Immunity, Vol. 7, 873–883, December, 1997, Copyright ©1997 by Cell Press

Genetic Control of Diabetes Progression

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Summary

Autoimmune diabetes in both the human and the nonobese diabetic mouse has elaborate genetics; in the latter case, the disease is influenced by at least 15–20 loci. We anticipated that the genetics would be simpler in the BDC2.5 T cell receptor transgenic mouse model of diabetes, wherein many T cells express a particular diabetogenic specificity. Initiation of insulitis in this model was the same on the two genetic backgrounds analyzed, but the kinetics and penetrance of diabetes were strikingly different, permitting us to focus on genetic influences during a defined window of disease progression. The differences correlated with variations in five genomic intervals, certain ones of which have been previously implicated in susceptibility to autoimmune disease. This reductionist approach indeed simplified the analysis of diabetes susceptibility loci.

Introduction

Insulin-dependent diabetes mellitus (IDDM), in human patients as well as in the nonobese diabetic (NOD) mouse model, has become a standard for the genetic dissection of polygenic diseases. This autoimmune disorder is mediated primarily by T lymphocytes reactive to antigens expressed by pancreatic islet β cells (Bach, 1994; Tisch and McDevitt, 1996). In an initial phase termed insulitis, T cells and other leukocytes infiltrate the islets; after a substantial delay, destruction of the insulin–producing β cells ensues, leading to loss of glucose homeostasis and progression to diabetes. The etiology and pathogenesis of IDDM are clearly complex, perhaps explaining why they remain largely a mystery. We still do not know why self-tolerance and immunoregulatory systems break down—why antigens normally ignored by the immune system seem so provocative to the lymphocytes of diabetic individuals.

Correspondingly, the genetics of IDDM are very complicated. This disorder is known to have an important genetic component, because 38% of an affected twin's siblings are also diabetic (Verge et al., 1995), but no culprit genes have been identified definitively. The main exception is the major histocompatibility complex (MHC), whose class II genes represent the dominant susceptibility factor in both the human and murine disorders. The influence of another 15–20 susceptibility loci has been documented. Polymorphism in a minisatellite within the human insulin gene locus has been identified (Bennett et al., 1995), and the analysis of some of the murine loci by constructing congenic strains is underway (Yui et al., 1996; Denny et al., 1997; Podolin et al., 1997). Yet most of these loci alone have a very limited impact on disease, and a comprehensive understanding of the full genetic influences on diabetes will be extraordinarily difficult (Wicker et al., 1995; Owerbach and Gabbay, 1996; Vyse and Todd, 1996).

Several groups have developed simplified models of IDDM via transgenesis (reviewed in Benoist and Mathis, 1993; Mueller and Sarvetnick, 1995), focusing on one particular player or event in the disease process. To highlight factors influencing the production and behavior of autoreactive T cells, we created the BDC2.5 line of transgenic (Tg) mice, which carries the rearranged T cell receptor (TCR) genes from a diabetogenic T cell clone reactive to a β cell–specific antigen (Haskins et al., 1989; Katz et al., 1993). There are no signs of tolerance induction in these mice but early and extensive leukocyte infiltration into the islets; insulitis persists for several months before a transition to terminal islet destruction and diabetes. This terminal phase can be provoked rapidly by the transfer of BDC2.5 T cells activated in vitro under conditions favoring differentiation to the T helper (Th)-1 phenotype (Katz et al., 1995). It is not clear what factors keep the autoimmune attack in check for such prolonged periods or what eventually disrupts the equilibrium (André et al., 1996). It is important to resolve these issues given that the human and NOD diseases appear to have a similar biphasic course.

The BDC2.5 Tg line is a potentially powerful tool for analyzing the genetics of the disease process. It allows one to bypass events involved in the generation and expansion of the repertoire of autoimmune T cells, which are preformed in the Tg mice, and to focus on the later steps of diabetes development. Our hope was that this reductionist approach would reduce significantly the number of genetic loci for which to account. Here, we have exploited the BDC2.5 TCR Tg model to identify and map loci that control the penetrance and time course of diabetes.

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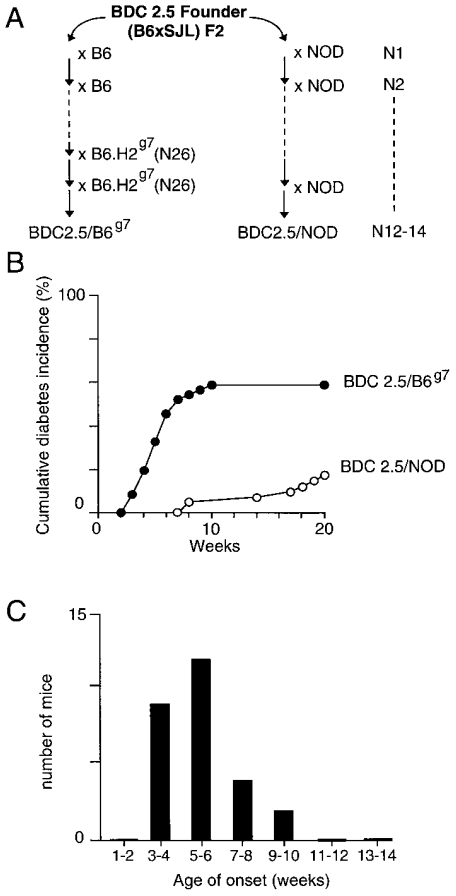

Figure 1. Genetic Background Effect on Diabetes of BDC2.5 TCR Tg Mice

(A) Parallel backcrosses of the BDC2.5 transgene onto the NOD and B6.H2^{g7} backgrounds.

(B) Cumulative incidence of diabetes in BDC2.5/NOD (n = 41) and BDC2.5/B6^{g7} mice (n = 46). All of the mice examined were BDC2.5 Tg and H2^{g7} homozygotes.

(C) Histogram showing the age of onset of diabetes in the BDC2.5/B6^{g7} mice (same cohort as in [B]).

Results

C57Bl/6 Genes Promote Accelerated Diabetes in BDC2.5 TCR Tg Mice

Many of the T cells in BDC2.5 TCR Tg mice express the transgene-encoded receptor, specific for an unknown pancreatic islet β cell antigen presented by the MHC class II A^{g7} molecule (the NOD allele). A^{g7} is required for thymic positive selection of cells expressing the BDC2.5TCR as well as for their peripheral reactivity (Katz et al., 1993). Preliminary data suggested that the genetic background on which the TCR transgenes were carried influenced the character of the disease, prompting us to investigate genetic influences more systematically (Figure 1A). Parallel backcrosses onto the NOD/Lt and C57Bl/6(B6) backgrounds were performed for 12 generations, resulting in greater than 99.95% NOD and B6 genotypes, respectively. Mice on the B6 background were then crossed for two generations with the MHC-congenic line B6.H2^{g7}, which carries the g7 haplotype of the MHC but has all other genes from the B6 strain. Thus, the final lines carried the BDC2.5 TCR transgenes together with selecting H2^{g7} MHC alleles but had different alleles at all other loci; hereafter, they will be referred to as BDC2.5/NOD and BDC2.5/B6^{g7}.

The two lines showed thymic positive selection of T cells displaying the BDC2.5 TCR specificity as well as peripheral seeding. (We did note a somewhat more effective selection on the B6 background, the relevance of which is discussed at length below.) A plot of the incidence of spontaneous diabetes in the two Tg lines is shown in Figure 1B. BDC2.5/B6^{g7} animals had a very fast onset of diabetes. Some were already diabetic at 3 weeks of age, and the the most frequent age of onset was 6 weeks; the incidence of diabetes in several independent cohorts already averaged 58.7% at 10 weeks. The animals that were free of diabetes at 10 weeks of age essentially remained resistant thereafter (Figure 1C). In contrast, disease appeared very slowly in BDC2.5/NOD mice; only 4.9% were diabetic before 12 weeks, and fewer than 20% were diabetic by 20 weeks of age (Figure 1B). No sex bias was apparent in either case.

These results are consistent with our initial observations on early NOD backcrosses of the BDC2.5 TCR Tg line (Katz et al., 1993), in that disease also appeared after 15–20 weeks. However, the incidence of disease has decreased significantly. This could be due to the more complete backcross onto the NOD background (see below) or to some degree of selection against transgene potency in successive crosses.

The present comparison suggests that polymorphic background genes that differ in B6 and NOD mice control the installation of stable insulitis versus a rapid transition to diabetes (previously termed checkpoint 2 [Katz et al., 1993; André et al., 1996]). As discussed at length below, B6 mice, otherwise not prone to autoimmune disease, carry the susceptibility alleles.

Early-Onset Diabetes Reflects a Rapid Evolution of Insulitis

In the original description of BDC2.5/NOD mice, we reported that insulitis follows an interesting time course: absent before 3 weeks of age then appearing abruptly (Katz et al., 1993). Thus, two scenarios might have been envisaged to explain the early diabetes in BDC2.5/B6^{g7} mice: a precocious insulitis, starting at birth and raging destructively in the young pancreas, or an insulitis with the same time of onset but of a more destructive nature. To distinguish between the two, we performed a histological analysis of hematoxylin–eosin-stained pancreata from BDC2.5/B6^{g7} and BDC2.5/NOD mice of different ages. For the NOD background animals, insulitis was absent at day 12, appeared at day 18, and was more extensive at day 24 (Figure 2A). It progressed smoothly, taking an organized appearance with a well-defined border between β cells and the large homogeneous masses of infiltrating cells (Figures 2Ba and 2Bb).

For the B6 background animals, the kinetics were essentially the same, with insulitis first detected at day 18 in the majority of mice (Figure 2A). However, the aspect of the infiltrate was markedly different: the onset

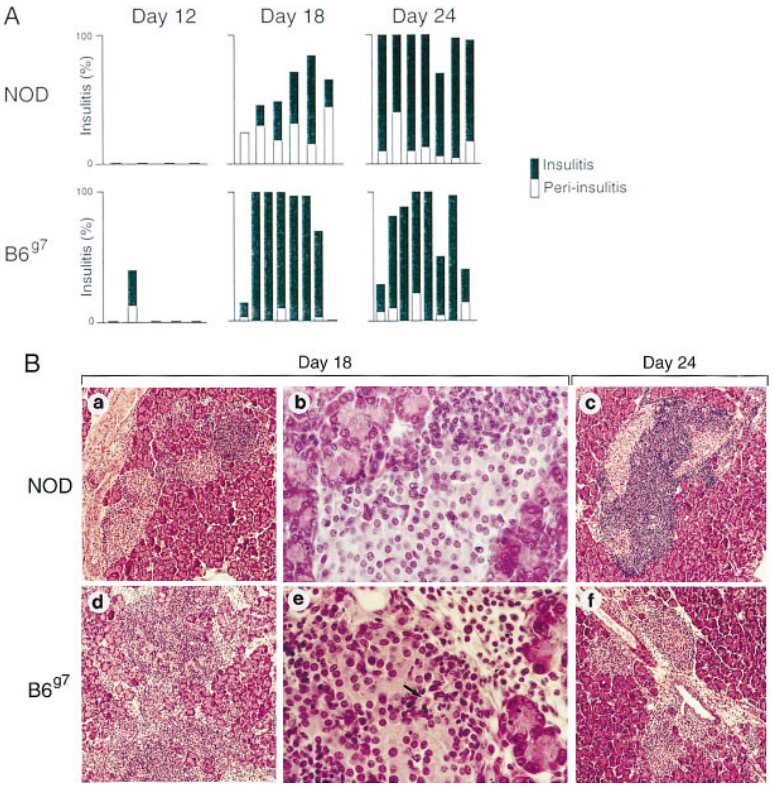

Figure 2. Effect of the Genetic Background on the Intensity but Not the Timing of Insulitis in BDC2.5 TCR Tg Mice

(A) Percentage of pancreatic islets showing mononuclear infiltrates in BDC2.5 Tg mice on the NOD and B6.H2^{g7} backgrounds at 12, 18, and 24 days of age.

(B) Representative hematoxylin-eosin–stained sections of pancreata from 18- and 24-day-old BDC2.5/NOD and BDC2.5/B6^{g7} mice. Arrows in (e) point to apoptotic figures. 10× objective, (a), (c), (d), and (f); 40× objective, (b) and (e).

was much more aggressive, appearing as a severe inflammatory reaction with marked edema around the islets and large numbers of lymphocytes and inflammatory cells in swollen connective spaces, spreading toward the exocrine acini (Figure 2Bd); the attacking cells penetrated the islets more aggressively, either dividing them by tracts of infiltration or appearing as a "swarm of bees" on the sections (Figures 2Bd and 2Be); apoptotic condensations within the β cells were common (e.g., Figure 2Be); and the strong inflammatory process was also deleterious to exocrine acinar cells in the vicinity of the islets (Figure 2Bd). Curiously, this extreme picture was less frequent in day-24 sections (Figure 2Bf), as though an initial burst of inflammatory activity was coming at least partially under control. (Note, however, that the BDC2.5/B6^{g7} day 24 group is somewhat biased, given that a proportion of the animals had already become diabetic and were not included in the histological study.) These images of hyperaggressive insulitis seen with BDC2.5/B6^{g7} mice were never seen on the NOD background. However, they are very reminiscent of what we observed after transfer of activated Th1-type BDC2.5 cells into neonatal NOD recipients (Katz et al., 1993) or after treating BDC2.5/NOD mice with cyclophosphamide (André et al., unpublished data).

Thus, it seems that in BDC2.5/NOD mice some elements are capable of restraining the insulitis for months, keeping it nonaggressive and inhibiting the transition to diabetes. In BDC2.5/B6^{g7} animals, insulitis is far more explosive and damaging from the outset, as if the processes that can restrain the aggressiveness of infiltrating T cells are absent or at least substantially muted.

A Few Recessive B6 Genes Confer Susceptibility to Early-Onset Diabetes

Given the striking difference in the diabetes phenotypes of BDC2.5/NOD and BDC2.5/B6^{g7} mice, we performed a genetic analysis to identify the loci controlling disease aggressiveness. As a first step, F1 mice were generated by mating BDC2.5/NOD females with B6.H2^{g7} males (Figure 3A). Sixty Tg mice of the resulting progeny were monitored for diabetes development (Figure 3B). Few F1 mice became diabetic, and the delayed onset mirrored that of parental BDC2.5/NOD animals (12% incidence by 20 weeks, predominantly in females). Therefore, the B6 alleles at the loci that determine early-onset diabetes are recessive in the F1 generation.

Subsequently, we backcrossed onto the susceptible B6 background. After mating BDC2.5 Tg F1 mice with B6.H2^{g7} animals, we obtained 665 BC1 offspring, of which 338 were Tg (Figure 3A). The BC1 Tg mice were monitored for diabetes starting from 2 weeks of age. A significant fraction (23.1%) were diabetic by 10 weeks of age; the most frequent time of onset was 6 weeks, just as in BDC2.5/B6g7 animals (Figure 3C). Thus, the parental early-onset phenotype seemed to reemerge rapidly upon backcrossing. The high frequency of early diabetes development in the BC1 generation suggests that one or only a few recessive B6 alleles are responsible for this phenotype.

Identification of the B6 Loci that Promote Early-Onset Diabetes

Next, we performed a genome-wide scan, assaying simple sequence length polymorphisms (SSLPs) to localize

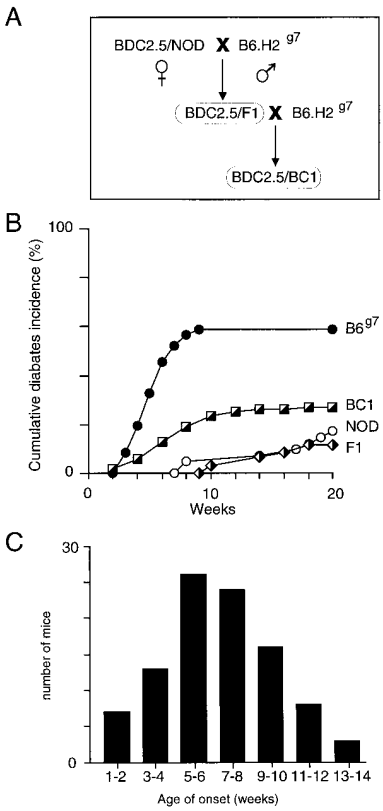

Figure 3. A Few B6 Genes Conferring Susceptibility to Early-Onset Diabetes

(A) Breeding setup: BDC2.5/NOD females were crossed with B6.H2^{g7} males, and the resulting TgF1 progeny were backcrossed with B6.H2^{g7} mice to yield BC1 offspring.

(B) Cumulative incidence of diabetes in BDC2.5/F1 mice (n = 60) and in BDC2.5/BC1 mice (n = 338). Data for the parental strains (Figure 1) are included for comparison.

(C) Histogram showing the age of onset of diabetes in the BDC2.5/BC1 mice (same cohort as in [B]).

genetic elements that cosegregate with the early-onset diabetes phenotype in the BC1 cohort. This was accomplished in three steps. Initially, we used 80 polymorphic SSLP markers that distinguish the NOD and B6 alleles; the markers covered approximately 95% of the 19 autosomes with a mean interval of 20 cM and included all of the chromosomal regions associated previously with diabetes in NOD mice (*Idd* loci). We analyzed the segregation of this set of markers in 40 BC1 mice that had succumbed to diabetes before 12 weeks; cosegregation was reflected by preferential transmission of the B6 allele in the diabetic mice. Linkage of a given microsatellite to a locus favoring early-onset diabetes should show a preponderance of the homozygous (*b/b*) over the heterozygous (*b/n*) genotype in diabetic animals; unlinked loci should show an even distribution. Thirty nondiabetic Tg BC1 mice were also examined to exclude transmission distortions unrelated to the diabetic phenotype. In the next phase, 38 microsatellite markers that showed some segregation bias (chosen so that the probability of missing a significant linkage would be <5%) were studied in an additional cohort of early-onset diabetic BC1 animals (up to a total of 89 mice). Finally, 12 loci that still showed some degree of linkage after testing of this second cohort (with very loose criteria; pointwise $P < 0.05$) were investigated further by examining 29 additional microsatellites in the vicinity of the initial markers to ensure that linked loci were not falling between the first-round markers. In addition, the sampling of nondiabetic animals was extended. The data from this scan are reported in Table 1; results and statistical analyses for the strongest linkages are compiled in Table 2 and Figure 4.

Four regions showed an association with early-onset diabetes, exhibiting a preponderance of *b/b* genotypes spanning several neighboring markers. The strongest association (pointwise $\chi^2 = 17.1$; $P = 3 \times 10^{-5}$) was found with a stretch on chromosome 7, covering a wide plateau between markers *D7Nds6* and *D7Mit232* (22 cM according to the Massachusetts Institute of Technology [M.I.T.] database, see below). The statistical P values at these positions put them well within the range of significant linkage, according to the stringent criteria proposed by Lander and Kruglyak (1995). This bias was reversed in the nondiabetic cohort, where the heterozygous genotypes were more frequent, confirming the significance of the linkage (Table 2). The width of this region, coupled with the 28 recombination events between *D7Nds6* and *D7Mit232* among the 89 diabetic mice (with 14 single homozygotes at either of the loci), suggests strongly that there are two closely linked loci on chromosome 7. This possibility was confirmed by logistic regression analysis (Table 2, and see below). These loci will tentatively be referred to as aggressive insulin-dependent diabetes (*aid*) *1* and *2*.

Another region of association resided on chromosome 1, the maximum bias with marker *D1Mit22* at 32 cM. Association of this locus was strong (pointwise $\chi^2 = 12.24$; $P = 5 \times 10^{-4}$), with a level of significance well within the suggestive linkage and close to the significant linkage range according to the same criteria. Regions of weak association were found on chromosomes 2 and 15, centered around *D2Mit305* at 60 cM and *D15Mit235* at 33 cM (pointwise $\chi^2 = 9.45$; $P = 2 \times 10^{-3}$, suggestive of linkage).

No other region of significant association was found. Several markers (e.g., on chromosomes 4 and 6) showed a preponderance of *b/b* homozygotes, but only at a level of significance that falls in the range of the false positive outcomes expected during such genome-wide searches. A locus on chromosome 3 (in the vicinity of *D3Mit57*), which also failed to attain the suggestive range of significance, showed a heterozygote bias among diabetic mice, possibly indicative of a NOD susceptibility locus. The strength of association of these three regions with early-onset diabetes is insufficient to affirm or exclude their involvement; thus, they will not be considered further in this report.

This genome-wide search also uncovered the location of the integration site of the BDC2.5 TCR transgene, which was in chromosome 13 near the *D13Mit125* marker. A strong bias to the heterozygous genotype was found in Tg mice, whether diabetic (3:26) or nondiabetic (6:25), reflecting that the transgene originated from the BDC2.5/NOD line. Since the BDC2.5 TCR Tg line is being used

Table 1. Results of the Genome SSLP Scan for Loci Associated with Early-Onset Diabetes

Chr	cM	Marker	Ho	He	χ^2	Chr	cM	Marker	Ho	He	χ^2
1	9.8	D1Mit372	52	29	6.53	8	14.2	D8Mit291	26	26	
	19.7	D1Mit321	57	32	7.02		38.3	D8Mit249	26	26	
	32.6	D1Mit22	61	28	12.24		57.9	D8Mit316	25	27	
	41.5	D1Mit24	60	29	10.80		71.0	D8Mit324	25	27	
	52.5	D1Mit48	60	29	10.80						
	60.1	D1Mit386	56	33	5.94	9	12.0	D9Mit297	25	27	
	69.9	D1Mit286	52	37			23.0	D9Nds6	21	19	
	82.0	D1Mit33	28	24			41.5	D9Mit133	37	52	
	104.9	D1Mit221	23	27			60.1	D9Mit212	22	30	
2	5.5	D2Mit2	25	24		10	9.8	D10Mit16	29	22	
	28.4	D2Mit297	29	23			33.9	D10Mit115	25	27	
	50.3	D2Mit44	51	34			50.3	D10Mit95	24	28	
	55.7	D2Mit395	56	32	6.55		69.9	D10Mit14	25	27	
	60.1	D2Mit305	59	30	9.45						
	64.5	D2Mit209	57	32	7.02	11	9.8	D11Mit152	27	25	
	68.9	D2Mit47	54	35	4.06		31.7	D11Mit260	29	23	
	91.8	D2Mit346	40	49			40.4	D11Nds1	26	22	
							48.1	D11Mit212	21	19	
3	9.8	D3Mit203	29	23			74.3	D11Mit61	18	22	
	14.2	D3Mit21	29	23							
	30.6	D3Mit49	40	49		12	9.8	D12Mit136	27	25	
	33.9	D3Nds11	37	52			28.4	D12Mit156	23	26	
	38.3	D3Mit103	35	54	4.06		51.4	D12Mit141	52	36	
	40.4	D3Mit57	36	52							
	45.9	D3Mit291	35	54	4.06	13	13.1	D13Mit61	3	26	
	60.6	D3Mit147	37	48			26.2	D13Mit125	3	17	
							47.0	D13Mit262	6	14	
4	10.9	D4Mit171	48	41							
	18.6	D4Mit23	53	36		14	2.2	D14Nds1	43	45	
	30.6	D4Mit178	54	35	4.06		3.3	D14Mitll	44	45	
	39.3	D4Mit145	51	38			31.7	D14Mit234	40	49	
	54.6	D4Mit12	45	44			47.0	D14Mit193	40	47	
	61.2	D4Mit71	43	46			63.5	D14Mit75	33	39	
	71.0	D4Mit160	36	53							
	74.0	D4Nds16	37	52		15	10.9	D15Mit180	50	37	
	82.0	D4Mit59	35	53			16.4	D15Mit255	52	37	
							17.5	D15Mit206	52	36	
5	0.0	D5Mit346	22	20			24.0	D15Mit121	54	32	5.63
	5.5	D5Mit61	23	18			32.8	D15Mit235	59	30	9.45
	28.4	D5Mit113	48	41			35.0	D15Mit71	57	32	7.02
	51.4	D5Mit210	39	45			49.2	D15Mit159	57	32	7.02
	77.6	D5Mit223	31	37			55.7	D15Mit147	55	34	4.96
							57.9	D15Mit193	51	35	
6	9.8	D6Mit223	24	28							
	29.5	D6Mit261	26	26		16	6.6	D16Mit131	27	25	
	51.4	D6Mit111	52	37			26.2	D16Mit195	32	31	
	55.7	D6Mit291	54	35	4.06		33.9	D16Mit140	51	37	
	63.4	D6Mit14	54	35	4.06		40.4	D16Mit178	52	37	
	66.7	D6Mit374	48	41			50.3	D16Mit128	50	37	
7	0.0	D7Mit152	61	28	12.24	17	0.0	D17Mit19	36	39	
	4.4	D7Nds6	64	25	17.09		7.7	D17Mit28	NA	NA	
	5.5	D7Mit245	64	25	17.09		12.0	D17Mit176	NA	NA	
	12.0	D7Mit308	64	25	17.09		29.5	D17Mit87	40	43	
	16.4	D7Mit228	63	26	15.38		42.5	D17Mit207	26	25	
	18.6	D7Nds5	62	27	13.76						
	26.2	D7Mit232	64	25	17.09	18	9.8	D18Mit120	27	23	
	29.1	D7Mit30	60	29	10.80		31.7	D18Mit188	25	27	
	33.9	D7Mit123	55	29	7.88						
	41.5	D7Mit253	52	36		19	8.7	D19Mit79	26	26	
	49.2	D7Mit105	49	36			32.8	D19Mit27	22	30	
	64.5	D7Mit242	38	46			55.7	D19Mit71	25	27	

The Ho and He columns indicate, for each of the 109 markers used, the number of diabetic BDC2.5/BC1 mice that were homozygous or heterozygous, respectively, for the B6 alleles. χ^2 values of the goodness-of-fit test are shown only when the distribution of genotypes differs from the expected 50:50 with $P < 0.05$. Shaded areas highlight the four regions segregating with the early-onset diabetes. The boxed region on chromosome 13 corresponds to the insertion site of the BDC2.5 transgenes indicated by a marked increase in the NOD alleles. Map positions (in centimorgans) were taken from the Whitehead Institute/M.I.T.-Center for Genome Research.
NA, nonapplicable because both the NOD and B6.H2^{g7} parental mice carried the NOD alleles for these markers.
Chr, chromosome.

Table 2. Statistical Data for the Loci Associated with Early-Onset Diabetes

	Diabetics					Nondiabetics		Combined Data			Multivariate Analysis[a]
Locus Marker	Ho	He	χ^2 [b]	P[c]	μ(T)[d]	Ho	He	χ^2 [e]	P[c]	μ(T)[d]	P[f]
Significant linkage											
aid1 D7Nds6	64	25	17.09	0.00003	0.017	33	54	20.53	6×10^{-6}	0.004	0.03
aid2 D7Mit232	64	25	17.09	0.00003	0.017	29	58	26.07	3×10^{-7}	0.00025	0.0001
Suggestive linkage											
D1Mit22	61	28	12.24	0.0005	0.20	40	47	9.16	0.0025	0.77	0.002
D2Mit305	59	30	9.45	0.0021	0.67	33	54	14.18	0.0002	0.09	0.0016
D15Mit235	59	30	9.45	0.0021	0.67	35	52	12.01	0.0005	0.20	0.005
Nonsignificant											
D3Mit103	35	54	4.06	0.044	6	39	48		NS		
D3Mit291	35	54	4.06	0.044	6						
D4Mit178	54	35	4.06	0.044	6	49	38		NS		
D6Mit291	54	35	4.06	0.044	6						
D6Mit14	54	35	4.06	0.044	6	45	42		NS		

[a] Logistic regression analysis of the combined data for the five markers showing significant bias.
[b] Goodness-of-fit test against the expected 50:50 distribution.
[c] Probability corresponding to the χ^2 value.
[d] Number of unlinked markers, which will show, by chance, a similar bias in a whole genome scan (Lander and Kruglyak, 1995).
[e] 2 $\times$ 2 contingency table comparing the genotypes in diabetic and nondiabetic BC1 mice.
[f] Probability corresponding to the t value for the parameters of the logistic regression model.
Ho, homozygous.
He, heterozygous.
NS, not significant.

by us and others in a variety of diabetes studies, it seemed worthwhile to confirm this localization by in situ hybridization on metaphase chromosome spreads. Splenocytes from a BDC2.5TCR Tg mouse were hybridized with a probe from the TCR-α constant segment. Consistent with the microsatellite analysis, hybridization signals were detected on chromosome 13C (as well as at the normal TCR-α locus on chromosome 14D) (data not shown).

The Identified Loci Account for Most of the Early-Onset Diabetes in Mice of the B6 Background

Once the putative susceptibility regions were identified, it was important to determine their relative importance, their contribution to the phenotype of early-onset diabetes in BDC2.5/B6^{g7} mice, and their interactions. Initially, we evaluated the genotypic risk ratio (GRR) (Risch et al., 1993) at the different loci. Pointwise GRRs were 0.69

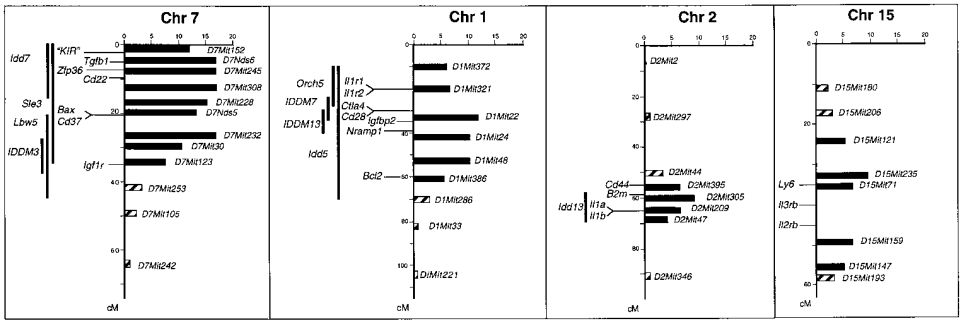

Figure 4. Schematic Representation of the Susceptibility Loci for Early-Onset Diabetes

Map of mouse chromosomes 7, 1, 2, and 15 showing the loci associated with the early-onset diabetes in the BDC2.5/BC1 mice. Length of the horizontal bars represents the association of the different SSLP markers with early-onset diabetes (χ^2 values in the goodness-of-fit test). Filled bars, linked markers; striped bars, unlinked markers. The relative positions (in centimorgans) of the SSLP markers are from the M.I.T. database, even in instances when the genetic distances in our crosses differed significantly. Potentially relevant genes are represented to the left of the chromosomes; for consistency, their relative map positions from the Mouse Genome Database (http://www.informatics.jax.org/mgd.html) are shown anchored on the scale of the M.I.T. genetic map. Further on the left, previously reported autoimmune susceptibility loci are indicated. The size of these regions is based on the published data: *Idd7* (Ghosh et al., 1993; McAleer et al., 1995), *Sle3* (Morel et al., 1994), *Lbw5* (Kono et al., 1994), *Orch5* (Meeker et al., 1995), *Idd5* (Cornall et al., 1991; Garchon et al., 1991; Ghosh et al., 1993), and *Idd13* (Serreze et al., 1994). When the available data were imprecise, we display 15 cM to either side of the position of the reported marker. Syntenic correspondences with human *IDDM* loci are approximate (*IDDM3* [Field et al., 1994], *IDDM7* [Copeman et al., 1995; Owerbach and Gabbay, 1995; Nistico et al., 1996], and *IDDM13* [Morahan et al., 1996]); the size of these loci has been set arbitrarily at 10 cM.

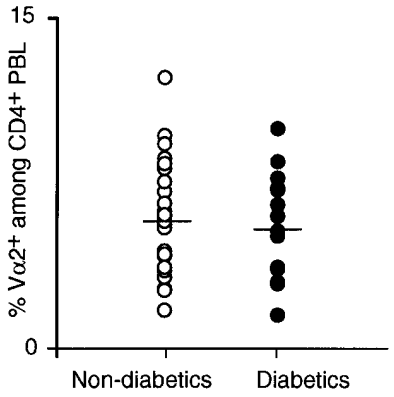

Figure 5. Equivalent Numbers of T Cells Bearing Endogenous TCRs in Diabetic and Healthy BC1 Mice

Percentages of $CD4^+$ T cells bearing a Vα2 chain in the blood of BDC2.5 Tg BC1 mice. Blood samples were taken prospectively at 28 days of age, and the mice were monitored for diabetes to 12 weeks. Mean values were 5.4% ± 2.4% in the 15 diabetic mice and 5.7% ± 2.4% in the 29 nondiabetic BC1 mice.

for both *D7Nds6* and *D7Mit232*, 0.73 for *D1Mit22*, and 0.75 for both *D2Mit305* and *D15Mit235*, indicating fairly similar overall contributions. GRRs of this magnitude place these loci among the most influential NOD *Idd* loci (Risch et al., 1993).

That the bulk of the susceptibility to early-onset diabetes in BDC2.5/B6^{g7} Tg mice can be accounted for by these five loci was indicated by the overall penetrance (78%) of early-onset diabetes when all are *b/b* homozygous. This frequency is as high as or higher than that in parental BDC2.5/B6^{g7} mice. That each locus contributes independently was also shown by logistic regression analysis (Table 2), which confirmed the independent effects of the two loci on chromosome 7. Homozygosity at any one of the loci was not an absolute condition for early diabetes, but B6 homozygosity on at least one of the three strongest loci, *aid1*, *aid2*, and *D1Mit22*, was required.

Analysis of combined haplotypes was performed to evaluate the interactions between these loci. The five loci interact in a cumulative way, diabetes incidence increasing with the number of B6-derived susceptibility alleles. Direct examination of the haplotypes of diabetic mice, as well as log-linear analysis, failed to demonstrate any significant epistatic interactions between these genes. However, the number of animals involved is too small, given the number of loci, and the applicable mathematical models are too general to allow a firm conclusion at this stage.

Differential BDC2.5 TCR Selection Does Not Correlate with the Diabetes Phenotype

As mentioned above, we found that the thymic selection of BDC2.5 T cells was more efficient on the B6 than on the NOD background. This was manifest as a higher proportion of $CD4^+CD8^-$ mature thymocytes (19.6% ± 1.0% in BDC2.5/B6^{g7} vs. 10.3% ± 2.8%) and as a lower proportion of mature thymocytes expressing endogenous rearranged TCR Vα chain genes: 1.1% ± 0.1% or 4.0% ± 0.5% of $CD4^+CD8^-$ thymocytes stain with anti-Vα2 on the B6.H2^{g7} or NOD backgrounds, respectively (we cannot measure the expression of the BDC2.5 Tg Vα chain directly, owing to a lack of a suitable reagent). Similar results were obtained with other reagents detecting other Vα chains of endogenous origin (data not shown). Since 10%–12% of T cells normally express Vα2 in NOD or B6.H2^{g7} mice, we can estimate that 60% of the $CD4^+$ mature thymocytes bear the BDC2.5 TCR on the NOD background, and about 90% bear it on the B6.H2^{g7} background.

This observation indicates that the genetic background influences the selection of BDC2.5 T cells. This does not correspond to a generally more efficient selection on the B6.H2^{g7} background, because non-Tg NOD and B6.H2^{g7} mice have roughly the same proportions of mature $CD4^+$ cells; however, it may correspond to the differential influence of peptides from each background in stimulating or antagonizing selection. More germane to the present study is whether this differential selection is the cause of the different susceptibility to early-onset diabetes. Such an influence of the frequency of Tg Vα chain expression on the autoimmune phenotype has been reported previously (Lafaille et al., 1994; Forster et al., 1995), in particular for BDC2.5 Tg mice, which have faster and more frequent diabetes when carrying the severe combined immunodeficient or TCRα null mutations (Kurrer et al., 1997) (André et al., unpublished data).

Could, then, the *aid* loci act by influencing the efficiency of BDC2.5 selection? To address this possibility, a cohort of BC1 mice, generated as for the genetic study described above, were analyzed prospectively for endogenous Vα2 usage in blood $CD4^+$ lymphocytes at 28 days of age and then followed for early-onset diabetes. As shown in Figure 5, we found no difference in Vα2 frequency between the mice that developed diabetes and those that remained healthy. We also failed to find any correlation between disease occurrence and the frequency of endogenous Vα rearrangement in thymocytes (data not shown). We also found no correlation between Vα2 levels on thymocytes and SSLP genotypes at the five loci described above (data not shown). Thus, the influence of B6.H2^{g7} background genes on provoking early-onset diabetes does not stem from an effect on the selection of the BDC2.5 TCR but rather on how the diabetogenic potential is expressed.

Discussion

In BDC2.5 TCR Tg mice on the NOD and B6.H2^{g7} genetic backgrounds, the early events of disease pathogenesis are fixed and equivalent: an anti-islet T cell repertoire is preformed, positive selection generates large numbers of islet-reactive T cells (Katz et al., 1993); the inciting islet cell antigen is similarly expressed (Haskins et al., 1989); and the kinetics of T cell invasion of the islets are comparable (Figure 2). However, progression to overt diabetes is markedly different on the two backgrounds: BDC2.5/B6^{g7} mice develop an IDDM typical in most features, but, in contrast to the disease in BDC2.5/

NOD or regular NOD animals, there is a quick transition from initial islet infiltration to massive destruction of the insulin–producing β cells. Thus, a genetic analysis of BDC2.5 Tg mice on the NOD versus B6.H2^{g7} background allowed us to focus specifically on a crucial window of disease: unfolding of the destructive potential of the insulitic lesion. No doubt a reflection of this narrowed focus, the genetic influences we observed were simplified compared with those impinging on NOD mice, as evidenced by the high incidence of aggressive disease in BC1 mice and that the five loci uncovered in the SSLP screen could account for essentially all of the B6/NOD differential.

At first it may appear paradoxical that the genome of the NOD mouse, arguably the best murine model of autoimmune disease, would provide a diabetes-resistant background, while the B6 genome conferred susceptibility. However, a balance of susceptibility and resistance alleles within a population is a characteristic of polygenic diseases, and no single inbred mouse strain would be expected to carry the full diabetes-predisposing complement of the *Mus* species. Indeed, diabetes-resistance loci in the NOD genome have been described previously (Ghosh et al., 1993; McAleer et al., 1995). Furthermore, the very nature of the NOD strain and the history of its isolation from an outbred Institute for Cancer Research stock (Tochino, 1986) almost guarantee that the *aid* elements would have been eliminated from the NOD genome; the selection of NOD as a defined strain by brother/sister matings imposed a strong selection pressure for diabetes susceptability but also for the ability to reproduce. Thereby, it was preordained that the resulting mouse would remain healthy during the primary ages of reproduction (6–15 weeks) but show frequent diabetes thereafter. Any *aid* alleles conferring early-onset disease would have been incompatible with survival of the NOD line as it was propagated. *aid* alleles become evident in this study because the Tg line was propagated on the B6 background under the protection of the H2^{b} MHC haplotype, which does not allow presentation of the relevant islet cell antigen to BDC2.5 T cells; only in the outcross to B6.H2^{g7} can the disease-accelerating alleles come into play. Therefore, it seems that NOD mice are susceptible to the formation, activation, and expansion of an autoimmune repertoire, but, at the same time, they are quite able to keep the autoimmune process in check.

It will be of great importance to decipher the cellular and biochemical events that are controlled by the *aid* loci. We have shown previously that diabetes in the BDC2.5 Tg model is influenced strongly by the Th1/Th2 phenotype of the invading T cells (Katz et al., 1995). However, an influence on the Th1/Th2 balance is unlikely to be the sole explanation for the effect of the *aid* susceptibility alleles; we have not found a shift toward the Th1 phenotype in splenocytes from B6 compared with NOD mice, judged from cytokine production after anti-CD3 stimulation in vitro (André et al., unpublished data). Furthermore, if anything, the NOD mouse has been reported to have defective Th2 cytokine production (Rapoport et al., 1993; Gombert et al., 1996).

What candidate genes are found in the vicinity of the *aid* loci? A number of genes appear to be possibilities (Figure 4), but it is important to keep in mind that our mapping data are still too coarse to argue strongly in their favor. In the *aid1* interval on chromosome 7, an appealing candidate is *Tgfb1* (coding for transforming growth factor-β1), because this cytokine is known to have anti-inflammatory activity and has been implicated in the dampening of autoimmune diseases, including IDDM (Pankewycz et al., 1992; Wahl, 1994; Hancock et al., 1995). Another gene with a potential role is *Zfp36* (encoding zinc finger–binding protein 36), given that a null mutation resulted in systemic autoimmunity, apparently through uncontrolled production of tumor necrosis factor α (Taylor et al., 1996). It has been reported recently that members of the killer inhibitory receptor family expressed on natural killer cells and on activated T cells and macrophages is encoded in a gene complex located in the proximal region of chromosome 7 (2–4 cM) (Wagtmann et al., 1997). These would make attractive candidates in an immunoregulatory context. Other genes coding for molecules involved in the immune system (e.g., those encoding CD22, CD37, and Bax), whose influence seems less easily explainable, also map in this region of chromosome 7.

On chromosome 1, enticing candidates are the genes for CD28 and CTLA-4, whose activities regulate reciprocally the activation of T cells. Signaling through CD28 enhances T cell stimulation, whereas CTLA-4–mediated signals can have a negative effect (Allison and Krummel, 1995). The *Nramp1* gene, whose allelic variants determine susceptibility or resistance to intracellular infections, also maps in the vicinity. *Nramp1* has pleiotropic effects on macrophages, regulating priming and activation for antimicrobial and tumoricidal activities. The implicated region on chromosome 2 contains the genes encoding the inflammatory cytokine interleukin-1. On chromosome 15, the *Ly6C* gene maps to the implicated region, and there are known polymorphisms between alleles in NOD and nonautoimmune strains (Philbrick et al., 1990).

Other alleles predisposing to autoimmune pathology have been reported to reside in the same genomic intervals as the *aid* loci (Figure 4). The most intriguing region is certainly that containing *aid1–2* on chromosome 7. Suggestively, it coincides with the NOD *Idd7* locus, which is one of the three diabetes resistance loci of the NOD genome mapped in backcross analyses of diabetes (Ghosh et al., 1993; McAleer et al., 1995). In addition, *aid2* maps in the region syntenic to that which contains the *IDDM3* locus, linked to diabetes in HLA-susceptible sib-pairs (Field et al., 1994). The *aid1–2* region also encompasses a similarly spaced pair of loci (*Sle3* and *Lbw5*) shown to influence the severity of glomerulonephritis in the NZB/W model of systemic lupus erythematosus (Morel et al., 1994; Kono et al., 1994). Such clustering suggests that different inbred strains may express allelic variants of proteins that have a role in pathological mechanisms common to several autoimmune models.

The chromosome 1 region is another that overlaps with loci implicated previously in diabetes. *Idd5* is probably not relevant because it is a susceptibility locus for NOD diabetes involved mainly with insulitis (Cornall et al., 1991; Garchon et al., 1991; Ghosh et al., 1993). On the other hand, two human diabetes susceptibility

loci have been localized to the syntenic region: *IDDM7*, in the area containing the *Cd28/Ctla4* gene pair (Copeman et al., 1995; Owerbach and Gabbay, 1995; Nistico et al., 1996), and *IDDM13*, slightly more distal, in the vicinity of *Igfbp2* (Morahan et al., 1996). The chromosome 2 region, which shows suggestive linkage, may overlap with the *Idd13* locus identified in a NOD × NOR intercross, although NOD mice carried the susceptible allele in this instance (Serreze et al., 1994).

This reductionist approach to the genetics of diabetes seems to have been fruitful. We have defined a relatively small number of loci that control a crucial window of diabetes pathogenesis—the progression from insulitis to aggressive β cell destruction. The relevance of these loci to IDDM in general is underscored by the fact that certain of them appear to correspond to genetic elements localized in previous studies of IDDM in standard NOD mice or human patients. The speed of the diabetes readout and the focus on the defined stage of diabetogenesis that the transgene allows should facilitate greatly the identification and understanding of the genes involved.

Experimental Procedures

Mice

BDC2.5 TCR Tg mice have been described (Katz et al., 1993). The founder was bred onto the NOD or B6 genetic background. The BDC2.5/NOD mice used in these experiments have been backcrossed for 12–14 generations onto the NOD background. After 11 generations of backcross onto the B6 background, BDC2.5 TCR Tg mice were crossed with B6.H2^{g7} congenic mice (backcrossed more than 26 generations onto the B6 background). The BDC2.5/B6.H2^{g7} line is maintained by breeding MHC heterozygotes (*b/g7*) to avoid genetic drift as a consequence of selective pressure due to early-onset diabetes (H2$^{g7/b}$ mice show a lower incidence of diabetes [Luhder et al., submitted]). All animals in the present analyses were H2$^{g7/g7}$ homozygotes. For the genetic analysis, F1 mice were produced by mating BDC2.5/NOD females with B6.H2^{g7} males; transgenic BDC2.5 F1 mice were mated with B6.H2^{g7} mice to produce the BC1 generation.

Mice were typed for the BDC2.5 transgenes either by Southern blot analysis of tail DNA with a Vα-specific probe (Katz et al., 1993) or by fluorescence-activated cell sorter analysis of blood after staining for Vβ4 and CD4.

Diabetes and Insulitis

BDC2.5 TCR Tg animals were monitored weekly for diabetes, starting from 2 weeks of age. They were tested either by blood glucose levels (glucofilm strips read in a Glucometer 3, Bayer Diagnostics) or by urine glucose levels (Uristix, Bayer Diagnostics). Mice were considered diabetic if they gave positive readings (blood glucose >300 mg/dl or urine glucose >10 g/l) in two successive tests. Positive urine scores were confirmed by blood glucose testing.

Thin sections from Bouin's-fixed, paraffin-embedded pancreata were examined for the presence of insulitis after hematoxylin-eosin staining. Sections were made at six different levels through the pancreas, and a minimum of 40 islets were examined for each mouse; in very young animals, all of the available islets were analyzed. The percentage of islets with insulitis or periinsulitis was recorded.

Antibodies and Fluorescence-Activated Cell Sorter Analysis

The following monoclonal antibodies were used for T cell analysis, with immunostainings done as described previously (Katz et al., 1993): phycoerythrin-conjugated anti-CD4 (Caltag, San Francisco, CA); biotin-conjugated anti-CD8 (Caltag) revealed with Cy5-labeled streptavidin (Jackson ImmunoResearch); fluorescein isothiocyanate–conjugated H57-597, specific for TCR-αβ (Kubo et al., 1989); and B20.1, specific for Vα2 (Gregoire et al., 1991), revealed with Texas–red-labeled anti-rat IgG (Jackson).

Genetic Mapping and Statistical Analysis

Tail DNA was used for polymerase chain reaction amplification with primers specific for microsatellite markers spanning the 19 autosomes. The 80 microsatellite markers used for the screening were selected from the Whitehead Institute/M.I.T.-Center for Genome Research database (http://www.genome.wi.mit.edu), spaced at about 20 cM intervals and discriminating between NOD and B6 alleles (a few errors were noticed in the M.I.T. database; the full listing of SSLP data for the markers used in this study can be consulted at http://www-igbmc.u-strasbg.fr/cbdm). Some markers were selected because they have been associated with diabetes susceptibility in NOD mice (Cornall et al., 1991; Garchon et al., 1991; Todd et al., 1991; Ghosh et al., 1993; Prins et al., 1993; Morahan et al., 1994; Ikegami et al., 1995; McAleer et al., 1995). Primers were obtained from Research Genetics (Huntsville, AL) or were synthesized in house. Polymerase chain reaction amplification was done with radiolabeled forward primers as described (Dietrich et al., 1992) and the radioactive products run on 6% polyacrylamide sequencing gels. Data were analyzed with the help of the MapManager software (a gift from K. F. Manly).

Evaluation of the association between genotype and diabetes phenotype was done initially with the χ^2 test for goodness of fit against the expected 50:50 distribution. In addition, 87 nondiabetic mice were studied in the regions that showed a significant bias, and the χ^2 values of the corresponding 2 × 2 contingency table were obtained. Significance levels were evaluated according to stringent guidelines for the interpretation of whole genome studies (Lander and Kruglyak, 1995). The chance of finding a marker at random that exceeds the significance level attained by each of the putative susceptibility loci in a whole genome screen [μ(T)] was estimated as described by Lander and Kruglyak (1995). GRRs were calculated as described (Risch et al., 1993); the maximum possible GRR for a recessive susceptibility locus is 0.5 and the minimum is 1.0.

An assessment of the contribution of the individual susceptibility loci to the early diabetes phenotype was made by stepwise logistic regression analysis (entry P values were set at 0.05, and exit P was set at 0.049). Analysis of the interaction of the susceptibility loci was done by analysis of the haplotypes and of their estimated penetrances. Penetrances were estimated assuming an unbiased Mendelian distribution of the haplotypes in Tg BC1 mice and adjusting for the recombination fraction between the two loci of chromosome 7. Also, a log-linear model was fitted to the haplotypes of the diabetic and nondiabetic BC1 mice to detect interactions between susceptibility loci and the early-onset diabetes. Statistical analysis was performed with the STATISTICA software (StatSoft).

Acknowledgments

We thank W. Magnant for great help in diabetes testing, P. Marchall for help with many microsatellites analyses, F. Fischer and V. Louerat for maintaining the mice, G. Molas and V. Duchatelle for advice on the interpretation of the pathology, and J. -L. Mandel for helpful discussions. This work was supported by a grant from the Juvenile Diabetes Foundation International (196078) and by institutional funds from the Institut National de la Santé et de la Recherche Médicale, the Centre National de la Recherche Scientifique, the Centre Hospitalier Universitaire Régional, and Bristol-Myers Squibb. A. G. was supported by fellowships from the European Union and the Ministerio de Educacion y Ciencia de Espana.

Received August 11, 1997; revised November 6, 1997.

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