

Identification of a New Susceptibility Locus for Insulin-dependent Diabetes Mellitus by Ancestral Haplotype Congenic Mapping

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Abstract

The number and exact locations of the major histocompatibility complex (MHC)-linked diabetogenic genes (*Idd-1*) are unknown because of strong linkage disequilibrium within the MHC. By using a congenic NOD mouse strain that possesses a recombinant MHC from a diabetes-resistant sister strain, we have now shown that *Idd-1* consists of at least two components, one in and one outside the class II A and E regions. A new susceptibility gene (*Idd-16*) was mapped to the < 11-centiMorgan segment of chromosome 17 adjacent to, but distinct from, previously known *Idd-1* candidates, class II A, E, and *Tap* genes. The coding sequences and splicing donor and acceptor sequences of the *Tnfa* gene, a candidate gene for *Idd-16*, were identical in the NOD, CTS, and BALB/c alleles, ruling out amino acid changes in the TNF molecule as a determinant of insulin-dependent diabetes mellitus susceptibility. Our results not only map a new MHC-linked diabetogenic gene(s) but also suggest a new way to fine map disease susceptibility genes within a region where strong linkage disequilibrium exists. (*J. Clin. Invest.* 1995. 96:1936-1942.) Key words: insulin-dependent diabetes mellitus • genetic susceptibility • major histocompatibility complex • linkage disequilibrium • non-obese diabetic mouse

Introduction

Insulin-dependent diabetes mellitus (IDDM)¹ is caused by autoimmune destruction of insulin-producing β cells of the pancreas in genetically susceptible individuals (1). Both in humans and animal models, strong genetic susceptibility to IDDM is closely linked to the major histocompatibility complex (MHC) (2, 3), but the number and precise locations of the MHC-linked susceptibility genes are unknown because of strong linkage disequilibrium within the MHC.

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Received for publication 17 March 1995 and accepted in revised form 7 July 1995.

1. Abbreviations used in this paper: IDDM, insulin-dependent diabetes mellitus; NOD, nonobese diabetic; NON, nonobese nondiabetic; Tap, transporter associated with antigen processing.

J. Clin. Invest.

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0021-9738/95/10/1936/07 \$2.00

Volume 96, October 1995, 1936-1942

In humans, certain alleles of *DR* and *DQ* loci of the HLA region (human MHC) have been shown to be associated with, and linked to, IDDM (4). Recent studies indicated that up to 50% of IDDM susceptibility is determined by genes in the HLA region (5, 6) and that genetic markers located as far as 20 centiMorgan (cM) away from the class II HLA region still show linkage with putative susceptibility genes (5). These data indicate the importance of MHC-linked genes in the predisposition to the disease.

The nonobese diabetic (NOD) mouse strain spontaneously develops IDDM (7) and, as in humans, strong genetic susceptibility to the disease has been mapped to the MHC region on chromosome 17 (2). The NOD strains that are congenic for the MHC from diabetes-resistant strains, such as C57BL/10 (B10), B10BR, and the nonobese nondiabetic (NON) strains, were reported to be completely resistant to IDDM (8-10), indicating that the NOD MHC is essential for the development of IDDM.

Two genes, a rare NOD allele of *Ab* gene and a defective *Ea* gene, have been implicated as candidates for the MHC-linked diabetogenic genes (*Idd-1*) of the NOD mouse (2, 11). Although suppression of IDDM was reported in transgenic NOD mice expressing normal alleles of *Ab* or *Ea* genes (12-14), there has been no direct evidence that NOD alleles of *Ab* and/or *Ea* genes actually cause IDDM. Moreover, the contribution of genes other than *Ab* and *Ea*, such as *Tap* (transporter associated with antigen processing) and class I genes, to IDDM susceptibility has also been suggested (15, 16), making interpretation of the contribution of MHC to IDDM susceptibility very complicated.

One way to fine map *Idd-1* is to find intra-MHC recombinant strains that possess a segment of the NOD MHC encoding candidate genes and to demonstrate that NOD mouse strains that are congenic for the recombinant MHC develop IDDM. The recombination frequency within the MHC, however, is too low to generate intra-MHC recombinant haplotypes by standard breeding between NOD and other inbred strains. Recombination events, however, have occurred during many historical meioses, giving rise to intra-MHC recombinant haplotypes in NOD-related strains. We previously found such a recombinant MHC in a NOD-related strain. The CTS mouse, a sister strain of the NOD mouse, is an intra-MHC recombinant strain that has the same class II MHC, including candidate genes A and E, as the NOD mouse, but different class I MHC from the NOD mouse (17, 18). Here, we report the molecular genetic dissection of *Idd-1* by using a congenic NOD mouse strain that contains a recombinant MHC from the CTS mouse. The results mapped a new susceptibility gene, *Idd-16*, to the region adjacent to, but distinct from, previously known *Idd-1* candidates, class II A and E genes. Our study also suggests a new way for fine structure mapping of disease susceptibility genes within a region where strong linkage disequilibrium exists.

Table I. Polymorphic Markers in the MHC Region Used in This Study

Locus	Marker	Polymorphism	Detection	Allele size
Oa	D17Dgm3	I/D* and MspI RFLP	PCR	C3H = 69 bp, NON = 75 bp, B6 = BALB = NOD = CTS = 52 + 23 bp
Lmp2 hot spot	D17Dgm1	(T) _n and TaqI RFLP	PCR	BALB = C3H = 129 bp, B6 = NOD = CTS = NON = 104 + 30 bp
Lmp2	D17Dgm4	Microsatellite	PCR	BALB = 134, C3H = 124, B6 = NOD = CTS = 122, NON = 112 bp
Lmp2	D17Dgm5	HhaI RFLP (exon 3)	PCR	B6 = 75 bp, BALB = C3H = NOD = CTS = NON = 42 + 33 bp
Lmp2	D17Dgm6	MspI RFLP (3' UT)	PCR	BALB = C3H = 127 bp, B6 = NOD = CTS = NON = 69 + 58 bp
Tap1		XbaI RFLP [‡]	Southern	
Ob	D17Dgm2	Microsatellite	PCR	B6 = C3H = BALB = 106, NON = 103, NOD = CTS = 88 bp
Ob	D17Mit21	Microsatellite	PCR	
Ab	D17Dgm7	I/D* and DdeI RFLP	PCR	B6 = BALB/c = 208 bp, C3H/He = NON = 202 bp, NOD = CTS = 118 + 84 bp
Eb		Microsatellite [§]	PCR	
Eb2	D17Mit22	Microsatellite	PCR	
Hsp70	D17Nds2	Microsatellite	PCR	
Hsp	D17Mit83	Microsatellite	PCR	
	D17Mit13	Microsatellite	PCR	
Tnfa		Microsatellite	PCR	
Tnfb	D17Nds3	Microsatellite	PCR	
Q4		Microsatellite [¶]	PCR	

* Insertion/deletion polymorphism; [‡] Reference 16; [§] reference 45; ^{||} reference 46; [¶] reference 47.

Methods

Mice. NOD/Shi, NON/Shi, and CTS/Shi mice were maintained by brother-sister mating at Shionogi Aburahi Laboratories. NOD.CTS-H-2 congenic strain was established at Shionogi Aburahi Laboratories by repeated backcrossing of CTS mice with NOD mice and selection for CTS MHC as reported previously (19, 20). CTS MHC homozygotes of NOD.CTS-H-2 mice at N16F2 generation were used in this study. C57BL/6, C3H/He, and BALB/c mice were obtained from Clea Japan, Inc. (Tokyo, Japan). The nuclei of the *Mus spretus* mice were originally obtained from Jackson Laboratory (Bar Harbor, ME), bred by brother-sister mating at the National Cardiovascular Center (Osaka, Japan), and raised at Shionogi Aburahi Laboratories. All mice were housed under sterile, specific pathogen-free conditions. Mice were monitored for the development of diabetes by testing for urinary glucose with Tes-Tape (Eli Lilly & Co., Indianapolis, IN) and were classified as diabetic after producing consistent Tes-Tape values of $\geq 3+$.

Typing of MHC region. To determine the region in the CTS MHC that is identical to the NOD MHC, polymorphisms in coding sequences, microsatellite polymorphisms, insertion/deletion polymorphisms, and restriction fragment length polymorphisms (RFLPs) were typed in the NOD, CTS, and NOD.CTS-H-2 mice. The polymorphisms used are listed in Table I. PCR-based assays for *Lmp2* hotspot (21), *Ob*, *Oa*, and *Lmp2* (three markers) loci were newly developed for this study and designated as D17Dgm1, D17Dgm2, D17Dgm3, D17Dgm4, D17Dgm5, and D17Dgm6, respectively. PCR primers were as follows: D17Dgm1: forward 5'-TGT AGA CAT GAC TTC TCT TC-3', reverse 5'-GCC TGC AGA TTC TGA ATT C-3'; D17Dgm2: forward 5'-AGA TAC ATC TAC AAC CCG GAG-3', reverse 5'-CAA GCC CCG CAG GGA GGT G-3'; D17Dgm3: forward 5'-GGT CCT TTT TGT GGA GCT GG-3', reverse 5'-GAT GGC CCT TAC TCC TCG G-3'; D17Dgm4: forward 5'-GTA GGT ACT CTA CCA CCT G-3', reverse 5'-CTC AGA TCA GTA AGT CAC TG-3'; D17Dgm5: forward 5'-GTG AAC CGC GTG TTC GAC AAG-3', reverse 5'-ATC AGC AGC GGA ACC CGA GAG-3'; D17Dgm6: forward 5'-TGA CTG ATC CCC AGA AGT CC-3', reverse 5'-GGG AGG ACG CTT CCC TCC-3'. D17Dgm1 consists of polymorphisms in the number of poly-T and a TaqI restriction site located in a recombinational hotspot at *Lmp2* locus (21). D17Dgm2 (ATT repeat) (22) and D17Dgm4 (CA repeat) (23) are microsatellite polymorphisms. D17Dgm3 consists of an insertion/deletion polymor-

phism and MspI RFLP in the coding sequences of *Oa* gene (24). D17Dgm5 and D17Dgm6 are RFLPs in exon 3 and the 3' untranslated region of the *Lmp2* gene (23, 25), respectively, and are detected by the digestion of PCR products with restriction enzymes HhaI and MspI, respectively. PCR-based detection of coding sequence variations in the *Ab* gene (18) was designated as D17Dgm7. PCR assays were performed essentially based on the protocol for MapPairs[®] (Research Genetics, Huntsville, AL) except that only unlabeled primers were used and PCR products were visualized by ethidium bromide staining. Size of CTS allele was determined by comparison with alleles of control and/or NOD strains when the size of these strains was previously reported, or approximated by comparison with a molecular size marker (pBR322-MspI digest) when allele sizes of other strains were unknown. Size of ambiguous bands was confirmed by PCR using ³²P-labeled primers with M13 sequencing ladder as the molecular standard.

Fingerprinting of background genes. The genome of NOD.CTS-H-2 mice was fingerprinted with locus-specific microsatellite markers (MapPairs[®]). To increase the possibility of finding polymorphisms between NOD and CTS strains, we screened a total of 114 microsatellites, most of which were reported to be polymorphic between NOD and closely related NON mice. Among these, 71 were found to be polymorphic between NOD and CTS strains and were used for fingerprinting. The following MapPairs[®] were used in this study: D1MIT24, D1MIT33, D1MIT49, D2MIT2, D2MIT15, D2MIT22, D3MIT10, D3MIT13, D3MIT17, D3MIT19, D3MIT22, D3MIT41, D3MIT44, D4MIT2, D4MIT54, D4Nds2, D5MIT13, D5MIT26, D5MIT30, D5MIT41, D5MIT48, D6MIT8, D6MIT14, D6MIT33, D7MIT25, D7MIT53, D7MIT65, D8MIT14, D8MIT15, D8MIT45, D8MIT46, D9MIT11, D9MIT18, D9MIT21, D9MIT46, D9MIT55, D10MIT2, D10MIT14, D10MIT40, D11MIT2, D11MIT41, D11Nds1, D12MIT2, D12MIT12, D12MIT17, D12MIT51, D13MIT13, D13MIT36, D13MIT74, D13MIT77, D14MIT1, D14MIT7, D14MIT18, D15MIT10, D15MIT29, D15MIT35, D15MIT51, D16MIT30, D16Nds2, D18MIT7, D18MIT9, D18MIT34, D18MIT35, D19MIT16, D19MIT32, D19MIT33, D19MIT45, D19MIT61, D19MIT90, D19MIT91, and DXMIT13.

To determine the region of CTS MHC that was retained in the NOD.CTS-H-2 congenic strain, chromosome 17 of NOD.CTS-H-2 congenic mice was fingerprinted with 32 microsatellite markers in comparison with that of NOD and CTS mice (Table II).

Table II. Chromosome 17 of NOD, CTS, NON, and Congenic Mice

Position	Locus	Marker	NOD	CTS	NON	Congenic CTS-H-2	Congenic NON.H-2
<i>cM</i>							
3.0		D17Mit19	174	180	180	174	174
7.1		D17Mit27	180	172	172	180	180
7.1		D17Mit99	93	89	89	93	ND
		D17Mit156	122	128	128	122	122
		D17Mit164	96	120	120	96	96
8.46		D17Mit113	137	133	133	137	137
10.0		D17Mit59	144	148	148	144	ND
10.0		D17Mit79	183	157	157	183	187
11.0		D17Mit144	148	152	152	148	ND
16.1		D17Mit61	196	166	166	166	166
16.1		D17Mit134	147	159	159	159	159
16.4		D17Mit81	114	132	132	132	132
17.28		D17Mit30	150	144	144	144	ND
		D17Mit192	148	146	142	146	142
18.18		D17Mit147	122	126	126	126	126
18.18		D17Mit16	88	118	110	118	110
18.18		D17Mit28	104	104	118	NI	118
18.44	K		d	CTS	NON	CTS	NON
18.52	Oa	D17Dgm3	55 + 23	55 + 23	75	NI	ND
18.59	Lmp2	D17Dgm1	104 + 30	104 + 30	104 + 30	NI	NI
		D17Dgm4	121	121	111	NI	111
		D17Dgm5	42 + 33	42 + 33	42 + 33	NI	NI
		D17Dgm6	69 + 58	69 + 58	69 + 58	NI	NI
18.60	Tap1	XbaI RFLP	6.9 + 3.7	6.9 + 3.7	10.6 kb	NI	ND
18.63	Ob	D17Dgm2	88	88	106	NI	ND
18.63		D17Mit21	124	124	126	NI	ND
18.64	Ab	D17Dgm7	118 + 84	118 + 84	202	NI	202
18.66	Eb		93	93	109	NI	109
18.67	Eb2	D17Mit22	157	157	177	NI	177
18.94	Hsp70	D17Nds2	121	121	103	NI	103
18.95		D17Mit83	146	146	135	NI	ND
18.95		D17Mit13	149	142	149	142	NI
19.05	Tnfa		103	120	103	120	NI
19.06	Tnfb	D17Nds3	140	145	140	145	NI
19.09	D		b	CTS	b	CTS	NI
19.16	Q4		160	204	220	204	220
20.36		D17Mit47	222	210	222	222	ND
21.65		D17Mit11	178	174	150	174	178
21.65		D17Mit105	129	121	117	121	129
		D17Mit176	154	182	172	182	154
22.33		D17Mit50	118	124	134	118	118
23.50	Tctel	D17Mit35	226	226	242	NI	226
24.30		D17Mit36	146	112	114	146	146
25.10		D17Mit10	148	150	150	148	ND
34.30	C3	D17Mit20	180	170	180	180	NI
38.50		D17Mit53	132	138	138	132	132
40.00		D17Mit153	137	151	149	137	137
48.50		D17Mit73	98	104	104	98	98
56.70		D17Mit123	133	137	155	133	133

Alleles identical to NOD alleles are indicated in bold. *NI*, not informative because allele of CTS and/or NON is identical to NOD allele; *ND*, not done.

Typing of candidate genes for *Idd-3* and *Idd-10*. Trinucleotide repeat polymorphism at *Il-2* locus, a candidate for a non-MHC gene on chromosome 3 (*Idd-3*) (26), was detected with the following intron primers: forward 5'-TCA AGC TCT ACA GCG GAA GC-3', reverse 5'-TAG ATG GGA TGG CTG TGC AC-3'. The expected length of the PCR products is 132 bp (C57BL/6 and BALB/c), 120 bp (NOD), or 111 bp (*Mus spretus*). 4-bp deletion in the *Fcgr1* gene, a candidate for a non-MHC gene on chromosome 3 (*Idd-10*), was detected by the method reported by Prins et al. (27) with slight modification. To increase the sensitivity of detection of a small deletion, a new forward primer was designed so that the length of the PCR products was reduced to 99 bp (deletion) or 103 bp (no deletion) instead of 184 bp (deletion) or 188 bp (no deletion) as described in the original method. The sequence of the new forward primer was 5'-TGT GAA AAT ACA CAG GCT GCA GA-3'. The PCR products were run on 9% polyacrylamide gel and visualized by staining with ethidium bromide. The CTS mouse had the same allele as the NOD mouse at *Il-2* locus but a different allele at *Fcgr1* locus.

Sequencing of a candidate gene. As a candidate gene in the MHC region, all exons of the *Tnfa* gene of NOD and CTS mice were sequenced. Genomic DNA was amplified by PCR with the following primers: exon 1: forward 5'-CAT CTC CCT CCA GAA AAG AC-3', reverse 5'-AGA ATA AGG GTT GCC CAG AC-3'; exons 2 and 3: forward 5'-GGT GAG TCT GTC TTA ACT AAC-3', reverse 5'-TCA AGG CAC ATG TAA AGA AATC-3'; exon 4: forward 5'-GTG ACA CTG ACT CAA TCC TC-3', reverse 5'-AGA ATG GAT GAA CAC CCA TTC-3'. Subsequently, 1/10 of the PCR products was subcloned into a pT7 vector (Novagen, Inc., Madison, WI) and both strands were sequenced by 373A sequencer (Applied Biosystems, Inc., Foster City, CA) with cycle-sequencing protocol. Several independent clones were sequenced to minimize the artifacts introduced by PCR amplification.

Results

MHC of CTS and NON mice. The CTS mouse has the same class II MHC, including a rare NOD allele of the *Ab* gene, as the NOD mouse, but different class I MHC from the NOD mouse at both *K* and *D* loci (17, 18). The same class II MHC was also found in one mouse from the original closed colony, Jcl:ICR, from which the NOD strain is derived (18). The existence of the same unique class II MHC of the NOD mouse in both a sister strain (CTS) and the original closed colony (Jcl:ICR) strongly suggests that the NOD MHC and the CTS MHC are the products of recombination among ancestral haplotypes that were contained in the original Jcl:ICR colony. To further characterize the identities and the differences between the CTS MHC and the NOD MHC, we typed the MHC of the CTS mouse in comparison with NOD and NON, another sister strain of the NOD mouse, using polymorphic markers in the MHC (Table I). All the markers in a 0.5-cM segment encompassing class II *Oa* to class III *Hsp70.1* loci, including a unique *XbaI* RFLP of *Tap1* gene which has been correlated with disease susceptibility (16, 28), were identical between the NOD and CTS strains, whereas the NON strain had different alleles from the NOD mouse at most loci (Table II). These data together with the identical *Ab* sequence between the NOD and CTS mouse (18) suggest that the 0.5-cM region encompassing class II *Oa* to class III *Hsp70.1* loci is identical in the NOD and the CTS strains and that this segment is derived from the same ancestral chromosome contained in the original Jcl:ICR mice.

Congenetic NOD.CTS-H-2 and NOD.NON-H-2 strains. To determine whether class II MHC is essential for the development of IDDM in the NOD mouse, the incidence of IDDM was monitored in congenic NOD strains that contain the MHC from

diabetes-resistant sister strains, CTS and NON mice. None of the NOD.NON-H-2 congenic mice developed IDDM, confirming the previous reports that NOD MHC is essential for the disease development. In contrast, NOD.CTS-H-2 congenic mice, which possess the CTS MHC in the homozygous state, developed IDDM, indicating that the CTS MHC is diabetogenic even in the absence of the NOD MHC.

Although NOD.CTS-H-2 congenic mice developed IDDM, the cumulative incidence of IDDM was much lower than that in the NOD parental strain. Among NOD.CTS-H-2 mice (N13-19F1), 3% (1/36) of males and 44% (11/25) of females became diabetic by 210 d of age as compared with 55% of males and 90% of females in the NOD parental strain in the same animal facility. To further confirm the low incidence of IDDM in NOD.CTS-H-2 congenic mice, MHC heterozygotes were intercrossed at N13-19 generations and the incidence of IDDM was monitored relative to the inheritance of the MHC. The cumulative incidence of IDDM in the CTS MHC homozygotes was significantly lower than that in the NOD MHC homozygotes: in CTS MHC homozygotes, 44% (11/25) of females and 3% (1/36) of males developed diabetes by 210 d of age as compared with 91% (40/45) of females and 55% (26/50) of males in NOD MHC homozygotes ($P < 0.001$ for both). Mean (\pm SD) age at onset of diabetes in females was 173 ± 40 d in CTS MHC homozygotes and 130 ± 29 d in NOD MHC homozygotes ($P < 0.001$). Age at onset of diabetes in a male CTS MHC homozygote was 210 d, whereas mean (\pm SD) age at onset of diabetes in male NOD MHC homozygotes was 157 ± 28 d.

These data suggest that *Idd-1* is encoded by two or more distinct chromosome 17 loci and that the CTS MHC contains a part, but not all, of the *Idd-1* encoding loci. These data map one component of *Idd-1* to a 0.5-cM segment within the MHC that is shared between NOD and CTS mice, and the second component to outside of this segment.

Genome of congenic strain. The similar cumulative incidence and mean age at onset of IDDM in NOD MHC homozygotes to those in the NOD parental strain in the same animal facility (91 vs. 90% in females and 55 vs. 55% in males by 210 d of age) suggest that the background genes of the congenic strain are completely replaced by the NOD genome. To further confirm that the reduction in incidence of IDDM in NOD.CTS-H-2 congenic mice is not due to the difference in background genes, the genome of the congenic mice was fingerprinted with 103 locus-specific microsatellite markers. To confirm that chromosomal intervals where *Idd* loci were previously mapped were replaced by the NOD chromosome, at least one marker located in each interval was included. All the markers except for those on chromosome 17 were of NOD origin, indicating that the background genes of the congenic mice are completely replaced by the NOD genome and that the lower incidence of IDDM in the congenic strain than that in the NOD parental strain is due to the difference in chromosome 17.

Chromosome 17 of congenic strain. To further localize MHC-linked diabetogenic genes, chromosome 17 of the NOD.CTS-H-2 mice was compared with those of NOD and CTS mice using 32 polymorphic markers. The results indicated that only the < 12 -cM segment between (but not including) D17MIT144 and D17MIT50 was of CTS origin and the rest of chromosome 17 was replaced by NOD chromosome by recombination (Table II and Fig. 1). Since the CTS MHC is identical to the NOD MHC in the 0.5-cM segment encompassing *Oa* to

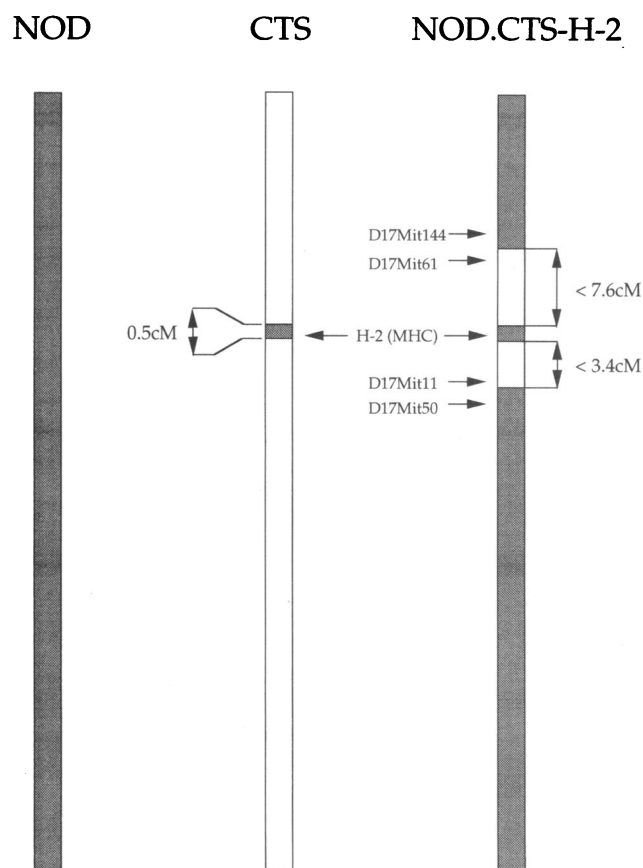


Figure 1. Chromosome 17 of NOD, CTS, and NOD.CTS-H-2 congenic strains.

Hsp70.1, these data place the second gene(s) that is responsible for up to 90% reduction in the incidence of IDDM in the < 7.6-cM segment proximal to *Oa* or the < 3.4-cM segment distal to *Hsp70.1* locus.

***Tnfa* locus.** Since the *Tnfa* gene is a major candidate gene for IDDM (29–31) and is located within the region where a second MHC-linked gene was mapped, we determined the nucleotide sequences of all the exons and exon-intron junctions of the NOD and CTS alleles of the *Tnfa* gene. Although differences in DNA sequences were found between the two strains, all were in noncoding sequences, and the coding sequences and splicing donor and acceptor sequences of NOD and CTS alleles were identical to the published sequences of the BALB/c allele (Fig. 2) (32, 33), indicating that the primary structure of the TNF molecule in NOD mice is identical to that in CTS as well as control mice and that amino acid changes in the TNF molecule are unlikely to be a determinant of IDDM susceptibility.

Discussion

With the aid of recent progress in genetic mapping techniques, it is relatively easy to map a gene responsible for complex traits such as IDDM to a chromosomal segment, but it is much more difficult to fine map the gene for subsequent positional cloning once strong linkage disequilibrium has been established between a genetic marker and a disease gene. Recombination frequency within such a region is too low to fine map the gene

BALB/c	687	CTCCTTCTTTTCTACACAG	AAGT	
NOD		-----*	-----	(exon 2)
CTS		-----	-----	
C57BL/6		-----*	-----	
BALB/c	901	CTAAAGGGCTCATCTTGCCTTTGTCTCTCCCTCCAG	GATC	
NOD		-----A	-----	(exon 3)
CTS		-----A	-----	
C57BL/6		-----A	-----	
BALB/c	1268	CCCCCCCC*TCAG	CAAA	1694
NOD		-----C	-----	TCTG
CTS		-----C	-----	TGAAGG
C57BL/6		-----C	-----	-----A

Figure 2. Nucleotide sequences of the *Tnfa* gene in NOD and CTS mice as compared with BALB/c and C57BL/6 mice. A dash indicates identity with BALB/c allele. An asterisk indicates a deletion. BALB/c sequence is from reference 32. C57BL/6 sequence and numbering is from reference 33.

by standard linkage analysis in breeding studies between two inbred strains. Recombination events, however, may have occurred, or are even occurring, in the original outbred colony from which an inbred strain, such as the NOD mouse, was derived, because the high frequency of heterozygosity in the outbred colony provides a high chance of recombination in each meiosis. Since the process of recombination events within the outbred colony starts long before the breeding of an inbred strain of interest is initiated, there is a high possibility to find recombinant haplotypes in the region of interest among the animals of the original colony and/or related inbred strains derived from the same colony. Based on this hypothesis, we have screened the MHC of NOD-related strains and found recombinant haplotypes in a sister strain, the CTS mouse, and in one mouse in the original colony, Jcl:ICR mice (18). Recombinant haplotypes were also found among other inbred strains derived from original Jcl:ICR mice (34) (Ikegami, H., S. Makino, E. Yamato, Y. Kawaguchi, and T. Ogiyara, manuscript in preparation), indicating the power of our method to identify recombinant haplotypes in a region where strong linkage disequilibrium exists. In fact, we found one recombinant in 10 Jcl:ICR mice (18) and three recombinants in 25 NOD-related strains (Ikegami, H., S. Makino, E. Yamato, Y. Kawaguchi, and T. Ogiyara, manuscript in preparation). In contrast, > 4,000 mice are reported to be needed to generate one recombinant between the class I *K* and class II *Ab* genes (35), and many more mice are necessary to generate a double recombinant as in the case of the CTS MHC. Identification of a recombinant MHC together with the establishment of a NOD MHC congenic strain enabled fine structure mapping of the MHC-linked diabetogenic genes. Our results are the first to demonstrate that an MHC allele other than the NOD allele confers susceptibility to IDDM and, more importantly, that the MHC-linked diabetogenic genes consist of two or more components, one of which is located in a region distinct from previously known *Idd-1* candidates, class II *A*, *E*, and *Tap* genes. These results not only map a new MHC-linked diabetogenic gene(s), but also suggest a new way, termed ancestral haplotype congenic mapping, for fine structure mapping and molecular dissection of genes responsible for a certain trait within a region where strong linkage disequilibrium exists.

A second MHC-linked diabetogenic gene (or genes) mapped in this study strongly affects the incidence of IDDM: 90% reduction in males and 50% reduction in females by 210

d of age. The effect is similar to that reported for other *Idd* loci, such as *Idd-3* and *Idd-10*, with respect to the degree of reduction and delay in the onset of diabetes (26, 27, 36). Initial studies on non-MHC genes showed the linkage of IDDM with a large interval on chromosome 3 (37), which subsequently turned out to be due to summation of at least two susceptibility loci, *Idd-3* and *Idd-10* (26, 27, 36). Since linkage of MHC with IDDM is also very strong, we predicted that there may be multiple susceptibility loci linked to the MHC. Introgression of a segment of NOD MHC in the congenic NOD strain as described in this study enabled genetic dissection of *Idd-1* and identification of a second component of *Idd-1*. We propose designation of the MHC-linked diabetogenic gene(s) located in the 0.5-cM segment of class II region as *Idd-1* and a second gene(s) newly mapped in this study as *Idd-16*. Previous studies suggested that *Idd-1* is encoded by at least two loci, *Ab* and *Ea* genes (2, 10, 38, 39). Similarly, the possibility that *Idd-16* may also be encoded by a number of loci cannot be ruled out. Furthermore, *Idd-16* may confer susceptibility to IDDM even in the absence of *Idd-1* in the class II region. This can be tested using the same strategy as used in this study: to identify a recombinant chromosome that contains the segment encoding a candidate for *Idd-16*, but not *Idd-1*, and to make NOD strains that are congenic for the recombinant chromosome.

A decrease in overall incidence of IDDM and delayed onset of IDDM, as observed in NOD.CTS-H-2 mice, was also reported for NOD strains congenic for *Idd-3*, *-10*, and *-13* (27, 36, 40). Although the incidence of diabetes was much lower in CTS MHC homozygotes than in the NOD MHC homozygotes, the phenotype of diabetic animals was similar regardless of MHC genotypes: all diabetic mice displayed polydipsia, polyuria, and weight loss, showed a similar natural course, and died unless treated with daily injection of insulin. Furthermore, a preliminary histological study of the pancreas suggested that the incidence and degree of insulinitis appear to be similar between the two groups. These data suggest that *Idd-16* may control the progression of insulinitis to overt diabetes.

There are several candidate genes for *Idd-16*. One is *Tnfa* gene. NOD mice are known to be low producers of TNF, and administration of recombinant TNF was reported to prevent IDDM in NOD mice (29, 30). Although the coding sequence of *Tnfa* gene is identical in NOD and CTS mice, there might be a difference in the regulatory region as suggested by different microsatellite alleles and intron sequences between the two strains. The difference in the regulatory region affects the production of TNF (41) and may thereby modulate the incidence of IDDM.

Another candidate is *Sod-2* gene, which encodes mitochondrial superoxide dismutase and is located ~10 cM centromeric to the MHC on chromosome 17 (42). Superoxide dismutase plays an important role in scavenging free radicals, which have been implicated as effector molecules in autoimmune β cell destruction (43). A recent whole genome search mapped *IDDM4* in the region linked to *SOD2* locus on human chromosome 6q (5). Our data, however, ruled out *Sod-2* as a candidate for *Idd-16* because the centromeric region of chromosome 17 including *Sod-2* locus was replaced by NOD chromosome in the congenic mice.

Other candidates are class I MHC genes. Our data suggest that allelic polymorphisms of class I *K* and/or *D* genes may be one of the mechanisms in the reduction of the incidence of IDDM in NOD.CTS-H-2 congenic mice. Consistent with this,

suppression of insulinitis was reported in transgenic NOD mice expressing *L^d* gene (15). Transgenic expression of NOD alleles of class I molecules (*K^d* and/or *D^b*) in NOD.CTS-H-2 congenic mice would clarify the contribution of class I gene polymorphisms to IDDM susceptibility.

Recent whole genome searches for human IDDM susceptibility genes excluded the existence of genes with as great an effect as the MHC genes (5, 6). These studies, however, cannot exclude the presence of a second major susceptibility locus linked to the MHC. Since genetic markers located as far as 20 cM away from the class II region still showed linkage with IDDM (5), it is likely that a number of genes linked to the MHC contribute to human IDDM as in the case of the NOD mouse as shown in this study. In fact, a contribution of class I HLA or *TAP* genes in addition to class II *DQ* and *DR* genes to IDDM susceptibility has been suggested recently (16, 28, 44). Identification and positional cloning of these genes will increase our understanding of the pathogenesis of IDDM and facilitate new therapeutic and preventive strategies.

Finally, ancestral haplotype congenic mapping can be applied to other *Idd* loci, as well as other complex traits. For example, NOD allele of *Il-2* gene, a candidate for *Idd-3*, was found in the CTS (this study) and in other NOD-related strains (Ikegami, H., S. Makino, E. Yamato, Y. Kamaguchi, T. Sakamoto, and T. Ogihara, manuscript in preparation). Since these strains have different microsatellite markers adjacent to *Il-2* locus, a segment of chromosome 3 flanking *Il-2* locus in these strains can be distinguished from the NOD chromosome. Introgression of these chromosomal segments in congenic NOD strains will enable molecular dissection and fine structure mapping of *Idd-3*. More generally, the method will provide a powerful tool for genetic dissection of complex, polygenic, and quantitative traits in inbred animal models that are established from outbred colonies.

Acknowledgments

We thank Dr. H. Kato for information about NOD-related strains and Miss Y. Ueno for skillful technical assistance.

This work was supported by a Grant-in-aid for Scientific Research from the Ministry of Education, Science and Culture, a Grant for Diabetes Research from the Ministry of Health and Welfare, Japan, the Japan Diabetes Foundation, a Grant for Diabetes Research from Otsuka Pharmaceutical Co., Ltd., and the Mochida Memorial Foundation for Medical and Pharmaceutical Research.

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