*Brief Genetics Report*

# Major Histocompatibility Complex–Linked Diabetes Susceptibility in NOD/Lt Mice

## Subcongenic Analysis Localizes a Component of *Idd16* at the *H2-D* End of the Diabetogenic $H2^{g7}$ Complex

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**The diabetogenic major histocompatibility complex (MHC) ($H2^{g7}$) of NOD mice comprises contributions from several class II loci collectively designated as *Idd1*. Introduction of the $H2^{gx}$ haplotype from the related but diabetes-resistant cataract Shionogi (CTS) strain demonstrated an additional MHC-linked locus designated *Idd16*. The NOD-related alloxan resistant (ALR)/Lt strain is also characterized by the $H2^{gx}$ haplotype, which does not differ from $H2^{g7}$ from the class I *H2-K*$^{d}$ gene distally through the class II and into the class III region. Polymorphisms distal to the heat shock protein 70 locus (*Hspa1b*) include a rare *H2-D*$^{dx}$ rather than the $H2^{g7}$ encoded *D*$^{b}$ allele. Two differential-length NOD.ALR-$H2^{gx}$ congenic stocks (D.R1 and D.R2), both containing *H2-D*$^{dx}$, significantly suppressed diabetogenesis. This protection was lost when ALR alleles between the class III region and *H2-D* were removed in a shorter interval congenic (D.R3). Because no differences were observed in the ALR-derived interval extending 0.41 mB proximal to *H2-K* in any of these congenic stocks, a component of what was originally designated "*Idd16*" was sited to an interval shorter than 7.33 mB, distinguishing D.R2 from D.R3. Evidence supporting the candidacy of the ALR/CTS-shared *H2-D*$^{dx}$ MHC class I variant present in both diabetes-resistant stocks, but not the susceptible stock, is discussed. *Diabetes* 54:1603–1606, 2005**

Full understanding of the genetic susceptibilities predisposing to type 1 diabetes is complicated by the fact that, in addition to "high-risk" *HLA-DR* and *-DQ* class II alleles, susceptible individuals must also inherit variable numbers of non–major histocompatibility complex (MHC) susceptibility contributors (1). Adding to this complexity, extended haplotype analysis of the HLA indicates the presence of additional type 1 diabetes susceptibility components in linkage disequilibrium with the high-risk class II alleles (2).

The immunogenetics of type 1 diabetes in NOD mice provides valuable insight into the multigenic contributions of the MHC and closely linked genes on mouse chromosome (Chr.) 17. The diabetogenicity associated with the $H2^{g7}$ haplotype of type 1 diabetes–prone NOD mice is collectively referred to as *Idd1*. This includes expression on antigen-presenting cells of the *H2-Ab*$^{g7}$ product (HLA-DQ8 subunit ortholog) and the lack of expression of an *H2-Ea*$^{b}$ gene product (HLA-DRα subunit ortholog). Class I alleles marking each end of the *H2* complex, the common *H2-K*$^{d}$ allele proximally and *H2-D*$^{b}$ allele distally, are also essential for diabetogenic targeting by $CD8^{+}$ T-cells. Their pathogenic potential can be epistatically modified by altering the β-2 microglobulin (*B2m*) gene product that pairs with them (3). The complex nature of $H2^{g7}$ susceptibility was first indicated by analysis of the cataract Shionogi (CTS)/Shi MHC, which suggests that *Idd1* comprised more than the class II alleles. The CTS/Shi strain was also derived from the Jcl:ICR (Institute for Cancer Research) outbred progenitors that originated the NOD/Shi strain. These two strains share identical class II alleles. However, serotyping suggested that both class I alleles were unique (4,5). Indeed, the *H2-D* allele in a CTS/Shi substrain sent to E.H.L. has previously been shown to be *H2-D*$^{dx}$ by cDNA sequencing (6). *H2-D*$^{dx}$ represents a rare allele shared with the related alloxan resistant (ALR)/Lt strain (7). Both the ALR/Lt and the CTS/ShiLt substrain at The Jackson Laboratory showed a cDNA sequence identical to the *H2-K*$^{d}$ class I allele expressed in NOD/Lt (6). However, sequencing of the *H2-K* allele in genomic DNA of CTS/Shi mice from the source colony in Japan revealed two amino acid differences (in a region uninvolved with antigen binding or presentation) between the CTS/Shi allele and the *H2-K*$^{d}$ allele expressed by NOD/Shi (8). The reason for the discrepancy in *H2-K* gene sequences between the two colonies was assumed to represent substrain divergence (8).

Congenic transfer in Japan of the CTS/Shi MHC onto the NOD/Shi genetic background significantly reduced diabe-

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ALR, alloxan resistant; CTS, cataract Shionogi; MHC, major histocompatibility complex.

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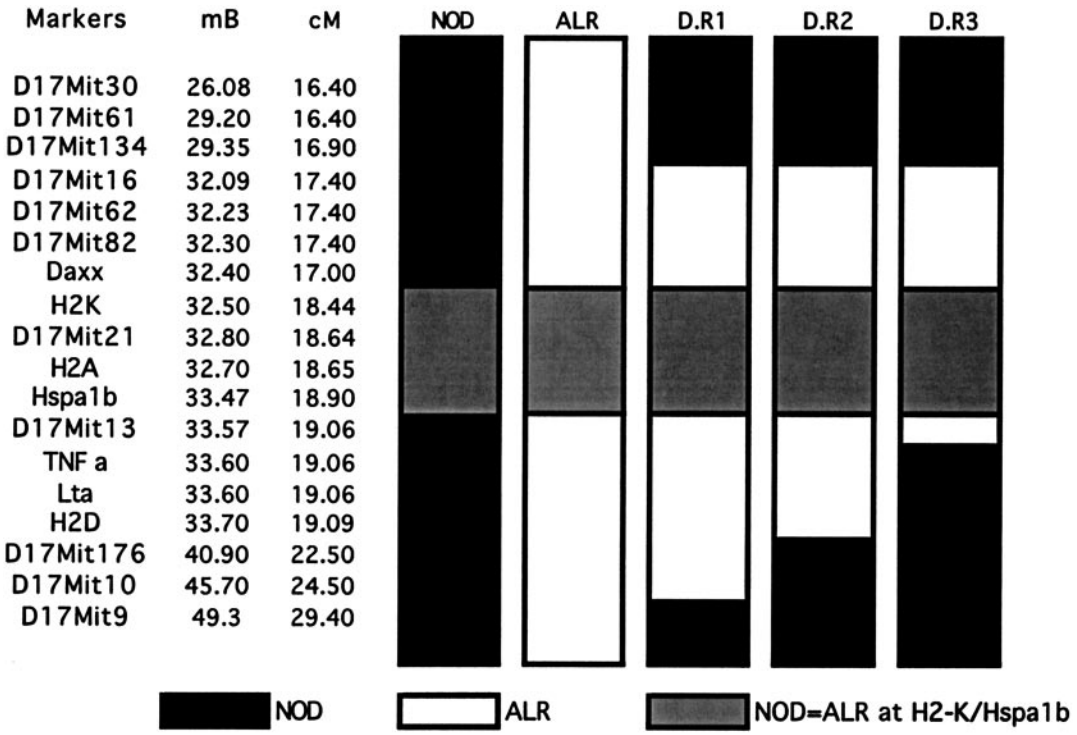


**FIG. 1. Chr. 17 map of MHC congenics used in incidence. DR indicates NOD.ALR. Mice were typed as NOD or ALR with markers shown and various other markers across the congenic intervals. Physical positions in megabases are from National Center for Biotechnology Information Ensembl build 33 and corresponding centiMorgan positions are from the Mouse Genome Informations database at The Jackson Laboratory.**

tes frequency and delayed time of onset (9). This resistance locus, provisionally designated *Idd16*, was not fine mapped but resided within a congenic segment extending 7.6 cM proximally and 3.4 cM distally from *H2*. Evidence suggesting that the CTS-defined *Idd16* locus might be proximal to *H2-K*$^{d}$ came from three sources. First, outcross of NOD/Lt with ALR/Lt identified an MHC-linked ALR resistance locus with peak linkage just proximal to *H2-K* (10). Subcongenic analysis of NOD stocks carrying segments of Chr. 17 from C3H-H2$^{R209}$ identified one new intra-*H2* resistance allele between *Lmp2* and *H2-K* and two additional resistance contributions proximal to *H2-K* (11). More recently, NOD interval-specific Chr. 17 congenic stocks generated by outcross of NOD with C57BL/6 (B6, *H2*$^{b}$ haplotype) also sited B6-derived resistance alleles proximal and distal to *H2-K* (12,13). Although the authors inferred that one of the proximal resistance loci was the CTS *Idd16* resistance locus, comparative CTS/B6 sequence data in the region was not reported.

We outcrossed NOD/Lt and ALR/Lt mice and produced NOD stocks congenic for specific intervals of the *H2*$^{gx}$ complex shared in common between ALR/Lt and CTS/ShiLt. Figure 1 shows the three interval-specific congenic stocks selected by screening for recombinations within the longest interval congenic stock (now designated D.R1) between N7 and N10 of backcrossing to NOD/Lt. All three stocks appeared identical in containing a short interval (0.41-mB minimum length to <2.15-mB maximum length) of ALR-derived alleles extending proximally from the common NOD/ALR H2-K allele to the polymorphic *D17Mit16* marker. The differences identified in the three stocks were all distal to the MHC class II and class III region alleles common to NOD and ALR. Two of these carried the entire ALR/Lt *H2*$^{gx}$ haplotype (Fig. 1, D.R1 and D.R2) but differed in the amount of ALR genome carried below the *H2-D*$^{dx}$ marker. D.R1 carried the longest distal congenic segment (Fig. 1), extending beneath the shared NOD/ALR class II/class III region from *D17Mit13* at 33.57 mB through *D17Mit10* (the recombination falling somewhere between *D17Mit10* at 45.7 mB and *D17Mit9* at 49.3 mB). A shorter (minimum 1.61 mB) congenic stock, designated D.R2, considerably reduced this distal segment to contain ALR alleles only from *D17Mit13* through *H2-D* (Fig. 1). An even shorter (<7.33 mB) interval-specific congenic stock, designated D.R3 (Fig. 1), was obtained by identification of an intra-*H2* recombinant wherein a very small segment of ALR/Lt genome was retained distally only around the *D17Mit13* marker. In this stock, the ALR *H2-D*$^{dx}$ allele and all genome distal to it were replaced by NOD-derived alleles.

Diabetes incidences for the NOD stocks homozygous for the three *H2*$^{gx}$-derived congenic intervals are shown in Fig. 2. Diabetes-free survival of D.R1 and D.R2 congenic mice of both sexes was significantly extended when compared with standard NOD/Lt (Fig. 2) or NOD littermates homozygous for NOD alleles through the congenic segments (data not shown). For each congenic stock, littermates homozygous for NOD alleles across each congenic segment were aged, and none developed diabetes at a rate significantly different from the standard NOD/Lt mice shown. Because there was no significant difference in the protection afforded by the D.R1 versus the D.R2 congenic interval, we concluded that the region containing the protective allele does not reside within the interval distally below *H2-D*$^{dx}$. In contrast, D.R3 congenic mice of either sex showed no significant diabetes protection compared with standard NOD/Lt mice (Fig. 2). Hence, the diabetes

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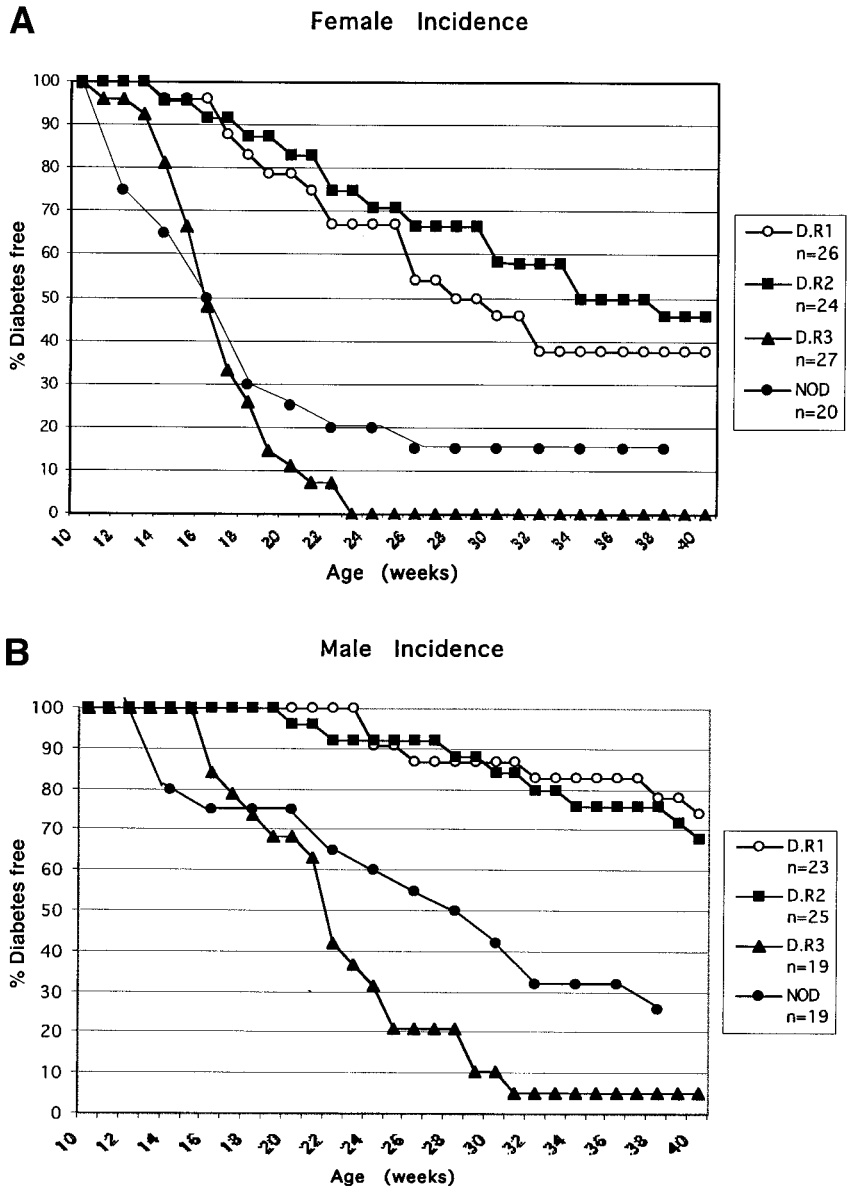


**FIG. 2. Incidence of diabetes in all DR congenic lines in females (*A*) and males (*B*) compared with NOD control mice. Data are shown as percentage of diabetes-free survival until 38 or 40 weeks of age. D.R1 and D.R2 are significantly different from both D.R3 ($P < 0.001$) and NOD ($P < 0.001$) in both female and male incidence. Survival curves for D.R1 and D.R2 did not differ from each other ($P > 0.5750$), and NOD and D.R3 did not differ from each other ($P > 0.9451$) in both male and female incidence.**

protection produced by introgression into NOD of the D.R1 and D.R2 intervals, but not the D.R3 interval, positions a component of the protective *Idd16* allele(s) to the short (maximum 7.33 mB) interval between *D17Mit13* in the class III region and *H2-D*$^{dx}$. This protection was associated with retardation of insulitis progression but not its prevention. Lower mean insulitis scores were recorded in a small sampling ($n = 3$ per group) of pre-diabetic 8-week-old D.R1 and D.R2 males compared with D.R3 males. However, histologic analysis of the pancreata from nondiabetic D.R1 survivors at 40 weeks showed widespread intra-islet insulitis, with 4 or 7 males and 5 of 7 females exhibiting mean insulitis scores ≥3.0 (over a range of 0–4).

At least two interesting candidate genes exist in this distal interval. One previously proposed candidate in this interval is *Tnfa*, in which coding sequences for NOD and CTS differ (9,14). However, basal and stimulated serum concentrations of tumor necrosis factor-α did not differ between these two strains (9). Given the essential role of CD8$^+$ T-cells in NOD diabetogenesis, another candidate for *Idd16*-mediated resistance is the ALR/CTS-shared *H2-D*$^{dx}$ allele. AI4 is a highly prevalent and pathogenic CD8 T-cell clonotype in NOD mice (15) and must engage peptides bound to both K$^d$ and D$^b$ molecules to mediate β-cell destruction (16). Complete chimerization with bone marrow from a NOD *Rag1* stock transgenically expressing the AI4 T-cell receptor induced diabetes in standard NOD but not in NOD.ALR-DR1 recipients (16). Thus, the D$^{dx}$ variant is a strong candidate for providing a component of *Idd16*-mediated resistance in the CTS and ALR strains.

The original segregation analysis for *Idd* loci, distinguishing NOD/Lt from the strongly diabetes-resistant ALR/Lt strain, identified a resistance contribution with peak linkage at *D17Mit16* just proximal to the common *H2-K*$^d$ allele (10). This was the same region where a B6 *H2-K*$^b$–linked resistance locus was also mapped and designated *Idd16* (12). The B6 *H2*$^b$–linked proximal component of *Idd16* differed from that produced by the ALR/Lt (and CTS/Shi) *H2*-linked distal component in its ability to completely suppress clinical disease (but not insulitis). In contrast, the distal *Idd16* component defined by the ALR MHC containing intervals in the D.R1 and D.R2 congenic stocks

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retarded but did not completely suppress diabetogenesis. Thus, what is currently termed as *Idd16* in the literature and defined by outcross with either B6 or CTS/Shi appears to comprise distinct *H2*-linked resistance at both ends of the MHC. Under these circumstances, we refer to the ALR/CTS-defined distal linkage as *Idd16.1* (because *Idd16* was first defined by the CTS MHC linkage) and the B6-defined proximal linkage as *Idd16.2*.

This B6-defined *Idd16.2* locus was mapped to a short interval just above *D17Mit16*. The same marker was linked with the highest ALR-contributed resistance on Chr. 17 to spontaneous type 1 diabetes development in backcross analyses (10). Because all three of our ALR-derived DR congenic intervals extended proximally only to the *D17Mit16* marker, we could not assess potential resistance contributions in an *Idd16.2* interval proximal to it or in the immediate vicinity of the *H2-K*$^{d}$ allele shared by the *H2*$^{gx}$ and *H2*$^{g7}$ haplotypes. However, given the results obtained by outcross with NOD-unrelated strains clearly showing resistance contributions just proximal to the MHC, it is entirely possible that similarly acting loci are present in the ALR/Lt and CTS/ShiLt strains.

In conclusion, we have demonstrated by interval-specific congenic analysis a type 1 diabetes resistance allele, provisionally designated *Idd16.1*, to be tightly linked to the distal end of the *H2*$^{gx}$ haplotype expressed by ALR/Lt and CTS/ShiLt mice. The MHC class I *H2-D*$^{dx}$ gene itself represents a strong *Idd16.2* candidate.

## RESEARCH DESIGN AND METHODS

NOD/Lt and the related ALR/Lt mice used in this study were bred and maintained in a pathogen-free research animal facility at The Jackson Laboratory. All mice were allowed free access to pasteurized food (National Institutes of Health–31 6% fat diet) and acidified drinking water.

**MHC congenics and incidence studies.** NOD/Lt and ALR/Lt mice were selected for specific H2$^{gx}$ and H2$^{g7}$ congenic regions, respectively, by PCR genotyping with polymorphic Massachusetts Institute of Technology microsatellite markers and by flow cytometric typing for *H2-D*$^{b}$ versus *H2-D*$^{dx}$ expression (mAb 28-24-8 and mAb 34-2-12, respectively; Pharmingen, San Diego, CA). Following outcross and nine backcrosses (N10) to NOD/Lt with selection for polymorphic *H2*$^{gx}$-linked markers introgressed into NOD/Lt, MHC heterozygous mice were intercrossed and inbreeding initiated to produce cohorts of mice homozygous for ALR markers across the intervals shown in Fig. 1. The D.R2 and D.R3 congenics were derived from recombinations within the D.R1 interval between N7–N10. Mice were typed for NOD homozygosity at the non-MHC loci on Chrs. 3 and 8 where ALR alleles contributed to diabetes resistance (10).

Incidence studies consisted of mice homozygous for the congenic segment of Chr. 17 mice and mice homozygous for NOD alleles through the interval. Males and females of each genotype in each strain were weaned at 4 weeks of age and started in an incidence study at 10 weeks of age. Each mouse was checked for diabetes using Diastix (kind gift of Bayer, Elkhart, IN) weekly between 10–40 weeks and was considered diabetic after two consecutive positive tests. Significance of differences in diabetes-free lifespan was estimated by Kaplan-Meier survival analysis using the StatView program (Abacus Concepts, Berkeley, CA).

**Insulitis scoring.** Insulitis was assessed histologically in a small group of nondiabetic males at 8 weeks of age and in nondiabetic D.R1 and D.R2 male and female incidence survivors at 40 weeks. Pancreata were fixed in Bouin's solution, stained with aldehyde fuchsin, and scored for insulitis. Scores were given for each islet beginning with 0 for no leukocytes in islet periphery, 1 for peri-insulitis, 2 for leukocyte penetration into <25% of the islet, 3 for leukocyte destruction of >25% of islet, and 4 for end-stage insulitis.

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