insight into this question. Currently, the products of over 60 generations of brother-sister matings, NOD mice inherit the same gender-specific set of susceptibility genes through the germline. Thus, IDDM development in standard NOD mice is presumably catalyzed by interactions among a fixed set of (homozygous) polygenes. Other inbred strains of mice may be homozygous for different alleleic variants of genes capable of reducing or increasing IDDM susceptibility. When the NOD-specific diabetogenic interactions are disrupted by outcross to various inbred strains, and then reassorted into diabetogenic combinations in an F_2 or backcross generation, the full set of susceptibility modifiers defining the NOD genome may not need to be fully reconstituted to elicit IDDM. Indeed, when *H2*^{g7} haplotype is homozygous, the subsets of non-MHC genes with which it must interact to effect pathogenesis may vary depending upon the genotype of the outcross partner strain. This is illustrated by the fact that the C57BL/10J and NON/Lt inbred strains apparently contribute not only *Idd* resistance alleles, but also alleles capable of synergizing with NOD alleles to promote diabetogenesis (2).

The F_2 cross-utilized in the present study identified a new locus on Chr 2 of NOR mice (*Idd13*) contributing strong IDDM resistance, but heretofore unrecognized in outcross with any other inbred progenitor. The existence of *Idd13* has been verified by demonstration of low diabetes incidence in a congenic stock. Thus, when homozygous, the *Idd13* resistance allele can block IDDM even in the presence of the strongly diabetogenic *H2*^{g7} and a host of previously reported *Idd* susceptibility genes. Such non-MHC linked genes with the ability to modify diabetes susceptibility only when homozygous cannot be identified by backcross analysis. If a human homolog exists, it would either be on human Chr 2q13-21 (marked by the *IL1* locus) or Chr 20pter-p12 (encompassing *PCNA*). These findings indicate that the nature of the specific genes contributing to IDDM susceptibility and the mode by which they do so vary according to the genomic composition of the strain to which NOD is outcrossed. This study in mice provides understanding as to why it has been difficult to establish fixed patterns of inheritance of non-MHC genes contributing to IDDM susceptibility in humans either at the population level or within isolated pedigrees.

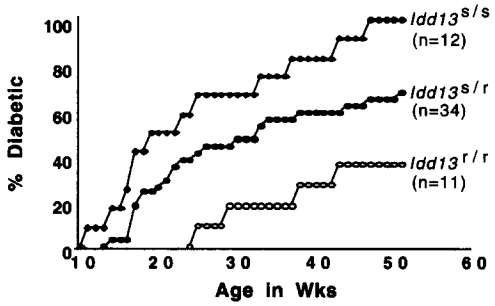

Figure 2. Diabetes resistance in a N_7F_1 stock of NOD mice congenic for ~18 cM of NOR/Lt Chr 2 encompassing *Idd13*^{r}. NOD mice homozygous for the congenic segment presumed to encompass the resistance allele (*Idd13*$^{r/r}$) were compared with segregants either homozygous (*Idd13*$^{s/s}$) or heterozygous (*Idd13*$^{s/r}$) for NOD genome in this region of Chr 2.

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