Diabetologia (2003) 46:1291–1295
DOI 10.1007/s00125-003-1168-7

Diabetologia

Genetic control of non obese diabetic mice susceptibility to high-dose streptozotocin-induced diabetes

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Abstract

Aims/hypothesis. Streptozotocin is a monofunctional alkylating agent that induces diabetes in a large variety of mammals. While multiple low doses of streptozotocin induce immune-mediated diabetes, a single high dose of streptozotocin causes a strictly toxic diabetes. Among mouse strains, non-obese diabetic (NOD) mice are characterized by an extreme susceptibility to high dose of streptozotocin-induced diabetes whereas C3H/Or mice are particularly resistant. We hypothesized that NOD genes involved in high dose streptozotocin-induced diabetes could be also involved in the autoimmune destruction of pancreatic beta cells that characterizes this mouse strain which is a model of Type 1 diabetes.
Methods. We carried out a whole genome linkage scan on a population of (C3H/Or × NOD) × NOD backcross 1 mice in order to identify the genetic loci involved in NOD susceptibility to high dose of streptozotocin-induced diabetes.
Results. Two loci, in chromosome 9 (D9Mit135 marker, 48 cM) and in chromosome 11 (D11Mit286 marker, 52 cM), were associated with NOD susceptibility to high dose streptozotocin-induced diabetes, the latter being co-localized with the autoimmune diabetes-predisposing *idd4* locus. Moreover, we report here that C57BL/6 mice deficient in Nitric Oxide Synthase 2 were as sensitive as wild-type C57BL/6 mice to high dose streptozotocin-induced diabetes.
Conclusion/interpretation. Although the Nitric Oxide Synthase 2 (*Nos2*) gene, localized at 45.6 cM in chromosome 11, is a good candidate gene, our results suggest that Nitric Oxide Synthase 2 activation might not be a crucial event for streptozotocin-induced destruction of pancreatic beta cells. [Diabetologia (2003) 46:1291–1295]

Keywords Non-obese diabetic mouse, diabetes, autoimmune, streptozotocin, pancreatic beta cells, genetic susceptibility, nitric-oxide synthase, C3H, C57BL/6, idd4, genome scan.

Streptozotocin (STZ) was first identified in 1956 from cultures of streptomyces achromogenes [1]. Shortly after, its anti-tumoural and diabetogenic properties were discovered, this drug was widely used to induce diabetes in various mammal species [2]. Among mouse strains, NOD and C57BL/6 are extremely susceptible to STZ diabetogenic action while C3H/Or is particularly resistant to this drug [2, 3]. In contrast to multiple low doses of STZ (MLDS), which eventually induce an immune-mediated diabetes [4, 5], a high dose of STZ (HDS) mediates diabetes by exercising only a direct toxicity on beta cells [6]. Although many researchers have studied STZ pancreatotoxicity, the exact mechanisms remain unknown [5, 7]. On the one hand, STZ is a potent alkylating agent which directly damages DNA [7, 8]. On the other hand, decomposition of STZ might generate free radicals such as nitric

Received: 2 April 2003 / Revised: 6 May 2003
Published online: 15 July 2003

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Abbreviations: BC, backcross; HDS, high dose of streptozotocin; MLDS, Multiple low doses of streptozotocin; NO, nitric oxide; NOD, non obese diabetic; NOS, nitric oxide synthase; PARP, poly (ADP-ribose) polymerase; STZ, streptozotocin.

oxide (NO) which is also involved in the pathogenesis of Type 1 diabetes [9, 10]

Type 1 diabetes is characterized by inflammatory infiltration of islets of Langerhans and autoimmune destruction of pancreatic beta cells. The NOD mouse develops a spontaneous disease similar to human Type 1 diabetes [11, 12]. Linkage analysis and construction of congenic strains revealed the implication of at least 20 insulin-dependent diabetes (*idd*) loci in the NOD mouse susceptibility to diabetes, of which the most influential is the Major Histocompatibility Complex region (*idd1*) [13, 14]. Although many genes are potential candidates, none of the susceptibility genes have been definitively identified. Type 1 diabetes is primarily due to a dysregulation of the immune system; however, many arguments suggest that pancreatic beta cells themselves could also be actively involved in their own immune destruction, essentially by regulating the expression of vulnerability genes [15, 16, 17].

Our purpose was to identify the genetic loci involved in the extreme sensitivity of the NOD strain to HDS-diabetes. We hypothesized that these loci could also be involved in the intrinsic vulnerability of the pancreatic beta cells of this mouse strain to the immune destructive process [17]. Moreover, we expected that the genetics of the susceptibility of the NOD mouse to toxic HDS-diabetes would be less complex than that of the spontaneous disease. A previous study suggested that a single major gene could determine the susceptibility of C57BL/6 mouse to HDS-diabetes [18].

Our segregation analysis of (C3H/Or × NOD) × NOD backcrosses allowed the identification of two genetic loci, on chromosomes 9 and 11, the latter being co-localized with the autoimmune diabetes-predisposing*idd4* locus [19]. These two loci were, however, insufficient to predict the resistance or sensitivity to HDS-diabetes of all the BC1 mice, suggesting that it is polygenic, and, unfortunately, probably at least as complex as the spontaneous autoimmune disease.

Materials and methods

Mice. Mice were maintained in a specific pathogen-free facility with free access to water and food. A genome scan was carried out on 102 (C3H/Or × NOD) × NOD BC1 mice. Linkage results were confirmed in a larger cohort composed of 223 BC1 mice. *Nos2–/–* C57BL/6 mice were provided by the Jackson Laboratory (Bar Harbor, Maine, USA). The use of animals was in accordance with international guidelines (NIH publication N° 85-23, revised 1985).

Tail DNA extraction. Tails were solubilized for 16 h at 50°C in a 250 mmol/l NaCl, 20 mmol/l Tris pH8, 2 mmol/l EDTA, 1% SDS and 250 µg/ml Proteinase K (Invitrogen, Groningen, The Netherlands) lysis solution. DNA was extracted by Phenol: Chloroform: Isoamyl Alcohol (25:24:1, v/v) (Invitrogen) and precipitated by Isopropyl Alcohol. DNA was dissolved in 10 mmol/l Tris pH 8, 0.5 mmol/l EDTA.

STZ administration and blood glucose measurement. NOD, C3H/Or, (C3H/Or × NOD) F1, (C3H/Or × NOD) × NOD BC1, C57BL/6 and *Nos2–/–* C57BL/6 mice received a single injection of STZ (Sigma, St Louis, Miss., USA) at a dose of 180 to 200 mg/kg body weight at 6 to 7 weeks of age. The dose of 180 mg/kg was eventually chosen for the genetic studies because it was the lowest dose able to render, 3 days after the injection, all the control male NOD mice diabetic. We exclusively used male mice which are more sensitive to STZ action than females [20]. STZ was dissolved in 0.1 mol/l sodium citrate buffer (pH 4.5) and was immediately injected intraperitoneally. Blood samples were taken from tail vein at days 0, 3, 7 and 14, and blood glucose concentrations were measured with a glucometer (Glucotrend; Roche Diagnostics, Basel, Switzerland).

Backcross genotyping. The backcrossed mice were genotyped for 74 microsatellite markers, polymorphic between NOD and C3H/Or mice, chosen from the MGD database (http://www.informatics.jax.org/) to cover the autosomal genome with a distance of 20 to 30 cM. Microsatellite sequences were amplified by the Polymerase Chain Reaction (PCR) for 35 cycles of 94°C for 1 min, 46 to 60°C (optimal annealing temperature depending on the marker) for 1 min and 72°C for 1 min. The PCR reaction mixture contained 20 mmol/l Tris-HCl (pH 8.4), 50 mmol/l KCl, 2 mmol/l $MgCl_2$, 0.2 mmol/l dNTP, 0.4 µmol/l primers, 0.4 U Taq DNA polymerase (Invitrogen) and 5 ng DNA. The size of the amplified products was determined by electrophoresis in ethidium bromide-stained 6% agarose gels.

Statistical analysis. Locus association was tested by comparing blood glucose concentrations in homozygous and heterozygous mice with the non-parametric Mann-Whitney U test. Alternatively, we used a simple χ^2 test to compare the frequencies of heterozygous and homozygous mice in diabetic and non-diabetic groups. Mice with glycaemia higher than 200 mg/dl were considered as diabetic. A p value less than 0.02 was considered as statistically significant.

Results

Identification of two susceptibility loci in chromosomes 9 and 11. Susceptibility or resistance to HDS-diabetes is highly variable among mouse strains. NOD and C57BL/6 male mice are indeed characterized by extreme susceptibility whereas C3H/Or mice are highly resistant to the diabetes induced by a single administration of STZ at a dose of 200 mg/kg body weight (Fig. 1). Following the administration of STZ (3 days), almost all NOD mice became diabetic whereas C3H/Or mice did not develop diabetes, at least not for 14 days. In addition, although basal blood glucose concentrations of (C3H/Or × NOD) F1 mice were higher than those of C3H/Or mice, (C3H/Or × NOD) F1 mice remained normoglycaemic and were as resistant as C3H/Or to HDS-diabetes, indicating that the NOD background encodes recessive susceptibility genes.

We carried out a genome scan on 102 BC1 (C3H × NOD) × NOD male mice to identify the genetic loci involved in NOD vulnerability to HDS-diabetes. Of the BC1 mice 45 (44.1%), 72 (70.6%) and 81 (79.4%) became diabetic at 3, 7 and 14 days, respectively after

the STZ injection. The distribution of the number of BC1 mice according to blood glucose at 3 days is shown in Fig. 2 and stands in sharp contrast with the diabetic state of all the STZ-injected control male NOD mice. Since all the male BC1 mice carried the same NOD X chromosome, we genotyped the 102 BC1 mice for 74 microsatellite markers regularly distributed along autosomes. Segregation analysis, assessed using both a non-parametric Mann-Whitney U test and a χ^2 test, disclosed two genetic regions, on chromosomes 9 and 11, associated with NOD susceptibility to HDS-diabetes. Linkage of these two genetic regions was confirmed by typing additional mice (Table 1). The first locus, localized on chromosome 9, is defined by the D9Mit135 marker. The second locus, defined by the D11Mit286 marker on chromosome 11, comaps with the*idd4* locus [19]. The susceptibility of BC1 mice to HDS-diabetes increased when both markers were homozygous for the NOD allele (p=0.003 using χ^2 test, and p=0.00005 using the Mann-Whitney U test).

Nos2 inactivation does not influence C57BL/6 susceptibility to HDS-diabetes. The HDS-interval on chromosome 11 notably includes the *Nos2* gene that drew our attention due to: (i) NO is an effector of inflammatory pancreatic beta-cell destruction and (ii) *Nos2* inactivation has been shown to prevent development of MLDS-diabetes in (C57BL/6×129SvEv) mice. We therefore analyzed the putative protective effect of *Nos2* gene inactivation in HDS-sensitive C57BL/6 mice. STZ was administered at a dose of 180 mg/kg to *Nos2–/–* C57BL/6 and C57BL/6 control mice.*Nos2–/–* C57BL/6 mice became hyperglycaemic at day 3 following the STZ administration as did control mice (Fig. 3). Our results thus suggest that Nos2 is dispensable for C57BL/6 susceptibility to HDS-diabetes.

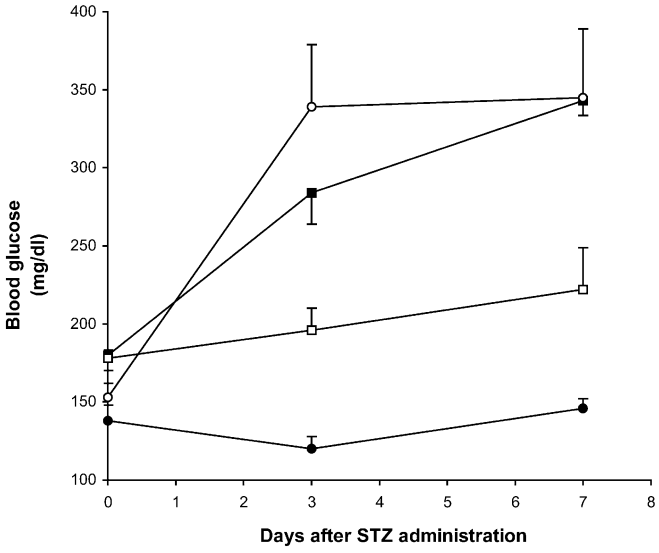

Fig. 1. Analysis of blood glucose concentrations in NOD (n=6; ○), C3H/Or (n=6; ●), (C3H/Or × NOD) F1 (n=6; □) and C57BL/6 (n=6; ■) mice. The glucose concentrations were determined 0, 3 and 7 days after a single intraperitoneal injection of STZ at 200 mg/kg body weight at 6 weeks of age. Values are means ± SEM

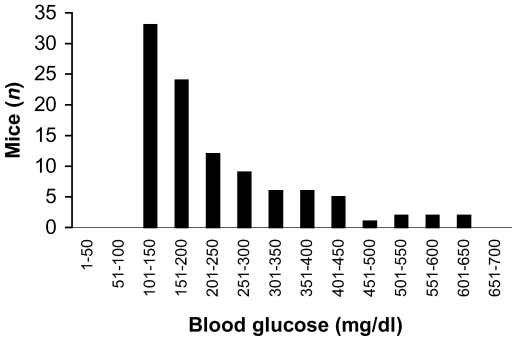

Fig. 2. Distribution of the 102 (C3H/Or × NOD) × NOD BC1 mice according to blood glucose concentrations. BC1 mice glycaemia was measured 3 days after a single intraperitoneal administration of STZ at 180 mg/kg body weight at 7 weeks of age

Table 1. Genetic segregation analysis of (C3H/Or × NOD) × NOD BC1 mice at 3 days using Mann-Whitney U test and χ^2 test

Marker	Chr	Position[a]	Blood glucose[b]		M.W.U. test	Ho	He	χ^2 test	
			Ho	He	p value	D:ND	D:ND	χ^2 value	p value
D9Mit229	9	28	250.9±15	229.8±13	0.13	41:38	37:56	2.53	0.11
D9Mit135	9	48	281.6±14	234±11	**0.0036**	62:44	52:65	**4.39**	**0.036**
D9Mit136	9	54	279±14	237.1±12	**0.012**	62:42	52:67	**5.63**	**0.018**
D9Mit17	9	62	278.5±14	236.4±11	**0.024**	61:46	53:63	2.85	0.09
D11Mit339	11	34	251.5±14	226.9±14	**0.047**	46:42	32:52	3.49	0.06
D11Mit286	11	52	253.3±14	225.7±14	**0.018**	45:41	33:53	3.38	0.07
D11Mit160	11	58	245.3±14	234.4±14	0.16	40:40	38:54	1.31	0.25
D9Mit135 and D11Mit286[c]			281.8±21	195.2±16	**0.000052**	27:19	12:34	**10**	**0.0016**

Chr: chromosome; M.W.U test: Mann Whitney U test; Ho: homozygous for the NOD allele; He: heterozygous for the NOD allele; D: diabetic; ND: non-diabetic. Boldface numbers: markers segregating with HDS-diabetes

[a] positions on chromosomes are indicated in centiMorgan (cM)
[b] blood glucose values are expressed in mg/dl ± SEM
[c] mice homozygous or heterozygous for both markers were taken into account

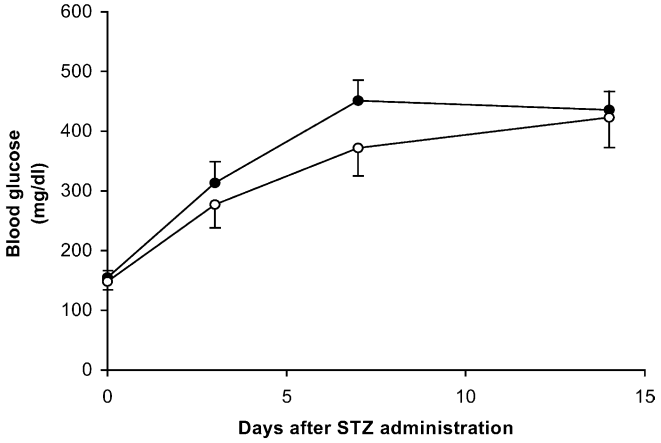

Fig. 3. Analysis of blood glucose concentrations in *Nos2–/–* C57BL/6 (n=11; ○) and C57BL/6 (n=10; ●) mice. The glucose concentrations were determined 0, 3, 7 and 14 days after a single intraperitoneal injection of STZ at 180 mg/kg body weight at 6 weeks of age. Values are means ± SEM

Discussion

Analysis of the genetic basis of the NOD susceptibility to HDS-diabetes was a strategy for the identification of genes involved in an intrinsic vulnerability of pancreatic beta cell to autoimmune destruction. Even if the increased STZ sensitivity of male mice contrasts with the higher incidence of spontaneous diabetes observed in female NOD mice, several arguments suggest that these two model diseases share some common mechanisms. Our previous work has indeed suggested that susceptibility of the NOD mouse to HDS-diabetes was related to a peculiarity of its pancreatic beta cells, which could be also involved in the pathogenesis of Type 1 diabetes [6]. Moreover, some of the known mechanisms of STZ toxicity, such as NO and free radical release [7, 9], are also implicated in autoimmune destruction of islets of Langerhans [10, 25]. In the ALR/Lt mouse, resistant to alloxan-induced diabetes, a locus in chromosome 3 has indeed been recently linked both to reduction of superoxide production by neutrophils and to spontaneous diabetes resistance [26], suggesting that a gene involved in resistance to alloxan-induced diabetes could also protect against autoimmune diabetes.

We have shown that two genetic loci, on chromosomes 9 and 11, are linked to NOD HDS-diabetes. The chromosome 11 locus corresponds to the previously described NOD diabetogenic locus *idd4*, located in a 5.2 cM interval, between the D11Nds1 (43.8 cM) and D11Mit38/D11Mit325 (49 cM) markers [19]. This observation suggests that *idd4* could be involved in a common vulnerability pathway leading to STZ or autoimmune-mediated pancreatic beta-cell destruction.

Although HDS-diabetes susceptibility or resistance are very clear-cut phenotypes in the NOD or C3H strains, our study indicates that its genetics are more complex than expected. Indeed, in contrast to a previous study which claimed that a single major gene controlled the difference in susceptibility to HDS-diabetes between C57BL/6 J and C3H/Hej [18], our work indicates that NOD susceptibility to HDS-diabetes is controlled by many genes, only two of which were detected by our genome scan.

Whereas PARP-1 activation and subsequent NAD^+ depletion have been implicated as a major mechanism of HDS-induced pancreatic beta-cell destruction [27, 28, 29], we did not detect any linkage of NOD HDS-susceptiblity to the D1Mit36 (92.3 cM) and D1Mit407 (101.5 cM) markers surrounding the corresponding gene at 98.6 cM in chromosome 1. This result is not really surprising since our previous studies showed that, in the NOD mouse, PARP-1 activation was not required for HDS-induced diabetes [6]. We did not detect linkage with genes encoding the heat-inducible proteins (Mr=70000) that have been involved in the heat-induced resistance of rat beta cells to STZ [30].

The genomic region, linked to NOD HDS-diabetes in chromosome 11, contains a very plausible candidate gene, *Nos2*. NO seems to be a major effector of both autoimmune and STZ-mediated pancreatic beta-cell destruction [9, 10, 21, 22, 23]. However, we reported here that *Nos2–/–* mice, which were previously shown to be resistant to MLDS-diabetes [24], were not protected from HDS-diabetes. This result, which is in agreement with the observed STZ sensitivity of *Nos2–/–* isolated islets [24], suggests that *Nos2* inactivation does not modify pancreatic beta cell intrinsic vulnerability to STZ but is rather involved in the immune component of MLDS-diabetes.

In conclusion, this work was undertaken as an attempt to reduce the genetic complexity of Type 1 diabetes of NOD mouse by analysing a subphenotype related to this disease. However, our genome scan detected two genetic loci which do not allow the prediction of the sensitivity or resistance to HDS-diabetes of all the BC1 mice. Genetics of NOD HDS-diabetes seems to be therefore as complex as that of the spontaneous autoimmune disease of this mouse strain. The generation of congenic strains will allow: (i) to confirm the implication of these two genetic regions in NOD susceptibility to HDS-diabetes and (ii) to determine if the chromosome 9 locus is also effectively involved in the autoimmune spontaneous diabetes of the NOD mouse, as is the case for *idd4*.

Acknowledgements. C.G. was consecutively supported by fellowships from the Ministère de la Recherche and the Fondation pour la Recherche Médicale. We thank Dr. H.-J. Garchon for critical reading of the manuscript and for the gift of the C57BL/6 *Nos2–/–* mice. We acknowledge Dr. O. Boulard for his kind help in the statistical analysis of our data. We are grateful to I. Cisse and to the animal facility staff for excellent assistance with animal care.

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