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 NOR/Lt is a recombinant congenic strain (RCS) in which limited regions of the NOD/Lt genome have been replaced by genome from the C57BL/KsJ strain.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 H2g7 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)F2 segregation analysis was employed to identify chromosomal regions in NOR containing Idd resistance alleles. IDDM developed in 33% (10/30) of F1 females, and 29.3% (36/123) of F2 females aged to 1 yr. A previously unrecognized diabetes resistance locus (designated Idd13") strongly protective in homozygous state was identified on NOR Chr 2 in linkage with the Il1cr 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 H2g7 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 F1 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 F2 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)F1 and F2 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 F1 and F2 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.

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of 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)F2 Segregants.** Initial genotyping of 53 markers in NOR mice at the F3 and F4 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 genotypes (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[32P]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 F2 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 F2 progeny was balanced by a consequent 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 (χ2) analysis. In contingency χ2 analysis, the expected number of the three possible F2 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 χ2 values ≥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 = H2s), 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)F1 and F2 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 insulitis 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 F1 female progeny. Of 30 F1 females monitored for diabetes development through 1 yr of age, 10 (33%) developed IDDM. Widespread severe insulitis (index ≥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)F1 females which are heterozygous at all loci, including H-2 (5). Of 123 (NOD × NOR)F2 females produced, 36 (29.3%) became diabetic by 1 yr of age. As observed in nondiabetic F1 progeny, all stages of insulitis were observed in the majority of pancreases of the nondiabetic F2 segregants. Only 17/87 (20%) of these latter pancreases exhibited the insulitis-resistance profile characteristic of the NOR parental strain. The proportion of F2 diabetics (29%), together with a comparable proportion of insulin-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 F1 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 F2 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 I gene (IIFL) was typed as a Southern blot RFLP with the previously described IIFL-1 probe (24). The Nos2 locus was typed by Southern blot analysis as a BglII RFLP with the previously described rat iNOS cDNA (20). Xrav65 was typed as previously described (25) by Southern blot analysis as a HindIII RFLP with the JS-6, JS-10 oligonucleotide probes. The Pcna locus was typed as previously described (26) by HindIII/TaqI digestion of an intron 4 sequence amplified by PCR. Similarly, B2m was genotyped by BglII digestion of a PCR product spanning portions of exons 2 and 3 generated with the oligonucleotide primers 5'-CACGCCACCCACCGGAGAATG-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 x NOR)F2 Progeny

| Chr | Marker | Diabetic genotypes | Control genotypes |
|-----|--------|--------------------|-------------------|
|     |        | NOD:F2:NOR         | NOD:F2:NOR        | $\chi^2(p)^*$ |
| (cM)|        |                   |                   |               |
| (37)| D1Mit46| 9:24:3             | 19:41:27          | 7.4 (<0.0125) |
| (42)| D1Mit8 | 11:21:4            | 18:40:29          | 6.5 (<0.05)   |
| (54)| B2m   | 18:17:1            | 17:41:29          | 18.2 (<0.0001) |
| (56)| II1   | 19:16:1            | 16:42:29          | 20.5 (<0.0001) |
| (58)| Pcm   | 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) |
| (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 F2 segregants, and significant distortions in allele frequencies assessed by Contingency $\chi^2$ analysis. Only those markers showing $\chi^2$ values >6.5 are listed.
ment of NOR Chr 2 spanning BKs-derived linkage markers at β2-microglobulin, (B2m), Ilt, and proliferating cell nuclear antigen (Pena). In the F2 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 F2 segregants. Since F2 segregants either homozygous or heterozygous for the NOD alleles at these loci were equally IDDM susceptible, the susceptibility allele (Idd13) was assumed to be dominant over the NOR-derived resistance allele (Idd13). Homozygosity for NOR-derived alleles on the region of Chr 2 delineated by the Ilt linkage marker did not prevent development of severe insulitis in females remaining diabetes free to 1 yr of age. Only 5/29 of nondiabetic F2 females homozygous for NOR/Lt-derived alleles in this region had mean insulitis scores of <1.0, characteristic of the NOR/Lt parental strain. Similarly, of the 17/87 F2 segregants that remained insulitis resistant through 1 yr of age (insulitis scores of <1.0), 12 of these mice were either homozygous or heterozygous for the NOD/Lt-derived Ilt allele. Thus, resistance conferred by expression of the NOR-derived Idd13′ 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 Iltα and Iltβ 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 posttranscriptionally. This might result from failure of NOD macrophages to differentiate fully from hematopoietic precursors (11). Furthermore, 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 Ilt structural genes. Hence, the IL-1 secretion defect in NOD macrophages results from complex interactions between the Ilt structural genes and trans-acting 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 F2 males revealed that this immunodeficiency did not segregate with the Ilt 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 Ilt 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 2 was suggested by a marginally significant segregation distortion associated with the D4Mit11 and the natuiriopetide 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′, Idd11′, and Idd5′ 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 Ilt, D1Mit46, and Nppa in the 40% (35/87) of nondiabetic F2 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′), cosegregating with the acetycholine 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 F2 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′ and Idd9′ or Idd11′) was assessed by congenic transfer of the Ilt and adenosine deaminase (Ada) linkage markers from NOR Chr 2 to the NOD background. At the N1 generation of backcrossing to NOD/Lt, segregants heterozygous for both the Ilt and Ada linkage markers on Chr 2 were intercrossed. Mice homozygous for the NOR/Lt-derived Ilt allele were then selected from the resulting N1:F1 progeny and assessed for diabetes development through 1 yr of age. Controls consisted of N1:F1 progeny presumed homozygous or heterozygous for the NOD Idd13 susceptibility allele based upon Ilt 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 F2 segregation analysis, 100% (12/12) of the Idd13+/− segregants and 71% (24/34) of the Idd13−/− segregants became diabetic by 1 yr of age. In contrast, only 36% (4/11) of the
Idd13+/− segregants became hyperglycemic over the same period of time. Of these four diabetics homozygous for the NOR III 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 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 was incapable of completely suppressing diabetogenesis in N2F1 congenic females. Nevertheless, it should be noted that the first hyperglycemic Idd13+/− homozygote was not observed until 25 wk of age, when 41.2% (14/34) of the Idd13+/− and 66.7% (8/12) of the Idd13+/− segregants had developed IDDM (Fig. 2). The decrease in overall IDDM incidence, coupled with the delayed time of onset in Idd13+/− congenic mice becoming hyperglycemic, support the conclusion that Idd13 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 III and Ada were transferred onto the NOR/Lt background in an effort to elicit IDDM. To date, after the same number of backcrosses (N7) to NOR, IDDM developed in only 1/5 females heterozygous for NOD linkage markers at III 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 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 allelic 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 F2 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 H2q 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 F2 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 H2q 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](image-url)
These studies were supported by National Institutes of Health grants DK-36175, DK-27722 (to E. H. Leiter), and DK-46266 (to D. V. Serreze) and by a grant from The Juvenile Diabetes Foundation International. M. Prochazka was a fellow of The Juvenile Diabetes Foundation International.

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Received for publication 6 April 1994 and in revised form 22 July 1994.

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