SYSTEMIC EXPRESSION OF HEME OXYGENASE-1 AMELIORATES TYPE 1 DIABETES IN NOD MICE

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ABSTRACT
Heme oxygenase-1 (HO-1) is an enzyme with potent immunoregulatory capacity. To evaluate the effect of HO-1 on autoimmune diabetes, female NOD mice at 9 weeks of age received a single intravenous injection of a recombinant adeno-associated virus bearing HO-1 gene (AAV-HO-1; $0.5 \times 10^6$ - $2.5 \times 10^6$ viruses/mouse). In a dose-dependent manner, HO-1 transduction reduced destructive insulitis and the incidence of overt diabetes examined over a 15-week period. HO-1-mediated protection was associated with a lower type 1 T helper cell-mediated response. Adaptive transfer experiments in NOD.scid mice demonstrated that splenocytes isolated from AAV-HO-1-treated mice were less diabetogenic. Flow cytometry analysis revealed no significant difference in the percentages of CD4$^+$ CD25$^+$ regulatory T cells between saline and AAV-HO-1-treated groups. However, the CD11c$^+$ MHC II$^+$ dendritic cell population was much lower in the AAV-HO-1-treated group. A similar protective effect against diabetes was observed in NOD mice subjected to CO gas (250 ppm CO for 2 h, twice per week). These data suggest that HO-1 slows the progression to overt diabetes in prediabetic NOD mice by down-regulating the phenotypic maturity of dendritic cells and Th1 effector function. CO appears to mediate at least partly the beneficial effect of HO-1 in this disease setting.
INTRODUCTION

Type 1 diabetes (T1D) is a chronic autoimmune disorder characterized by the progressive destruction of pancreatic insulin-producing β-cells (1). Studies on NOD mice, which spontaneously develop T1D with features similar to the human disease, have demonstrated that the disease is initiated by infiltration of antigen-presenting cells, particularly dendritic cells (DCs), into pancreatic islets followed by recruitment of T and B lymphocytes (2,3). In these mice, insulitis appears around 3-4 weeks of age and is well established by 10 weeks without clinical symptoms. The progression to overt diabetes occurs in 80% of female mice by 30 weeks of age. Evidence suggests that β-cell destruction is mediated primarily by a skewed type 1 T helper cell (Th1)-mediated response and the production of proinflammatory cytokines (4). How the biased Th1 phenotype and the progression to destructive insulitis are regulated over the course of T1D development is not fully understood. Nevertheless, it is well documented that full activation of naive T cells requires presentation of antigen in the context of the MHC II complex and costimulatory signals from mature DCs (5-7). DCs from NOD mice have been shown to exhibit enhanced APC function and abnormally high costimulatory and Th1-inducing abilities (8-10). Furthermore, increasing evidence suggests that the autoimmune process is caused by the failure of immunosuppressive mechanisms (11-13). A naturally occurring CD4^+ CD25^+ regulatory T (Treg) cell population with suppressive function has been shown to control the progression from nondestructive insulitis to aggressive islet destruction in NOD mice (14). The CD4^+ CD25^+ Treg cell population declines during autoimmune diabetes (14,15). In view of the complexity of T1D etiology, the identification of endogenous molecules that modulate Th1 induction or immunosuppressive function may facilitate the design of effective therapies for preventing or delaying disease onset or progression.

Heme oxygenase-1 (HO-1) is a stress-response enzyme that catalyzes the degradation of heme to free iron, carbon monoxide (CO), and biliverdin in mammalian cells (16,17). Over the past few years, accumulated evidence has supported an immunoregulatory function for HO-1. It has been shown that HO-1 overexpression prevents graft rejection in organ transplantation (18). HO-1 and CO exhibit potent antiproliferative effects on T cells, and HO-1 promotes activation-induced cell death of alloreactive T cells (19-22). A study on HO-1-deficient mice revealed that the absence of HO-1 correlates with a Th1-weighted shift in cytokine responses, suggesting a pivotal role for HO-1 in regulating the immune response (23).
functional importance of HO-1 in immune regulation.

The aforementioned properties of HO-1 provoked our interest in investigating the impact of HO-1 on the development of TID in NOD mice. To determine the effect of HO-1 on the progression to overt diabetes in prediabetic mice with insulitis, we performed systemic gene transfer of HO-1 mediated by adeno-associated virus (AAV) in female NOD mice at 9 weeks of age. The effect of HO-1 gene transduction on the onset of diabetes was then assessed over a 15-week period. Our results clearly showed that systemic HO-1 expression suppressed the progression of insulitis and delayed the onset of hyperglycemia.

RESEARCH DESIGN AND METHODS

Construction of recombinant adeno-associated virus vector. We used the AAGACCAGAGTCCTCACAGATGG CG-6-carboxy-tetramethyl-rhodamine-3’.

Animals. Female NOD and NOD.scid mice were purchased from Jackson Laboratory, and kept in specific pathogen-free conditions. Female NOD mice at 9 weeks of age were subjected to retro-orbital plexus injection of saline or the indicated amounts of AAV-HO-1 in 50 µl saline. Some NOD mice at 8-9 weeks of age received two intraperitoneal injections of cyclophosphamide (200 mg/kg body weight) with one-week interval to facilitate the onset of diabetes. To test the effect of CO, animals were exposed to 250 ppm CO gas as described previously (28) for 2 h twice per week. Animals were monitored twice per week for hyperglycemia as defined by two consecutive nonfasting blood glucose levels > 240 mg/dl. Mice were sacrificed at 24 weeks of age. All animal procedures were approved by the institutional animal care and utilization committee of Academia Sinica, Taipei, Taiwan. production. For flow cytometric experiments, splenocytes (1 x10^6 cells/ml) were preincubated with purified rat anti-mouse CD16/CD32 (eBioscience) to block Fc receptor binding, followed by staining with FITC-conjugated anti-CD4 antibody (BD Biosciences), PE-conjugated anti-CD25 antibody (BD Biosciences), APC-conjugated anti-CD11c antibody (BD Biosciences), or
FITC-conjugated anti-I-A<sup>g7</sup> MHC II antibody (BD Biosciences) as indicated. To examine intracellular Foxp3 expression, splenocytes were immunostained with an APC-anti-mouse Foxp3 staining set (eBiosciences) according to the manufacturer’s instruction. Flow cytometry data were acquired and analyzed using a FACS Calibur (Becton Dickinson) and Cell Quest<sup>TM</sup> software (version 3.3, Becton Dickinson).

Adoptive transfer. Freshly isolated splenocytes from 24-week-old saline- or AAV-HO-1- (2.5 x 10<sup>10</sup> genome particles) treated NOD mice were injected at 1 x 10<sup>7</sup> cells/mouse into 5-week old female NOD.scid mice via the retro-orbital plexus. Hyperglycemia was then assessed twice per week.

Cytokine measurements. Cytokines IL-2, IL-4, IL-10, and IFN-γ were measured by ELISA kits (R&D Systems).

Histopathology. The pancreas was removed, fixed in Bouin’s solution (Sigma) for 16 h, embedded in paraffin, and sectioned at 5 µm. Sections were hematoxylin/eosin stained and evaluated on a blinded basis by two individuals using a scoring criteria described by others (29): stage 0 = normal islet, stage 1 = peri-insulitis, stage 2 = insulitis in less than 50% of the islet, stage 3 = insulitis in greater than 50% of the islet. At least 20 islets from each pancreas were examined.

Immunohistochemistry. Sections were deparaffinized, rehydrated, and pretreated with target retrieval solution (Dako) at 95 °C for 30 min. Endogenous peroxidase was blocked in a solution of 3% H<sub>2</sub>O<sub>2</sub> for 10 min at room temperature. After incubation with 5% bovine serum albumin in phosphate-buffered saline (PBS) at 37 °C for 30 min, sections were incubated with rabbit polyclonal anti-HO-1 (1:100; Stressgen) at 37 °C for 1 h, followed by three washes with PBS containing 0.1% Tween-20 (PBST). Sections were then incubated with horseradish peroxidase-conjugated secondary antibody (1:200) for another 1 h at 37 °C. After three washes, antigen was visualized after incubation with diaminobenzidine/H<sub>2</sub>O<sub>2</sub> followed by counter stain with hematoxylin reagent. Negative control was performed by using rabbit normal IgG as the primary antibody.

Western blot analysis. Tissues were homogenized in lysis buffer containing 25 mM Tris-HCl, pH 7.9, 1% Triton X-100, 10 mM EDTA, 1 mM sodium vanadate, 1 mM PMSF, and 1 µg/ml leupeptin. After centrifugation at 14,000 rpm for 30 min, the supernatant was removed, and protein concentration was determined by Bio-Rad protein assay. An equal amount of protein (50 µg) from each sample was separated by SDS-PAGE and transblotted onto nitrocellulose membranes. After blocking for 12 h with 5% nonfat milk in PBST, membranes were incubated with rabbit anti-HO-1 antibody (Stressgen), rabbit anti-HO-2 antibody (Santa Cruz), or rabbit anti-GAPDH antibody (Santa Cruz) for 1 h at 37 °C. After three washes with PBST, membranes were incubated with horseradish peroxidase-conjugated secondary antibody for 1 h. HO activity measurement. Tissues were homogenized in ice-cold 0.1M potassium phosphate buffer pH7.4 containing 1 mM EDTA and 0.5 mM PMSF, and
centrifuged at 13,000 rpm at 4 °C for 10 min. Supernatant proteins (1 mg) were then incubated in dark with 200 µl of reaction mixture containing mouse liver cytosol (1 mg protein), 50 µM hemin, 1 mM NADPH, 2 mM glucose-6-phosphate and 0.2 U glucose-6-phosphate dehydrogenase in 0.1M potassium phosphate buffer pH7.4 at 37 °C for 1 h. Bilirubin was then extracted with 1 ml chloroform and measured by the absorbance difference between 464 and 530 nm with an extinction coefficient of 40 mM⁻¹·cm⁻¹.

Statistical analysis. Data were presented as mean ± SD and analyzed using the Mann-Whitney-Wilcoxon test. Hyperglycemia frequencies and survival compared to young counterparts (Fig.1C). In order to evaluate the potential effect of HO-1 on T1D development, we constructed a recombinant AAV vector carrying mouse HO-1 gene for the in vivo gene transfer in NOD mice. AAV-mediated transgene expression was first confirmed in HT1080 cells (data not shown). To assess the effect of HO-1 on T1D, 9-week-old female NOD mice received saline or various doses of AAV-HO-1 via retro-orbital plexus injection. The incidence of hyperglycemia in each group was examined for 15 weeks. As shown in Fig. 2A, 10% of the saline-treated control mice developed hyperglycemia in the following 1-3 weeks. In contrast, the onset of diabetes was substantially delayed in mice receiving AAV-HO-1. The salutary effect of AAV-HO-1 was dose-dependent, and 85% of mice receiving the highest dose of AAV-HO-1 (2.5 x 10¹⁰ virus particles/mouse) remained normoglycemic at the time of sacrifice (P < 0.001 vs control group). Histological assessment of pancreatic sections from the animals revealed that the extent of insulitis was inversely correlated with the dose of AAV-HO-1 received (Fig. 2C). In contrast to the control group in which only about 15% of the examined islets were devoid of lymphocyte infiltration, over 50% of the examined islets in the group treated with the highest dose of AAV-HO-1 were normal (P < 0.01 vs control group). To confirm that this protective effect resulted from sustained transgene expression, Western blot analysis was performed to examine HO-1 expression in various tissues 15 weeks after gene delivery. As shown in Fig. 3A & B, the levels of HO-1 protein in liver and spleen, which express high basal levels of HO-1, were not significantly enhanced by AAV-HO-1 administration. In contrast, HO-1 expression in lung, heart, and pancreas was significantly higher in mice receiving AAV-HO-1 (2.5 x 10¹⁰ virus particles/mouse) compared to control counterparts. Likewise, HO activities determined in these tissues were also higher in AAV-HO-1-treated mice.
When immunostaining experiment was performed with 9 noted that HO-1 was primarily expressed in β–cells of islets and was much prominent in mice receiving AAV-HO-1 (Fig.3D). Together, these results support an inverse association between HO-1 expression and the onset of autoimmune diabetes in NOD mice.

Effect of HO-1 transduction on diabetogenic properties of T lymphocytes. To examine whether HO-1 expression affects the balance of Th1/Th2 responses, we measured Th1 and Th2 cytokines in serum samples and in media from Con A-activated splenocytes isolated from surviving animals 15 weeks after saline or AAV-HO-1 (2.5 x10^{10} particles) administration. As shown in Fig. 4A, significant decreases in serum levels of IL-2 and IFN-γ were observed in mice receiving AAV-HO-1. However, the levels of IL-4 and IL-10 were not significantly different between the saline- and AAV-HO-1-treated groups. Likewise, the production of IL-2 and IFN-γ, but not of IL-4 and IL-10, by activated splenocytes was substantially lower in AAV-HO-1-treated mice (Fig. 4B). To further confirm that HO-1 overexpression reduced the diabetogenicity of lymphocytes, an adoptive transfer experiment was performed in female NOD.scid mice. Splenocytes isolated from 24-week-old NOD mice with or without AAV-HO-1 treatment for 15 weeks were transferred into 5-week-old NOD.scid mice. Results showed that 9 of 10 mice receiving splenocytes from control donors became diabetic by 4 weeks after transfer, whereas the onset of diabetes was significantly delayed in recipients receiving splenocytes from AAV-HO-1-treated donors (P < 0.05) (Fig. 4C).

Effect of HO-1 transduction on immunoregulatory cells. To determine whether systemic HO-1 overexpression affects the level of CD4^{+}CD25^{+} Foxp3 cells between saline and AAV-HO-1 treated groups (3.74 ± 0.35% vs. 3.57 ± 0.11%). We next examined the population of CD11cDCs, which are crucial for the activation of autoreactive T cells. The percentage of CD11c^{+}CD4^{+}CD25^{+}Foxp3^{+} cells in total splenocytes of the AAV-HO-1-treated group (2.60 ± 0.12%, n=3) was not significantly different from that of the saline-treated group (2.73 ± 0.28%, n=5) (Fig.5A). When the frequencies of Foxp3 expression were examined, again there was no significant difference in the percentage of CD4^{+}CD25^{+}Foxp3^{+} cells between the control and AAV-HO-1-treated groups (0.70 ± 0.14% vs. 0.74 ± 0.26%). However, when the expression level of MHC II, an index of DC maturation, in this cell population was analyzed, it was found that the percentage of CD11c MHC II cells was reduced by 35% in the AAV-HO-1-treated group compared to the saline-treated control (Fig. 5B & C).

Effect of CO exposure on type 1 diabetes. To determine whether CO, a byproduct of heme degradation, has a role in HO-1-mediated T1D protection, we exposed 9-week old NOD mice to a low dose of CO gas (250 ppm for 2 h) twice
As shown in Fig. 6A, CO treatment for a 15-week period did not significantly affect the expression levels of HO-1 in tissues examined. However, the incidence of diabetes in the CO-treated group was significantly reduced compared with that of the control group (P < 0.001) (Fig. 6B). Consistently, insulitis was less severe in CO-treated animals (Fig. 6C). In contrast to control mice in which only about 12% of the examined islets were normal, about 40% of examined islets were normal in CO-treated mice (P < 0.01 vs control group). To examine whether CO treatment also affects the Th1/Th2 response, cytokine expression in ConA-activated splenocytes isolated from control and CO-treated mice was assessed. As shown in Fig. 7, the animals died at the first week after diabetes onset, all CO-treated animals were still alive at 6 weeks after the disease onset (P < 0.05 vs control group).

DISCUSSION

Decreased HO-1 expression has been shown in human patients with diabetes (30,31). Studies on experimental diabetes also revealed lower levels of HO-1 expression and HO activity in streptozotocin-induced diabetic rats compared to non-diabetic controls (32,33). In the present study, we did not observe significant changes in HO-1 and HO-2 protein expression along with aging process in NOD mice. However, a significant reduction in net HO activity was noticed in old NOD mice. This finding appears to be similar to a recent report showing that hyperglycemia did not affect HO-1 and HO-2 expression, but caused a decrease in HO-1 activity (33). It is conceivable that hyperglycemia developed in old NOD mice may have an impact on HO activity. Earlier studies by others have demonstrated that HO-1 overexpression can attenuate vascular endothelial damage and prevent cardiac ischemia/reperfusion injury in experimental diabetes (32,34-36). Furthermore, overexpression of HO-1 in islets provided protection from apoptosis and increased allograft survival (37-40).
HO-1 in prediabetic NOD mice significantly attenuated the progression of destructive insulitis and the onset of diabetes. The protection conferred by AAV-HO-1 appears to be correlated with the systemic induction of HO-1 in several tissues, including the pancreas. Immunostaining of pancreatic sections revealed that HO-1 was predominantly expressed in β-cells. Because we did not specifically investigate whether pancreatic HO-1 overexpression can prevent the autoimmune destruction of β-cells in situ, this possibility cannot be completely ruled out in the present experimental setting. Nevertheless, we provide evidence showing that systemic HO-1 gene transduction resulted in suppression of the Th1 cell response. AAV-HO-1 administration significantly reduced the levels of the Th1-type cytokines IL-2 and IFN-γ in circulation and in activated splenocytes, without affecting the levels of the Th2-type cytokines IL-4 and IL-10. An influence of HO-1 on the Th1/Th2 cytokine profile has also been reported in a study of liver allografts after HO-1 gene transfer (41). A recent study on HO-1-deficient mice revealed a profound Th1 response following T cell stimulation (23). The preferential suppression of Th1 has been implicated in the suppressive capacity of CD4 CD25+ Treg cells (44).

To examine whether HO-1-mediated T1D protection in NOD mice involves regulation of CD4 CD25+ Treg cells, we analyzed the percentage of CD4 CD25+ Treg cells in splenocytes. Our results showed that the AAV-HO-1-treated group did not show a higher proportion of CD4 CD25+ Treg cells in splenocytes, indicating that HO-1-induced suppression of diabetogenic T cells is not achieved by elevating the Treg population. As the present study did not assess the suppressive activity of CD4 CD25+ Treg cells, it is unclear whether HO-1 affected CD4 CD25+ Treg function in our experimental setting. Further study is required to clarify this issue. Along with Treg cells, DCs play an integral role in controlling autoimmunity and self tolerance. Recently, it has been demonstrated that immature DCs induce peripheral tolerance by either increasing Treg cells or inducing T cell anergy (45).
splenocytes was not altered by AAV-HO-1 administration in NOD mice. However, the percentage of CD11c+ cells expressing I-A^MHC II was reduced by 35% in AAV-HO-1-treated mice compared to the control group. This finding supports the idea that HO-1 overexpression in NOD mice suppressed the functional maturation of DCs, which in turn limited Th1 induction and disease progression. NOD mice have abnormally high immunostimulatory and Th1-inducing abilities (8-10). Down-regulating the phenotypic maturation of DCs suppresses the immune response and induces peripheral tolerance in NOD mice (46). A recent study showed that HO-1 expression inhibits DC maturation and proinflammatory and allogeneic immune responses while preserving IL-10 production (26). It is of great interest to determine whether DC maturation is affected by HO-1 in NOD mice. Flow cytometry analysis revealed that the total number of CD11c+ cells

HO-1 is an endoplasmic reticulum-anchored protein, and its protective functions are primarily mediated through its reaction byproducts (16,17). Recently, studies have shown that both CO and bilirubin can prevent endothelial cell sloughing and prolong the survival of allogeneic islets in T1D likely through different mechanisms (33,47,48). In the present study, we specifically assessed the potential role of CO in HO-1-mediated T1D protection in NOD mice. Animals were subjected to a 2-h exposure of low dose CO twice per week for a period of 15 weeks. We admit that this treatment may not exactly resemble the same situation in AAV-HO-1-treated mice in which CO was constantly produced by HO-1 in situ. Nevertheless, it was interesting to note that the prediabetic mice subjected to the periodic CO treatment had a significantly lower incidence of diabetes compared to the untreated control mice. Similar to what we observed in the AAV-HO-1-treated mice, Th1 cytokines produced by activated splenocytes were markedly reduced in the CO-treated group. Previous studies on various disease settings in animals have revealed multiple cellular mechanisms underlying the protections conferred by CO inhalation. For example, CO has been shown to activate heme-containing soluble guanylyl cyclase to increase cellular cGMP level or modulate p38 kinase-mediated signaling transduction (17). Recently, there are reports showing that CO treatment can protect animals against liver failure and chronic colitis through induction of HO-1 expression (49,50). We did not observe changes in level of HO-1 protein expression in NOD mice after CO treatment, suggesting that the beneficial effect of CO observed in present experimental setting is unlikely due to up-regulating HO-1 expression. Nevertheless, to completely rule out the implication of HO-1, additional experiments with condition of HO-1 activity inhibition or HO-1 deficiency in NOD mice will be needed. The detailed mechanism responsible for the protective effect of exogenous CO treatment on T1D remains to be determined. Further experiment demonstrated that the periodic CO treatment also prolonged the survival of NOD mice with chemical-induced overt diabetes. Together, these results suggest a possible role for CO in the protective function of HO-1 in NOD mice. In conclusion, the present study provides convincing evidence to support an immunosuppressive function of HO-1 during the course of T1D. The beneficial
effect of HO-1 is most likely through
down-regulating DC maturation, thereby
limiting T cell activation and Th1
response. These findings suggest a new
therapeutic approach for treating T1D by
manipulating the expression level of HO-1
with pharmacological agents or gene-
based therapy.

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REFERENCES
31. Bruce CR, Carey AL, Hawley JA, Febbraio MA: Intramuscular heat shock protein 72 and heme oxygenase-1 mRNA are reduced in patients with type 2 diabetes:
evidence that insulin resistance is associated with a disturbed antioxidant defense mechanism. Diabetes 52:2338-2345, 2003


40. Tu CF, Kuo CH, Juang JH: Effects of heme oxygenase-1 transgenic islets on transplantation. Transplant Proc 37:3465-3467


FIGURE LEGENDS

FIG. 1. HO expression in NOD mice. A: HO Western blots in indicated tissues of young (10 weeks) and old (24 weeks) mice. B & C: Quantitative results of Western blots and HO activities in young (closed bar) and old (open bar) mice. *P < 0.05 vs. young group.

Figure 1
FIG. 2. HO-1 overexpression delayed T1D in NOD mice. A: Animals received an administration of saline (closed circle; n=18) or AAV-HO-1 at doses of $0.5 \times 10^{10}$ (open circle; n=10), $1 \times 10^{10}$ (closed triangle; n=10), or $2.5 \times 10^{10}$ (open triangle; n=20) viruses/mouse. The percentage of normoglycemic mice in each group was determined. B: Representative H&E-stained pancreatic sections showing various stages of insulitis. C: Histogram depicting percentages of normal islets (stage 0, black) and islets with insulitis at various stages (stage 1, dark gray; stage 2, light gray; stage 3, white) in different groups.
FIG. 3. HO-1 expression in mice after receiving saline or AAV-HO-1 (2.5 x 10^10 viruses/mouse) administration. A: HO-1 Western blots in indicated tissues. B & C: Quantitative results of Western blots and HO activities in saline- (closed bar) and 23 AAV-HO-1-treated (open bar) groups. *P < 0.05 vs. saline-treated group. D: Representative images showing HO-1 immunostaining in pancreatic sections. Magnification: X 200.
FIG. 4. Effect of HO-1 on T cell response. A: Serum cytokine levels in mice receiving saline (n=5) or AAV-HO-1 (n=9) for 15 weeks. *P < 0.05 vs. saline-treated group. B: Cytokine levels in culture medium of splenocytes from saline- (n=6) and AAV-HO-1- (n=8) treated groups in the absence (closed bar) or presence (open bar) of Con A activation. *P < 0.05 vs. saline-treated group. C: NOD.scid mice received splenocytes from 24-week-old NOD mice previously treated with saline (closed circle) or AAV-HO-1 (open circle) (n=10 for each group). Recipients were monitored for diabetes every other day.
FIG. 5. Effect of HO-1 overexpression on splenocytes. Flow cytometry analysis of Treg population (A) and CD11c⁺ MHC II⁺ DC population (B) in spleens of NOD mice treated with saline or AAV-HO-1. C: The percentage of CD11c⁺ MHC II⁺ DCs in saline (n=5) or AAV-HO-1-treated (n=6) group. *P < 0.05 vs. saline-treated group.

FIG. 6. Effect of CO on T1D progression. NOD mice were untreated (closed circle; n=18) or treated with CO gas twice per week (open circle; n=20) for 15 weeks. A: Representative HO-1 Western blots. B: The percentage of normoglycemic mice in each group was assessed. C: The severity of insulitis in each group was scored.
FIG. 7. Effect of CO on splenocyte function. Splenocytes from control (n=4) and CO-treated (n=6) NOD mice were incubated with (open bar) or without (closed bar) Con A for 48 h. Cytokine levels in culture medium were determined. *P < 0.05 vs. 24 control group.

**Figure 7**

- **IL-2 (pg/ml)**
  - Control: 150, CO gas: 400
  - *P < 0.05 vs. Control

- **IFN-γ (ng/ml)**
  - Control: 0, CO gas: 2

- **IL-4 (pg/ml)**
  - Control: 10, CO gas: 10

- **IL-10 (pg/ml)**
  - Control: 100, CO gas: 100

FIG. 8. Effect of CO on the survival of diabetic mice. Chemical-induced diabetic mice were untreated (closed circle; n=6) or treated with CO (open circle; n=3) starting from day 3 after disease onset. The percentage of survival in each group over a period of 6 weeks was determined.

**Figure 8**

- Survival (%)
  - Post diabetes onset (weeks)
  - 0 1 2 3 4 5 6
  - Survival: 100, 60, 40, 20, 10, 0