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Original Contribution

Commonalities of genetic resistance to spontaneous autoimmune and free radical-mediated diabetes

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ABSTRACT

ALR/Lt, a NOD-related mouse strain, was selected for resistance to alloxan free radical-mediated diabetes (ALD). Despite extensive genomic identity with NOD (>70%), ALR mice display strong resistance to autoimmune type 1 diabetes (T1D) due to both an unusual elevation in systemic antioxidant defenses and a reduction in cellular ROS production that extends to the beta cell level. Reciprocal backcross to NOD previously linked the ALR-derived T1D resistance to Chr. 3, 8, and 17 as well as to the ALR *mt-Nd2*^{a} allele encoded by the mitochondrial genome (mtDNA). To determine whether any of the ALR-derived loci protecting against T1D also protected against ALD, 296 six-week-old F2 mice from reciprocal outcrosses were alloxan-treated and assessed for diabetes onset, and a genome-wide scan (GWS) was conducted. GWS linked *mt-Nd2* as well as three nuclear loci with alloxan-induced diabetes. A dominant ALR-derived ALD resistance locus on Chr. 8 colocalized with the ALR-derived T1D resistance locus identified in the previous backcross analysis. In contrast, whereas ALR contributed a novel T1D resistance locus on Chr. 3 marked by *Susp*, a more proximal ALR-derived region marked by *Il-2* contributed ALD susceptibility, not resistance. In addition, a locus was mapped on Chr. 2, where heterozygosity provided heightened susceptibility. Tests for alloxan sensitivity in ALR conplastic mice encoding the NOD *mt-Nd2*^{c} allele and NOD mice congenic for the protective Chr. 8 locus supported our mapping results. Alloxan sensitivity was increased in ALR.mt^{NOD} mice, whereas it was decreased by congenic introduction of ALR genome on Chr. 8 into NOD. These data demonstrate both similarities and differences in the genetic control of T1D versus ROS-induced diabetes.

Introduction

Pancreatic beta cells are particularly sensitive to killing via reactive oxygen species (ROS), whether generated by the complex proinflammatory environment of an autoimmune cellular infiltrate (insulitis), or by the free radical generating toxin alloxan [1,2]. This exquisite sensitivity has been proposed to play a role in the pathogenesis of T1D [3–12]. Production of ROS and down-regulation of antioxidant defenses characterized by a reduced glutathione [GSH] level and a progressive decline in the transcripts for catalase [CAS], superoxide dismutase [SOD], and thioredoxin [TRX] have been observed in apoptotic processes [13–15]. Hence, the ability to maintain redox potential in beta cells may counteract apoptotic signaling, protecting beta cells and inhibiting T1D [4,5,16,17]. Indeed, GSH, SOD, and TRX have all been shown to inhibit apoptotic signaling by blocking the actions of apoptosis signal-regulating kinase [ASK1], AP-1, and NF-kB in islets [18–20]. Recombinant TRX has been shown to protect cells against apoptosis mediated through TNF and Fas pathways [21], and when overexpressed in beta cells, TRX protects against T1D in transgenic NOD mice [22]. The rapid rejection of islet grafts in diabetic NOD recipients has also been attributed in part to damage mediated by ROS, and treatment with ROS scavengers slowed allograft rejection [23,24]. Likewise, increasing the stress response in NOD islets can prevent or reduce immune-mediated damage including rejection of syngeneic islet grafts in recurrent autoimmune disease [22,25,26]. Therefore, identification of genetic elements that reduce cellular ROS production or increase anti-radical defenses could allow for better protection of islets both before and after diabetes onset. Studies with the ALR mouse have strongly supported this concept [3–6,16,17,27,28].

ALR mice were selected for resistance to alloxan-mediated diabetes [29]. Alloxan is a diabetogenic agent that destroys pancreatic beta cells by free radical damage [30,31]. Alloxan, a glucose mimetic, is selectively transferred into beta cells where it decomposes to produce

Abbreviations: ALD, alloxan free radical-mediated diabetes; Chr., chromosome; Q-RT-PCR, quantitative real-time polymerase chain reaction; ROS, reactive oxygen species; SOD, superoxide dismutase; T1D, type 1 diabetes; TRX, thioredoxin.

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hydroxyl radicals. Inbred mouse strains are not equally susceptible to alloxan-induced diabetes. ALR/Lt represents an ICR-derived strain selected for resistance to alloxan at doses that are diabetogenic to related strains [29]. One of the most closely related strains is NOD, sharing >70% genetic identity, including the diabetogenic MHC class II alleles. Previous studies between these two related strains have demonstrated an unusually strong ability of ALR islets to resist both cellular and cytokine-mediated beta cell killing that typifies T1D pathogenesis in NOD mice [5]. This resistance to autoimmune T1D development was correlated with the ALR strain's ability to dissipate ROS systemically and at the beta cell level. Genetic analysis has demonstrated that resistance was, in part, due to an interaction between nuclear and mitochondrial genes [3]. ALR-contributed loci on Chr. 3 and 8 (*Idd22*), as well as an MHC-linked locus on Chr. 17 (*Idd16*), were linked to T1D resistance in segregating populations following outcross of NOD and ALR [28]. The ALR-contributed linkage on Chr. 3 was correlated with both heightened superoxide dismutase 1 (SOD1) activity as well as suppression of superoxide generation in activated neutrophils and designated *Susp* [28]. In addition, reciprocal outcrosses between these two strains identified a single nucleotide polymorphism in *mt-Nd2* encoded by the mitochondrial genome (mtDNA) unique to ALR [3].

While this prior analysis allowed for the association of loci with autoimmune diabetes, this analysis was not able to delineate the linkages that protected at the beta cell level from linkages that controlled dysfunction at the level of the immune system. Outcrosses between T1D-susceptible NOD mice with TID-resistant strains have identified well over 30 chromosomal loci exerting control of autoimmune diabetes development. There is little information as to what chromosomal loci distinguish mouse strains susceptible or resistant to chemically induced diabetes, and whether there may be genetic overlap. Because beta cells in NOD and ALR islets are diametric opposites in terms of both the susceptibility to spontaneous T1D development in vivo and the ability to withstand stress mediated by cytokines and dissipate ROS in vitro, we initiated a genetic analysis to identify loci controlling diabetogenic responsiveness to alloxan. We were particularly interested in determining which, if any, of these overlapped with the loci identified to confer resistance to spontaneous T1D.

Methods

Mice

Breeding colonies of NOD/ShiLtJ and ALR/Lt were maintained at The Jackson Laboratory and the Children's Hospital of Pittsburgh. Reciprocal F1 crosses were initiated (NOD females crossed to ALR males, F1 denoted as DR, and ALR females crossed to NOD males, F1 denoted as RD hereafter). From these reciprocal F1 mice, the four classes of F2 genotypes shown in Table 1 were produced. Mitochondrial conplastic mice, NOD/ShiLtJ.mt$^{ALR/LtJ}$/Mx (henceforth designated NOD.mt^{ALR}) and ALR/LtJ.mt$^{NOD/ShiLtDvs}$ (ALR.mt^{NOD}), were produced and maintained as previously described [6]. The NOD.mt^{ALR} mice carried the ALR allele of *mt-Nd2* that results in a L to M amino acid substitution at residue 276 of this complex I subunit. Alloxan sensitivity of these conplastic mice was tested at N12F2. To test whether the ALR locus on Chr. 8 contributing to T1D resistance (provisionally designated *Idd22*) also controlled alloxan responsiveness, NOD.ALR-(*D8Mit205-D8Mit137*)/Mx marker-assisted congenic mice (henceforth designated as NOD.ALRc8) were created by crossing NOD/ShiLtJ to ALR/LtMx followed by 10 generations of successive backcrossing as previously described [6]. Homozygous congenic mice were then obtained by intercrossing N10 mice and testing progeny for alloxan resistance at F4.

Table 1
Incidence of alloxan-induced diabetes in F2 crosses

F1 intercross	Total incidence	Female incidence	Male incidence	Y Chr.	mtDNA
DR×DR	23.6%	19.1% (9/47)	28.6% (12/42)	R	D
RD×RD	12.3%	10.3% (3/29)	13.6% (6/44)	D	R
DR×RD	35.8%	18.2% (4/22)	44.4% (20/45)	D	D
RD×DR	11.9%	11.9% (5/42)	12.0% (3/25)	R	R

D, NOD; R, ALR. For cross directions the female breeders appear first.

All mice used were bred and maintained in the specific pathogen-free research animal facility at either The Jackson Laboratory, Bar Harbor, Maine, or the Children's Hospital of Pittsburgh, Pittsburgh, Pennsylvania. Mice were allowed free access to acidified drinking water and NIH-31 diet (Purina Mills, Richmond, IN), containing 6% fat for NOD and both F1 and F2 progeny, but only 4% fat for ALR breeders. All procedures involving in the use of animals were approved by the Animal Care and Use Committees at both institutions.

Alloxan treatment

Alloxan was prepared in PBS immediately prior to injection and kept on ice during the procedure. F2 mice at 6 week of age were treated with alloxan at 52 mg/kg body weight [3,16]. For dose-dependent sensitivity tests for conplastic and congenic male mice, alloxan was injected at different doses as indicated. Alloxan was administered via tail vein injection.

Plasma or blood glucose measurement

For determination of plasma glucose, blood was drawn, without previous fasting, immediately prior to the injection and then 1 week after alloxan treatment. Mice that did not develop hyperglycemia by 7 days were screened at 2 weeks after alloxan injection. Plasma glucose was determined using a Beckman Glucose Analyzer II (Beckman Instrument, Fullerton, CA). Alloxan-induced diabetes was diagnosed on nonfasting plasma glucose values ≥ 250 mg/dl. Blood glucose in congenic and conplastic mice was measured using a glucometer (Freestyle Flash, Alameda, CA) before as well as 7 and 14 days after treatment with different alloxan doses.

DNA extraction and genome-wide scan

All F2 mice were sacrificed after two sequential blood glucose readings greater than 250 mg/dl or if diabetes did not occur, 2 weeks after alloxan administration. At time of sacrifice, a kidney was removed from each mouse and frozen immediately in liquid nitrogen and then kept at −80°C until DNA was extracted. DNA was purified from kidney using standard methods [27,28]. Genome-wide scan was performed using a total of 152 markers (71 microsatellite and 81 SNP markers) that distinguish NOD and ALR (Supplemental Table 1). Microsatellite primer pairs were used as previously described [27,28]. Fifty-eight of the SNPs were typed by KBioscience (Hoddesdon, UK). The other 23 SNPs were typed via pyrosequencing as previously described [3,6]. The primers for pyrosequencing (Supplemental Table 2) were designed using PSQ Assay Design (Biotage AB, Uppsala, Sweden) and Integrated DNA Technology software (Coralville, IA). Marker positions are based on Ensembl (*http://www.ensembl.org/Mus_musculus/index.html*) and NCBI (http://www.ncbi.nlm.nih.gov/projects/SNP/).

Linkage analysis and statistics

The association of nuclear and mitochondrial genotypes with alloxan-induced diabetes incidence is determined by χ^2 test using the JMP software (SAS Institute, Inc.) using $P<0.01$ for suggestive and $P<0.001$ for significant linkages. QTL linkage analysis is performed with R/qtl (The Jackson Laboratory, *http://www.rqtl.org/*) [32].