Unexpected Sensitivity of Nonobese Diabetic Mice With a Disrupted Poly(ADP-Ribose) Polymerase-1 Gene to Streptozotocin-Induced and Spontaneous Diabetes

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Poly(ADP-ribose) polymerase-1 (PARP-1) is a nuclear enzyme that consumes NAD in response to DNA strand breaks. Its excessive activation seems particularly deleterious to pancreatic β-cells, as exemplified by the complete resistance of PARP-1–deficient mice to the toxic diabetes induced by streptozotocin. Because of the possible implication of this enzyme in type 1 diabetes, many human trials using nicotinamide, an inhibitor of PARP-1, have been conducted either in patients recently diagnosed or in subjects highly predisposed to this disease. To analyze the role of this enzyme in murine type 1 diabetes, we introgressed a disrupted PARP-1 allele onto the autoimmune diabetes-prone nonobese diabetic (NOD) mouse strain. We showed that these mice were protected neither from spontaneous nor from cyclophosphamide-accelerated diabetes. Surprisingly they were also highly sensitive to the diabetes induced by a single high dose of streptozotocin, standing in sharp contrast with C57BL/6 mice that bear the same inactivated PARP-1 allele. Our results suggest that NOD mice are characterized not only by their immune dysfunction but also by a peculiarity of their islets leading to a PARP-1–independent mechanism of streptozotocin-induced β-cell death. *Diabetes* 51:1470–1476, 2002

Type 1 diabetes is characterized by an inflammatory infiltration of Langerhans islets and autoimmune destruction of pancreatic β-cells. Nonobese diabetic (NOD) mice develop a spontaneous disease similar to human type 1 diabetes that is attributed to a dysregulation of its effector immune system (1–4). Less attention, however, has been paid to the role of the target β-cells and their intrinsic vulnerability to the immune-mediated destructive process (5).

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MEM, minimum essential medium; PARP, poly(ADP-ribose) polymerase; SPF, specific pathogen free.

To address this issue, we produced a poly(ADP-ribose) polymerase-1 (PARP-1)–deficient NOD mouse (NOD *Adprt1* −/−). This nuclear enzyme was chosen because its excessive activation seems particularly deleterious to pancreatic β-cells. It is normally activated by DNA strand breaks and catalyzes the transfer of poly(ADP-ribose) groups from its substrate, NAD, onto numerous nuclear proteins (6). PARP-1 has been implicated in DNA repair, control of genome integrity, apoptosis, and NF-κB regulation (7–12). However, in drastic stress conditions resulting in important DNA damage, PARP-1 activation becomes paradoxically deleterious to certain cell types, leading to depletion of the intracellular ATP pool and, eventually, to necrosis (10,13–18).

The role of PARP-1 in the death of pancreatic β-cells has been demonstrated by numerous experiments using either mutant mice or pharmacologic inhibitors. PARP-1–deficient mice are thus completely resistant to toxic diabetes elicited by a single high dose of streptozotocin, an alkylating agent that is specifically toxic to β-cells from several mouse strains (19–21). PARP-1–deficient mice are also less susceptible to the diabetes induced by multiple low doses of this same drug (22). Moreover, PARP-1–deficient islets (or islets treated with a specific PARP-1 inhibitor) are resistant in vitro to the toxic effects of nitric oxide or reactive oxygen intermediates (23–25), suggesting that PARP-1 deficiency could protect β-cells from inflammatory damage induced by activated macrophages. Consequently, nicotinamide, a weak PARP-1 inhibitor, has been reported to prevent both spontaneous and cyclophosphamide-accelerated diabetes in NOD mice (26,27). These latter studies prompted clinicians to launch nicotinamide trials in subjects either recently diagnosed or highly predisposed to diabetes (28–30). In addition, the *Adprt1* genetic locus, on the distal part of human chromosome 1 (1q42), has recently been linked to diabetes susceptibility (31).

Taken together, these observations strongly suggest that downregulation of PARP-1 should result in increased resistance of β-cells to autoimmune attack and therefore should protect against autoimmune diabetes. However, our results showed that NOD *Adprt1* +/+, +/−, and −/− mice developed spontaneous diabetes with the same high incidence, demonstrating that, at least in the NOD strain, activation of this enzyme is not mandatory for immune-mediated β-cell death. More surprising, NOD *Adprt1*−/− mice were also sensitive to toxic diabetes induced by a

single high dose of streptozotocin. This unexpected observation stands in sharp contrast with previous results showing that, in other mouse strains, *Adprt1* inactivation completely protects against streptozotocin-induced β-cell toxicity (19–21). We conclude from our results that NOD mice are characterized not only by their immune dysfunction but also by a peculiarity of their islets leading to a PARP-1–independent mechanism of streptozotocin-induced β-cell death.

RESEARCH DESIGN AND METHODS

Mice. Mice were maintained in a specific pathogen–free (SPF) facility with free access to water and food. *Adprt1* −/− mice (7), on a hybrid genetic background (129/Sv × C57BL/6), were backcrossed with NOD mice for five generations using the speed congenic strategy (32). Mice were genotyped for the *Adprt1* allele by Southern blot analysis of *Eco*RI-digested tail DNA using a probe that hybridized to a 9.6-kb wild-type fragment and a 3.3-kb mutant fragment (7). The heterozygous backcrossed littermates were genotyped for 70 microsatellite markers, polymorphic between NOD and the original *Adprt1*−/− mice (hybrid background 129/Sv × C57BL/6). *Adprt1* +/− littermates of the fifth generation were then intercrossed to produce large cohorts of NOD *Adprt1* +/+, +/−, and −/− mice. C57BL/6 *Adprt1* +/− mice, obtained from the same *Adprt1* −/− founder (7) after six backcrosses with C57BL/6 mice, were intercrossed to produce C57BL/6 *Adprt1* −/− and C57BL/6 *Adprt1* +/+ animals.

Streptozotocin administration and blood glucose measurement. Male mice received a single injection of streptozotocin (Sigma) at a dose of 180 mg/kg body wt, 175 mg/kg, or 5× 30 mg/kg at 6–8 weeks of age. Streptozotocin was dissolved in 0.1 mol/l sodium citrate buffer (pH 4.5) and was injected intraperitoneally immediately. Blood samples were taken from tail vein, and glucose levels were measured with a glucometer (Glucotrend; Roche Diagnostics).

Monitoring of spontaneous diabetes. Female mice were tested for glycosuria once a week and males were tested every other week using glukotest strips (Roche Diagnostics) from 10 to 42 weeks of age. Mice were considered diabetic and killed after two consecutive positive readings. Comparison of the frequencies of diabetes in NOD *Adprt1* +/+, +/−, and −/− cohorts was performed with the log rank test.

Cyclophosphamide administration and diabetes monitoring. Female NOD and NOD *Adprt1* −/− mice received a single intraperitoneal injection of 300 mg/kg body wt of cyclophosphamide (Endoxan-ASTA) at 9 weeks of age. Diabetes incidence was monitored by testing mice for glycosuria at day 0 and twice a week after the administration.

Adoptive transfer of diabetes. Spleens from freshly diabetic NOD mice and from prediabetic NOD and NOD *Adprt1* −/− mice at 9–11 weeks of age were dissociated in sterile 2% FCS-minimum essential medium (MEM). Erythrocytes were lysed for 5 min at 4°C in a solution containing 0.155 mol/l NH_4Cl, 0.01 mol/l $KHCO_3$, and 0.1 mmol/l EDTA. Splenocytes were washed twice and resuspended in 2% FCS-MEM, and an aliquot was stained for 30 min with a FITC-conjugated–anti-CD3 monoclonal antibody (145-2C11; provided by Prof. Lucienne Chatenoud, Hôpital Necker). Splenocytes were washed in a solution containing Hanks' balanced salt solution without calcium and magnesium, 5% FCS, and 0.02% sodium azide in PBS. Cells were fixed, and the percentage of T-cells in total splenocytes was determined using a FACScan (Becton Dickinson). Total splenocytes samples containing 2×10^6 T-cells were injected intravenously into 4-week-old NOD *Rag1* −/− and NOD *Rag1* −/− *Adprt1*−/− double mutant recipient mice that were tested weekly for glycosuria.

Isolation of pancreatic islets of Langerhans. Pancreata from C57BL/6, C3H, NOD, and NOD *Adprt1*−/− mice were perfused with 1.5 mg/ml collagenase P (Roche Diagnostics) dissolved in Dulbecco's modified Eagle's medium containing 100 units/ml penicillin G and 100 μg/ml streptomycin. They were then incubated for 10 min at 37°C, washed three times in a 5% FCS-PBS medium, and centrifuged for 17 min at 2,300 rpm on a ficoll gradient (Sigma) (40%; 23, 20, and 11% ficoll) dissolved in Eurocollins solution (Fresenius Kabi). Material sedimenting at the level of the 20–23% and 23–40% interfaces was pooled and washed in 10% FCS-MEM. Isolated islets were collected under a binocular microscope.

Western blot analysis of PARP expression. Spleen or islet samples were sonicated and boiled in a lysis solution containing 1% SDS, and the protein concentration was determined by the Bicinchoninic Acid Protein Assay (Pierce). Thirty micrograms of protein per splenocyte sample and 20 μg protein per islet sample were separated by 10% SDS-PAGE, blotted to Hybond ECL nitrocellulose membrane (Amersham Pharmacia Biotech), and incubated either with rabbit anti–human PARP-1 polyclonal antibody (1:2,500 dilution) (7) or with rabbit anti–murine PARP-2 polyclonal antibody (1:2,000 dilution) (33). After subsequent incubation with goat anti–rabbit IgG horseradish peroxidase–conjugated antibody (Pierce), PARP-1 or PARP-2 proteins were visualized using the Enhanced Chemiluminescence (ECL) Plus Western blotting detection system (Amersham Pharmacia Biotech).

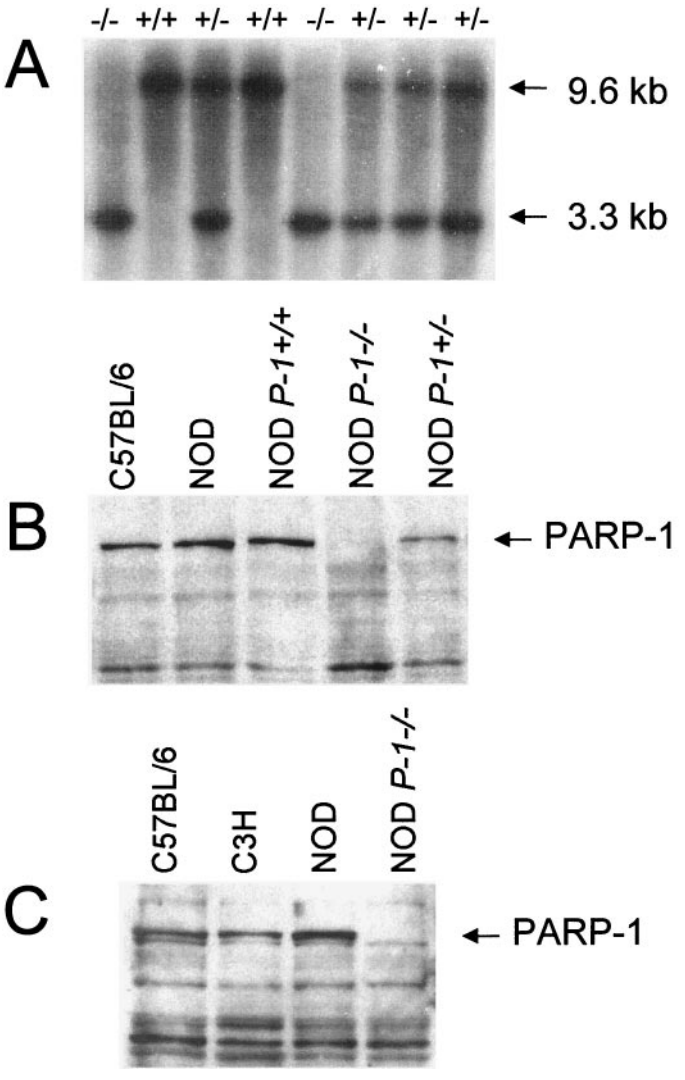

FIG. 1. Characterization of NOD *Adprt1* −/− mice. *A*: Southern blot analysis of *Eco*RI-digested DNA from offspring of NOD *Adprt1* +/− intercrosses. The probe detected a 9.6-kb wild-type fragment and a 3.3-kb mutant fragment. *B*: Western blot analysis of PARP-1 expression in splenocytes from NOD and C57BL/6 control mice and from NOD *Adprt1* +/+, +/−, and −/− littermates using a polyclonal antibody against human PARP-1 (113 kDa). *C*: Western blot analysis of PARP-1 expression in islets from C57BL/6, C3H, NOD, and NOD *Adprt1* −/− mice using a polyclonal antibody against human PARP-1 (113 kDa).

RESULTS

Generation of speed congenic NOD *Adprt1* −/− mice. We used the speed congenic strategy to accelerate the backcross transfer of the *Adprt1* mutant allele onto the NOD background (32). At each generation, offspring were selected not only for the presence of the heterozygous *Adprt1* mutant allele by Southern blot analysis (Fig. 1*A*), as usually done when generating congenic strains, but also for the lowest degree of heterozygosity throughout the rest of the genome. After five backcrosses, mice were homozygous for 70 evenly spaced background polymorphic markers. NOD *Adprt1* +/− littermates were intercrossed to

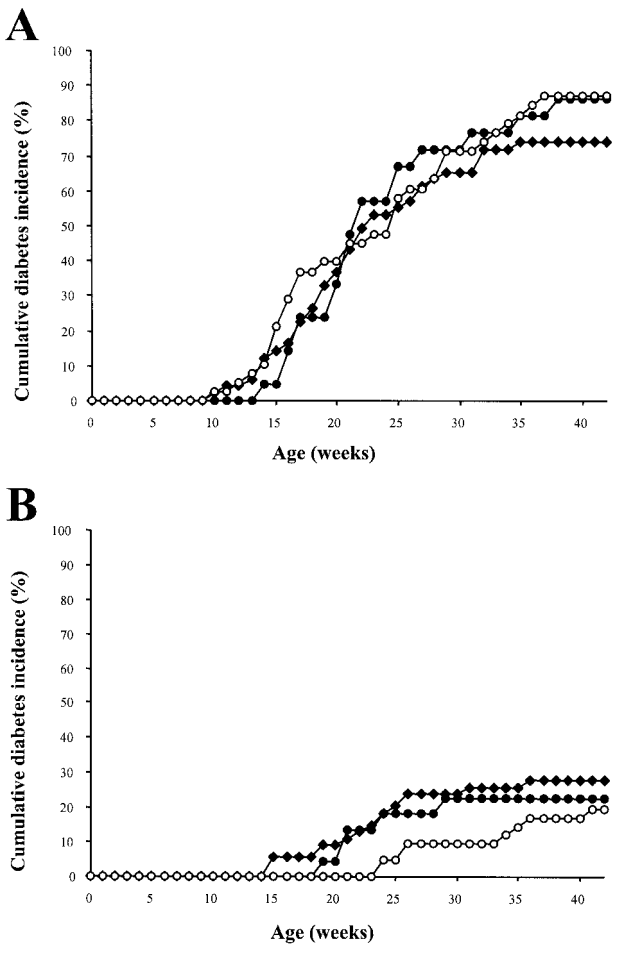

FIG. 2. Cumulative incidence of spontaneous diabetes. *A*: Female NOD *Adprt1* +/+ (n = 21, ●), NOD *Adprt1* +/− (n = 49, ♦), and NOD *Adprt1* −/− (n = 38, ○) littermates were tested weekly for glycosuria from 10 to 42 weeks of age. *B*: Male NOD *Adprt1* +/+ (n = 22, ●), NOD *Adprt1* +/− (n = 54, ♦), and NOD *Adprt1* −/− (n = 41, ○) were tested for glycosuria every other week from 10 to 42 weeks of age. The log rank test indicated that diabetes incidence was not significantly different for the three genotypes within male or female littermates.

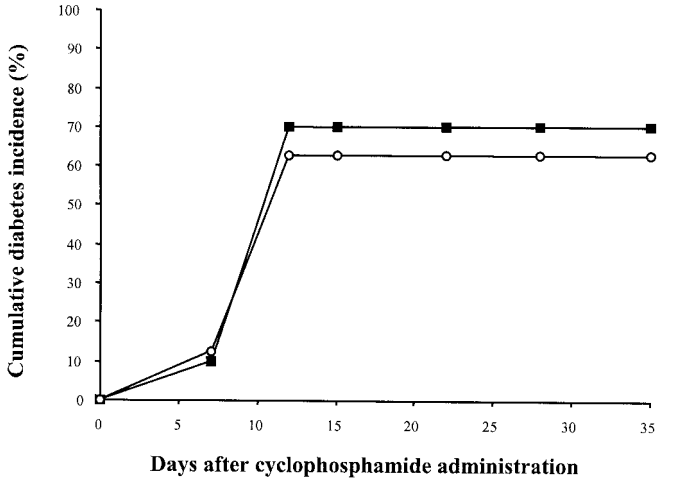

FIG. 3. Cumulative incidence of cyclophosphamide-accelerated diabetes. NOD *Adprt1* −/− (n = 10; ○) and NOD mice (n = 8; ■) received an injection of 300 mg/kg body w of cyclophosphamide at 9 weeks of age. Mice were tested weekly for glycosuria from 10 to 35 days after injection. The log rank test indicated that Kaplan-Meier curves were not significantly different.

obtain NOD *Adprt1* +/+, +/−, and −/− littermates and to establish the novel NOD *Adprt1* −/− strain. PARP-1 deficiency was then confirmed in splenocytes and pancreatic islets from NOD *Adprt1* −/− congenic mice by Western blot analysis (Fig. 1*B* and *C*). An intermediate level of PARP-1 expression was detected in splenocytes from the heterozygous littermates (Fig. 1*B*), and a slightly increased level of PARP-1 expression was detected in NOD islets compared with C57BL/6 or C3H islets (Fig. 1*C*). At variance with the results of Burkart et al. (19), intermediate levels of PARP-1 expression were observed by Western blot in the islets of NOD × C57BL/6 *Adprt1* +/− F1 mice (data not shown). NOD *Adprt1* +/+ control mice showed a normal diabetes incidence (Fig. 2) similar to that usually observed in our SPF facility, confirming that most of 129/Sv and C57BL/6 diabetes resistance genes had been eliminated from the NOD *Adprt1* −/− strain. To characterize precisely the genetic interval derived from 129/Sv and flanking the defective *Adprt1* gene (located at 98.6 cM on chromosome 1), we genotyped 26 additional microsatellite markers surrounding *Adprt1* locus. NOD alleles for the NRLi3A and D1Mit407 delimited a maximum 129-derived interval of 8.9 cM according to the MGD database (www.informatics.jax.org/).

NOD *Adprt1* −/− mice develop spontaneous and cyclophosphamide-induced diabetes. We analyzed the incidence of spontaneous diabetes in male and female NOD *Adprt1* +/+, +/−, and −/− littermates. Females of the three genotypes developed spontaneous diabetes from 10 weeks of age (Fig. 2*A*). Their incidence of diabetes was not different and was comparable to that observed in our SPF facility for female NOD mice. Similarly, NOD *Adprt1* +/+, +/−, and −/− male mice developed diabetes with the same characteristic relatively low incidence (Fig. 2*B*). In addition, because nicotinamide had been reported to protect NOD mice from cyclophosphamide-accelerated diabetes (27), we analyzed the susceptibility of NOD *Adprt1* −/− females to this accelerated disease (34). NOD *Adprt1* −/− and control NOD mice developed diabetes from 10 days after intraperitoneal administration of a single high dose of cyclophosphamide (Fig. 3). In conclusion, PARP-1 deficiency does not modify the course of spontaneous or cyclophosphamide-accelerated diabetes in NOD mice.

PARP-1 deficiency neither exacerbates the diabetogenic potential of splenocytes nor protects β-cells against autoimmune aggression. We conducted transfer experiments to evaluate in our NOD *Adprt1* −/− mice the diabetogenic potential of splenocytes and the resistance of islets of Langerhans to autoimmune attack. The normal diabetes incidence of the NOD *Adprt1* −/− mice could indeed result from a concomitant exacerbation of these two parameters. Because PARP-1 deficiency has been reported to rescue the lymphopenia in NOD scid/scid mice (35), we decided to use NOD *Rag1* −/− mice as recipients (36). Figure 4*A* shows the ability of prediabetic NOD *Adprt1* −/− splenocytes to transfer diabetes to NOD *Rag1* −/− recipients from 8 weeks of age as efficiently as NOD control splenocytes. Figure 4*B* shows that NOD *Rag1* −/− *Adprt1* −/− double mutant mice, used as recipients, were as sensitive to diabetes as control NOD *Rag1* −/− mice after transfer of diabetogenic splenocytes. Thus, PARP-1

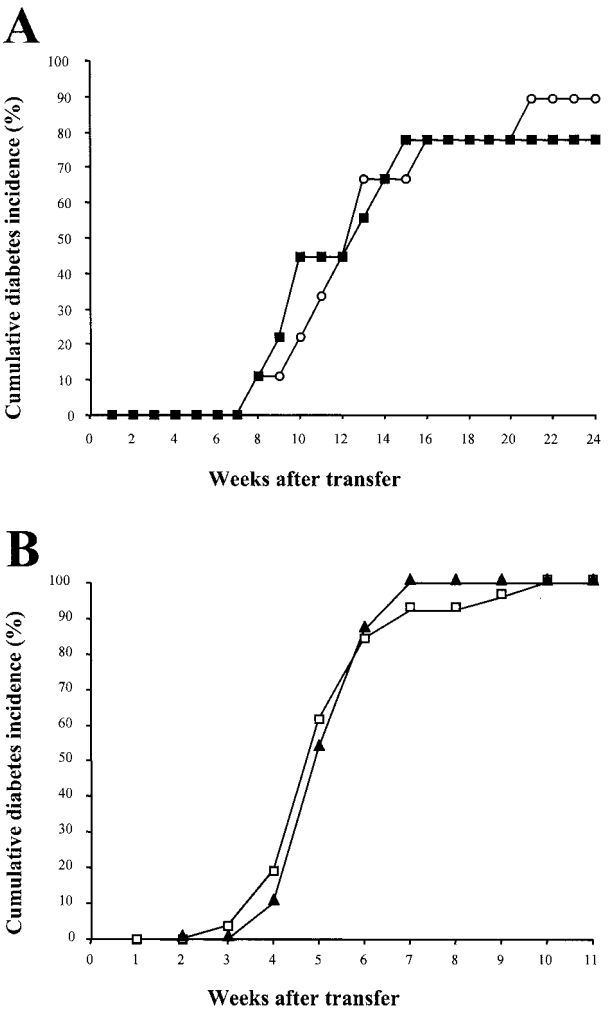

FIG. 4. Adoptive transfer of splenocytes. *A*: Splenocytes containing 2 × 10^6 T-cells from prediabetic NOD (■) and NOD *Adprt1* −/− (○) mice at 9 weeks were injected into NOD *Rag1* −/− mice (n = 9 for each type of splenocyte). *B*: NOD *Rag1* −/− (n = 30; ▲) and NOD *Rag1* −/− *Adprt1* −/− double mutant (n = 26; □) mice at 4 weeks of age received splenocytes containing 2 × 10^6 T-cells from NOD mice. Recipient mice were tested weekly for glycosuria. Kaplan-Meier curves were not significantly different as deduced from the log rank test.

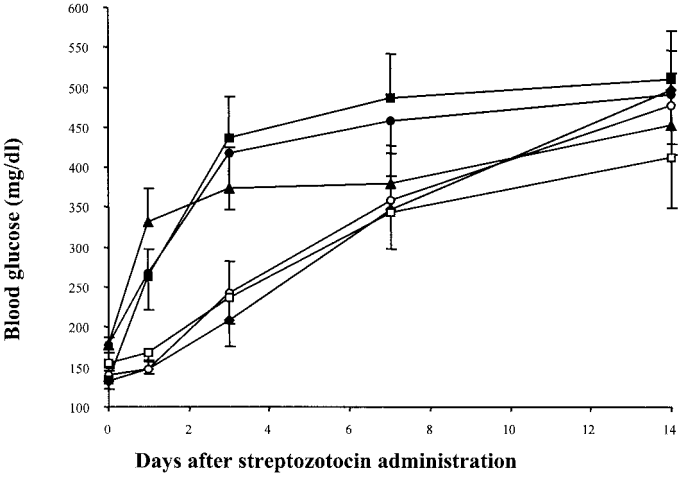

FIG. 5. Streptozotocin sensitivity of NOD *Adprt1* −/− male mice. NOD *Adprt1* +/+ (n = 8; ●), NOD *Adprt1* +/− (n = 8; ♦), and NOD *Adprt1* −/− (n = 8; ○) littermates and NOD (n = 10; ■), NOD *Rag1* −/− (n = 10; ▲), and NOD *Rag1* −/− *Adprt1* −/− double mutant (n = 10; □) male mice received an intraperitoneal injection of streptozotocin (180 mg/kg body wt) at 6 weeks of age. Values are means ± SE

deficiency affects neither the aggressivity of diabetogenic splenocytes nor the susceptibility of target islets. Moreover, pancreatic sections at 11 weeks of age showed similar insulitic lesions in NOD *Adprt1* −/− and NOD control mice (data not shown), confirming therefore that PARP-1 deficiency does not modify the migration or the activation of diabetogenic T-cells.

NOD *Adprt1* −/− mice are susceptible to streptozotocin-induced diabetes. With the expectation to confirm the previously reported resistance of *Adprt1* −/− mice to the diabetogenic action of a single high dose of streptozotocin (19–21), we monitored glycemia in male NOD *Adprt1* +/+, +/−, and −/− littermates after a single injection of streptozotocin (Fig. 5). Whereas NOD *Adprt1* +/+ control littermates developed diabetes from the day after the injection, diabetes onset was slightly delayed in NOD *Adprt1* −/− and NOD *Adprt1* +/− mice (3 days). Surprisingly, after 1 week, glycemia was similar in the three groups. Our NOD *Adprt1* −/− strain was therefore sensitive to high-dose streptozotocin-induced diabetes, standing in sharp contrast to the two previously described streptozotocin-resistant *Adprt1* −/− mouse strains (19–21). To determine whether the cause of this difference was the nature of the *Adprt1* null allele or the NOD background itself, we injected a single high dose of streptozotocin into C57BL/6 *Adprt1* −/− mice derived after six backcrosses from the same *Adprt1* −/− founder strain (7). Figure 6 shows that these mice were protected against the streptozotocin-induced hyperglycemia during at least 1 month. PARP-1 activation is therefore required for high-dose streptozotocin-induced destruction of pancreatic β-cells in C57BL/6 but not in NOD mice. Because young NOD male mice are reportedly very sensitive to the diabetes induced by multiple low doses of streptozotocin (37), we also monitored glycemia in 6-week-old NOD *Adprt1* −/− and NOD male mice 16 days after the injection of five low doses of streptozotocin (5 × 30 mg/kg). As for the diabetes induced by a single high dose of streptozotocin, we found similar glycemia values (means ± SE) in the two groups of mice: 280 ± 41 mg/dl for the NOD mice (n = 10) and 318 ± 48 mg/dl for the NOD *Adprt1* −/− mice (n = 10).

Lymphocytes do not contribute to the susceptibility of NOD and NOD *Adprt1* −/− mice to streptozotocin. Because NOD islets are infiltrated by immune cells at the time of streptozotocin injection (6 weeks), the existence of an immune component in the high-dose streptozotocin-induced diabetes of NOD mice could not be excluded. To determine the contribution of lymphocytes in the high-dose streptozotocin-induced diabetes, we analyzed the susceptibility of immunodeficient NOD *Rag1* −/− and NOD *Rag1* −/− *Adprt 1* −/− double mutant mice, which are defective in B- and T-cells (Fig. 5). The observed toxic diabetes incidence was not reduced in these mice, demonstrating that the immune system is not required for high-dose streptozotocin-induced β-cell destruction in NOD and NOD *Adprt1* −/− mice.

PARP-2 does not compensate for the absence of PARP-1 activity in NOD *Adprt1* −/− mice. In PARP-1–deficient cells, a residual activity of poly(ADP-ribosyl)a-

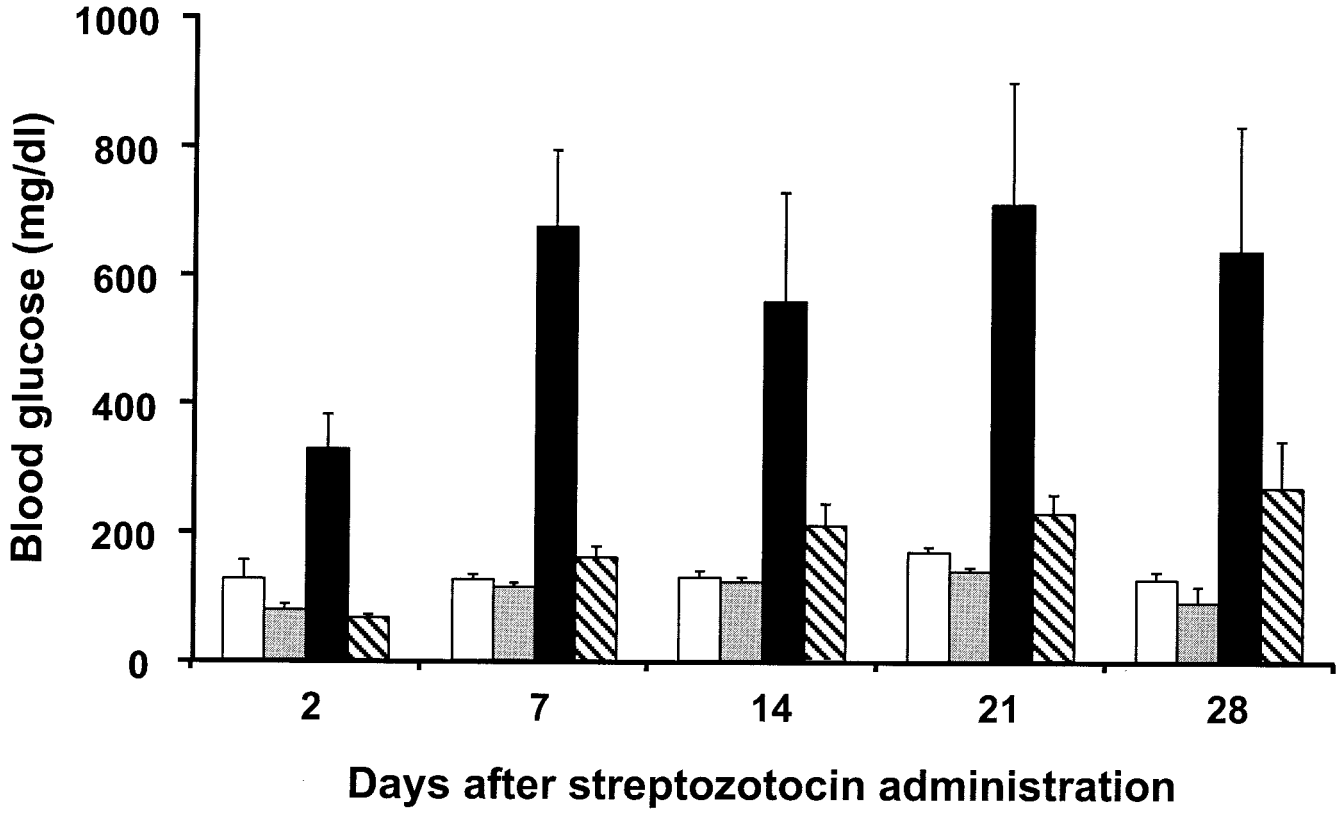

FIG. 6. Streptozotocin sensitivity of C57BL/6 *Adprt1* −/− mice. Blood glucose levels were measured in untreated C57BL/6 *Adprt1* +/+ (n = 4; white bars) and C57BL/6 *Adprt1* −/− (n = 4; gray bars) mice and in C57BL/6 *Adprt1* +/+ mice (n = 10; black bars) and C57BL/6 *Adprt1* −/− (n = 10; hatched bars) that received an injection of streptozotocin (175 mg/kg body wt). Mice were tested at days 2, 7, 14, 21, and 28. Values are means ± SE. Treated C57BL/6 *Adprt1* −/− mice were significantly protected against streptozotocin toxicity ($P < 0.05$ at days 2, 7, 14, 21, and 28).

tion was attributed to PARP-1 homologs such as PARP-2 (33,38). Detection, by Western blot analysis, of identical levels of PARP-2 in islets from C57BL/6, C3H, NOD, and NOD *Adprt1* −/− strains shows that a PARP-2 overexpression does not explain the streptozotocin susceptibility of NOD *Adprt1* −/− mice (Fig. 7).

DISCUSSION

We showed here that *Adprt1* gene disruption does not modify the incidence of spontaneous or cyclophosphamide-accelerated diabetes of NOD mice. This observation was unexpected because previous results were strongly suggestive of a wide islet protective effect of PARP inhibition or of *Adprt1* gene invalidation. However, the lack of diabetes protection that we observed in our NOD *Adprt1* −/− strain could alternatively be due to the neutralization of such expected islet-protective effect by a putative exacerbation of the lymphocyte diabetogenicity. The results of our transfer experiments clearly rule out such a hypothesis because diabetogenicity of NOD and NOD *Adprt1* −/− splenocytes were equivalent. Moreover, reciprocal transfer experiments indicated that immunodeficient NOD and NOD *Adprt1* −/− recipients were equally sensitive to the transfer of diabetogenic splenocytes.

These results suggest that the protective effect of nicotinamide, a known PARP-1 inhibitor, which was reported for the spontaneous and cyclophosphamide-accelerated diabetes of the NOD mouse (26,27), could also result from other pharmacologic properties, such as scavenging of free oxygen radicals (30). However, the observed protective effects in BALB/c mice of a benzopyrone PARP inhibitor in the multiple low doses of streptozotocin diabetes model (22) suggest that islet immune destruction could be PARP-mediated in other mouse strains. Our most surprising observation, however, is a new characteristic of the NOD mouse, which, unlike other strains, is not pro-

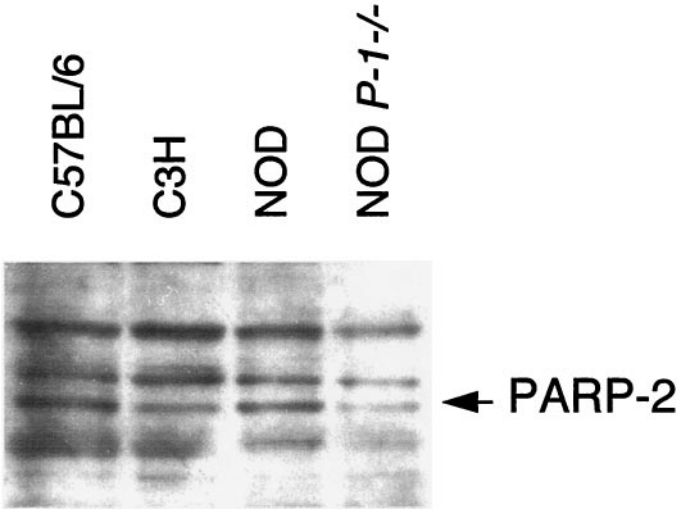

FIG. 7. Western blot analysis of PARP-2 islet expression. PARP-2 expression was detected using a polyclonal antibody against murine PARP-2 (62 kDa). The position of the PARP-2 band was determined on another Western blot by comparison of spleen extracts from PARP-2 −/− and wild-type mice.

tected against streptozotocin toxicity by an inactivation of its *Adprt1* gene (19–21).

We have indeed shown that 1 week after a single high-dose injection of streptozotocin or 16 days after multiple low doses of this drug, the NOD and NOD *Adprt1* −/− strains shared the same high sensitivity to its diabetogenic effect. This unexpected observation could be due to the presence of an inflammatory infiltration of NOD islets at the time of the streptozotocin injection, which could considerably amplify the initial destructive effect of the drug. To test this hypothesis, we compared the sensitivity of NOD, NOD *Rag1* −/−, and NOD *Rag1* −/− *Adprt1* −/− double mutant mice with a single high dose of streptozotocin. The results shown in Fig. 5 demonstrate that the lymphocytic infiltration of NOD pancreas does not contribute to the high streptozotocin sensitivity of this strain that is therefore due to an intrinsic characteristic of its islets.

Our study thus unexpectedly revealed that in the NOD strain, PARP-1 activation is not mandatory for the induction of diabetes by streptozotocin. The NOD genome must encode one or several genetic determinants that sensitize islets to streptozotocin, bypassing the usual requirement for PARP-1. PARP-2 is an obvious candidate because its putative overexpression in the NOD islets could replace the deficient PARP-1 activity of our NOD *Adprt1* −/− strain (33). However, we showed in Fig. 7 that NOD and NOD *Adprt1* −/− islets contain the same amount of PARP-2 as other mouse strains.

The central question raised by our study is the relevance of these PARP-1–independent, streptozotocin-sensitizing genetic determinants to the pathogenesis of spontaneous diabetes of the NOD mouse. Although such relevance seems very likely, we cannot eliminate the hypothesis of the existence of a NOD peculiar streptozotocin metabolism explaining the PARP-1–independent drug sensitivity of this mouse strain. We are actually trying to answer this question by searching for C57BL/6 genetic loci that are able to confer streptozotocin resistance to NOD *Adprt1* −/− animals. The colocalization of such loci with known diabetes protecting loci (idd) could demonstrate the functional implication of the PARP-1–independent streptozotocin sensitivity mechanism in the pathogenesis of the NOD spontaneous autoimmune diabetes.

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