Immunoregulatory Role of Nitric Oxide in Kilham Rat Virus-Induced Autoimmune Diabetes in DR-BB Rats

Israel I. Mendez, Young-Hwa Chung, Hee-Sook Jun and Ji-Won Yoon

*J Immunol* 2004; 173:1327-1335; doi: 10.4049/jimmunol.173.2.1327
http://www.jimmunol.org/content/173/2/1327

References This article cites 33 articles, 19 of which you can access for free at:
http://www.jimmunol.org/content/173/2/1327.full#ref-list-1

Subscription Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

Permissions Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Immunoregulatory Role of Nitric Oxide in Kilham Rat Virus-Induced Autoimmune Diabetes in DR-BB Rats

Israel I. Mendez, Young-Hwa Chung, Hee-Sook Jun and Ji-Won Yoon

Macrophages play a critical role in the pathogenesis of Kilham rat virus (KRV)-induced autoimmune diabetes in diabetes-resistant BioBreeding (DR-BB) rats. This investigation was initiated to determine the role of macrophage-derived soluble mediators, particularly NO, in the pathogenesis of KRV-induced diabetes in DR-BB rats. We found that the expression of inducible NO synthase (iNOS), an enzyme responsible for NO production, was significantly increased during the early phase of KRV infection. Inhibition of iNOS by aminoguanidine (AG) treatment resulted in the prevention of diabetes in KRV-infected animals. The expression of IL-1β, TNF-α, and IL-12 was significantly decreased in the spleen of AG-treated, KRV-infected DR-BB rats compared with PBS-treated, KRV-infected control rats. Subsequent experiments revealed that AG treatment exerted its preventive effect in KRV-infected rats by maintaining the finely tuned immune balance normally disrupted by KRV, evidenced by a significant decrease in the expression of IFN-γ, but not IL-4, and a decrease in Th1-type chemokine receptors CCR5, CXCR3, and CXCR4. We also found that iNOS inhibition by AG decreased the KRV-induced expression of MHC class II molecules and IL-2Rα-chain, resulting in the suppression of T cell activation, evidenced by the decreased cytokolytic activity of CD8+ T cells. We conclude that NO plays a critical immunoregulatory role by up-regulating macrophage-derived proinflammatory cytokines, up-regulating the Th1 immune response, and activating T cells, leading to type 1 diabetes after KRV infection, whereas suppression of NO production by AG treatment prevents KRV-induced autoimmune diabetes in DR-BB rats. The Journal of Immunology, 2004, 173: 1327–1335.

Copyright © 2004 by The American Association of Immunologists, Inc. 0022-1767/04/$02.00
diabetes. These results suggest that NO produced by KRV-activated macrophages plays a critical role in the regulation of immune responses such as the up-regulation of the Th1 immune response and the activation of β-cell-CTLs, resulting in the development of autoimmune diabetes in KRV-infected DR-BB rats.

**Materials and Methods**

**Animals**

DR-BB rats were produced from breeding stocks purchased from the University of Massachusetts (DR-BB/Wor; Worcester, MA). The animals were maintained under specific pathogen-free conditions at the University of Calgary (Calgary, Alberta, Canada) and used at 21–25 days of age. The use and care of the animals in this study were approved by the Animal Care Committee, Faculty of Medicine, University of Calgary.

**Cells and virus**

Normal rat kidney (NRK) cells, Chang (human liver) cells, H-1 virus, and KRV were obtained from American Type Culture Collection (Manassas, VA). NRK and Chang cells were cultured in complete medium, DMEM/RPMI 1640 supplemented with 10% heat-inactivated FBS, 5 mM sodium pyruvate, 5 mM L-glutamine, and 5 μg/ml gentamicin, at 37°C under 5% CO2. KRV and H-1 virus were cultivated in NRK and Chang cells, respectively, and harvested from the supernatant of infected cultures at 3 days after viral infection (multiplicity of infection (MOI) = 5). Anti-IFN-γ mAb (Sigma-Aldrich) was administered every 24 h for 3 days after infection. AG (100 μg/g of body weight, i.p.; Sigma-Aldrich) was administered to 21- to 25-day-old DR-BB rats, and poly(I:C) (5μg/g of body weight, i.p.; Sigma-Aldrich) or PBS (as a vehicle control) was given to DR-BB rats at 12 and 24 h before infection with KRV and every 12 h thereafter for 24 days or until sacrifice. The development of diabetes was monitored by blood glucose measurement (Lifescan, Burnaby, Canada) and used at 21–25 days of age. The use and care of the animals in this study were approved by the Animal Care Committee, Faculty of Medicine, University of Calgary.

Preparation of peritoneal macrophages

Peritoneal macrophages from DR-BB rats were harvested by lavage of the peritoneal cavity with 20 ml of RPMI 1640 at 4 days after i.p. administration of 2 ml of 3% thioglycolate. Cells were washed twice and suspended in RPMI 1640 medium (5 x 10^5 cells/ml; 2 ml/well) in six-well plates (Corning Glass Works), and nonadherent cells were removed 2 h later. Adherent cells were infected 24 h later with KRV or H-1 virus at a MOI of 5. Anti-FcγR-γ mAb (BD Pharmingen, San Diego, CA) was added at a final concentration of 2.5 μg/ml, and AG (Sigma-Aldrich, St. Louis, MO) was added at a final concentration of 1 μM.

**Nitrite assay**

The production of NO was measured as described previously (12). At various time points after in vitro infection of macrophages with KRV or H-1 virus, 50 μl of culture supernatant from KRV- or H-1 virus-infected macrophages was mixed with 100 μl of Griess reagent and incubated for 10 min at 37°C. The absorbance of the medium alone was subtracted from the value obtained for each sample.

**Infection of DR-BB rats with KRV or H-1 virus and AG treatment**

KRV or H-1 virus (3 x 10^5 TCID₅₀/rat, i.p.) was administered to 21- to 25-day-old DR-BB rats, and poly(I:C) (5 μg/g of body weight, i.p.; Sigma-Aldrich) was administered every 24 h for 3 days after infection. AG (100 mg/kg of body weight, i.p.; Sigma-Aldrich) or PBS was administered to DR-BB rats at 12 and 24 h before infection with KRV and every 12 h thereafter for 24 days or until sacrifice. Above normal levels of glucosuria (>2 g/L) and hyperglycemia (>167 mg/dL) were observed during these studies (6).

**RT-PCR analysis of cytokines, chemokines, and chemokine receptors**

Total RNA was extracted from either KRV- or H-1 virus-infected peritoneal macrophages from DR-BB rats or from isolated splenocytes of AG- or PBS-treated, KRV/poly(I:C)-injected DR-BB rats using the acid guanidine thiocyanate phenol-chloroform method (13). Three micrograms of total RNA was converted to cDNA using SuperScript RT II (Life Technologies, Burlington, ON, Canada), and conventional or quantitative real-time PCR was performed using specific primers described previously for several cytokines (5), chemokines (14), chemokine receptors (15), and iNOS (16). Hypoxanthine phosphoribosyltransferase (HPRT) mRNA was measured as an internal standard (5). After amplification, the products were subjected to electrophoresis on 1% agarose containing ethidium bromide and analyzed by UV light against DNA molecular markers to confirm correct amplicon size and purity. To determine that the efficiency of each reaction was greater than or equal to E = 1.85, standard curves were generated by serial dilution of the control templates and assigning relative concentrations. To generate standard curves, the crossing cycle numbers of the logarithmic-linear PCR phase (crossing points) of the standards were plotted against the logarithm of their concentrations (fit points method). The correlation coefficient of the standard curves generated in each experiment was always >0.95 or better, and the coefficient of variation of triplicate samples was <10%. Each gene was analyzed at least three times, and cDNA levels during the linear phase of amplification were normalized against constitutively expressed housekeeping gene HPRT controls. Each real-time PCR was performed with an initial denaturation step of 10 min at 95°C, followed by amplification for 45 cycles using previously described conditions and primers (5, 14–16).

**Flow cytometric analysis**

Flow cytometric analysis of splenocytes isolated from DR-BB rats at 3 and 7 days after KRV infection was performed as previously described (17). Briefly, cells were incubated for 30 min at 4°C with FITC-conjugated anti-CD3 mAb (RT1.B; Cedarlane, Hornby, ON, Canada) and PE-conjugated anti-CD4 mAb (CD11b/c; BD Pharmingen), or biotin-conjugated anti-CD3 mAb (CD5; Cedarlane) and FITC-conjugated anti-CD8 mAb (CD25; Cedarlane). The biotin was labeled with PE-streptavidin. The cells were then washed two times with FACS buffer (1% heat-inactivated FBS and 0.1% sodium azide in PBS (pH 7.2)) before fixation with 1% formalin, and 1% crystal violet solution to determine TCID₅₀. Four wells were used for each dilution.

**Measurement of viral titer**

NRK or Chang cells (2 x 10⁵ cells in 100 μl of complete DMEM medium) were seeded in 96-well microplates. An equal volume (100 μl) of a serially diluted KRV or H-1 virus sample in complete medium was added to each well and incubated for 5 days at 37°C under 5% CO₂. Cells were then fixed with 1% formalin and stained with 1% crystal violet solution to determine TCID₅₀. Four wells were used for each dilution.

**In vitro CD8⁺ CTL assay**

Splenocytes were isolated from AG-treated, KRV/poly(I:C)-injected, or PBS-treated, KRV/poly(I:C)-injected DR-BB rats at 7 days after KRV infection, and CD8⁺ T cells were isolated using rat CD8⁺ T cell-negative selection immunomagnetic columns using the manufacturer’s protocol (Cedarlane). The purity of the recovered CD8⁺ T cells was analyzed by flow cytometry and was found to be >90%. Subconfluent NRK cells (MHC matched with DR-BB rat) were infected with KRV (MOI = 5) for 24 h and labeled with 51Cr (200 μCi/2 x 10⁶ cells/ml) for 90 min. Purified CD8⁺ T cells (2 x 10⁵ cells/well) from AG- or PBS-treated, KRV/poly(I:C)-injected DR-BB rats were incubated in complete medium containing 10% FCS, 4 mM glutamine, and 50 μM/ml penicillin/streptomycin with 51Cr-labeled KRV-infected NRK cells (1 x 10⁵ cells/well). The culture supernatant was collected, and the released radioactivity was measured using a TopCount NXT microplate scintillation and luminescence counter (Packard, Meriden, CA). Spontaneous cell lysis was determined from the target cells without addition of effector cells, and total cell lysis was determined by lysing the cells with 5% Triton X-100.
Specific $^{51}$Cr release was calculated as follows: percent lysis = $100 \times \frac{\text{test cpm} - \text{spontaneous cpm}}{\text{total cpm} - \text{spontaneous cpm}}$.

**Histologic examination**

Pancreata were fixed with formalin, paraffin-embedded, serially sectioned at 5 μm, and stained with H&E. The insulitis lesions were classified as early, intermediate, late, or end stage according to morphological criteria described previously (18).

**Statistical analysis**

Statistical analyses were performed using Fisher’s exact test or Student’s t test. The level of significance was set at $p < 0.05$.

---

**Results**

**iNOS activity is increased in response to KRV infection in DR-BB splenocytes and isolated peritoneal macrophages**

To determine whether KRV can activate iNOS expression leading to NO production, we examined the expression of iNOS mRNA in the splenocytes of KRV/poly(I:C)-injected DR-BB rats. We found that the expression of iNOS was significantly increased at 3 days postinfection. When we administered AG, a selective inhibitor of iNOS, into DR-BB rats during KRV infection, the expression of iNOS mRNA was significantly decreased in the splenocytes compared with splenocytes from PBS-treated, KRV-infected control animals (Fig. 1A). To determine whether KRV infection directly
induces NO production in macrophages of DR-BB rats, we isolated peritoneal macrophages from DR-BB rats, infected them with KRV, and examined NO production at different times after infection. We found that KRV infection clearly induced NO production within 2 h, and NO production increased linearly for 24 h before leveling off. In contrast, AG treatment resulted in the inhibition of NO production in KRV-infected macrophages (Fig. 1B).

To determine whether KRV induces iNOS expression through KRV-induced IFN-γ production, we infected macrophages in the presence of anti-IFN-γ Ab and examined NO production. We found that KRV could induce NO production as efficiently as in the absence of anti-IFN-γ Ab (Fig. 1B), indicating that KRV can directly induce the expression of iNOS independent of IFN-γ.

To determine whether a nondiabetogenic virus has a similar NO-inducing effect, we next examined the production of NO in macrophages after infection with H-1 virus, which is a rat parvovirus in the same family as KRV but does not induce diabetes. We found that H-1 virus was unable to generate NO production as high as KRV in DR-BB rat peritoneal macrophages in vitro (p < 0.05; Fig. 1C). Macrophages are highly nonspecific, and most externally delivered pathogens are likely to induce some NO production. Thus, we examined whether H-1 virus was able to induce iNOS expression in vivo. We infected DR-BB rats with the same infectious dose of either KRV or H-1 virus in the presence of poly(I:C) and sacrificed them 3 days later. We found that H-1 virus was unable to elevate iNOS levels to that seen in KRV-infected animals, even though H-1 virus was able to induce some iNOS expression (p < 0.001; Fig. 1D). These in vitro and in vivo studies indicate that a nondiabetogenic virus has a significantly lower effect on the production of NO in DR-BB rats.

**iNOS activity is required for up-regulation of proinflammatory cytokine mRNA after KRV/poly(I:C) treatment**

To determine whether iNOS has any effect on the expression of proinflammatory cytokines in DR-BB rats, we injected DR-BB rats with KRV/poly(I:C), with or without AG treatment, and examined the expression of proinflammatory cytokine mRNA in the splenocytes 3 days later. Infection of DR-BB rats with KRV induced a high expression of proinflammatory cytokines such as IL-12, IL-1β, and TNF-α. When KRV-infected DR-BB rats were treated with AG, the expression of IL-12, IL-1β, and TNF-α mRNA was significantly decreased compared with KRV/poly(I:C)-injected controls that were not treated with AG (Fig. 2A). The expression of these proinflammatory cytokine mRNAs was closely correlated with iNOS gene expression.

To determine whether iNOS inhibition specifically reduced proinflammatory cytokine gene expression, as opposed to a general reduction in gene expression, we examined the expression of several chemokines in the splenocytes of AG-treated, KRV/poly(I:C)-injected DR-BB rats. We found that the expression of MIP-1α, MIP-1β, and RANTES were increased during KRV infection, but AG treatment did not alter the expression of these chemokines by day 7 (Fig. 2B), suggesting that iNOS inhibition selectively reduces proinflammatory cytokine expression, but not chemokine expression.

To directly determine whether KRV can induce cytokine and chemokine gene expression in macrophages, we infected isolated peritoneal macrophages with KRV in vitro and examined the expression of IL-1β and RANTES, as representatives. We found that KRV was able to induce the expression of both IL-1β and RANTES, with maximum expression occurring 12 h after infection and declining thereafter, whereas PBS treatment had no effect on gene expression (Fig. 2C).

**Inhibition of iNOS activity results in a decreased Th1 immune response**

To determine whether inhibition of iNOS activity influences Th1 and Th2 immune responses, we analyzed Th1- and Th2-type cytokine gene expression in splenocytes from KRV/poly(I:C)-injected animals, with or without AG treatment, at 3 and 7 days after infection. Splenocytes from KRV/poly(I:C)-injected animals showed an elevation of IFN-γ (Th1-type) gene expression as compared with uninfected controls, whereas AG treatment resulted in the inhibition of IFN-γ gene expression to a level comparable with splenocytes from uninfected rats (Fig. 3A). In contrast, inhibition of iNOS activity did not affect expression of the IL-4 (Th2-type) gene in KRV/poly(I:C)-injected animals (Fig. 3A).

We further examined the expression of Th1-type chemokine receptor genes (CCR5, CXC3, and CXCR4) and a Th2-type chemokine receptor gene (CCR4) in KRV/poly(I:C)-injected DR-BB rats, with or without AG treatment, at 7 days after infection. We

---

**FIGURE 2. iNOS inhibition reduces KRV-induced proinflammatory cytokine but not chemokine gene expression in DR-BB rats.** Splenocytes were isolated from DR-BB rats (n = 3/group) treated with PBS, AG, PBS and KRV/poly(I:C) (KRV + PBS), or AG and KRV/poly(I:C) (KRV + AG) either 3 or 7 days postinfection. Total RNA was extracted from splenocytes, and either proinflammatory cytokine (A) or chemokine (B) gene expression was analyzed by real-time RT-PCR. Lane M, 100-bp DNA ladder. C, Purified peritoneal macrophages (5 × 10^6/mL) were infected with KRV (MOI = 5) or treated with PBS and incubated at 37°C. At various times, cells were removed and total RNA was isolated. IL-1β and RANTES gene expression were analyzed by real-time RT-PCR. Gene expression was determined as the fold difference over the internal standard control, HPRT. Error bars represent SEM (n = 4).
found that AG-treated, KRV/poly(I:C)-injected rats showed a significant decrease in the expression of Th1-type chemokine receptor genes CCR5, CXCR3, and CXCR4, compared with PBS-treated, KRV/poly(I:C)-injected DR-BB rats (Fig. 3B). In contrast, AG treatment did not change the expression of CCR4, a Th2-type chemokine receptor (Fig. 3B). Collectively, these results suggest that inhibition of iNOS activity results in a decrease primarily in the Th1 immune response in KRV/poly(I:C)-injected DR-BB rats.

To determine whether the diminished Th1 immune response was a result of inhibiting an increase in the Th1-type cell population, we examined the proportion of Th1-like CD4+CD45RC- T cells in splenocytes of the infected DR-BB rats treated with AG by

FIGURE 3. Inhibition of iNOS reduces Th1-type cytokine and chemokine receptor gene expression in KRV-infected DR-BB rats. A, Splenocytes were isolated from DR-BB rats (n = 3/group) treated with PBS, AG, PBS and KRV/poly(I:C) (KRV + PBS), or AG and KRV/poly(I:C) (KRV + AG) 3 days postinfection. Total RNA was extracted from splenocytes, and IFN-γ, IL-4, and HPRT gene expression were analyzed by RT-PCR. Lane M, 100-bp DNA ladder. B, Splenocytes were isolated from DR-BB rats (n = 4/group) treated with PBS and KRV/poly(I:C) (KRV + PBS) or AG and KRV/poly(I:C) (KRV + AG) 7 days postinfection. Total RNA was extracted from splenocytes, and cytokine and chemokine receptor gene expression was analyzed by real-time RT-PCR. Values were normalized against HPRT and expressed as the fold difference between genes. * p < 0.005; **, p < 0.0005, compared with the KRV + PBS group. Error bars represent SEM (n = 4).

FIGURE 4. iNOS activity is required for the amplification of the Th1-type CD4+CD45RC- T cell population in KRV-infected DR-BB rats. A, Splenocytes were isolated from DR-BB rats (n = 2–4/group; representative data from one animal shown) treated with PBS and KRV/poly(I:C) (KRV + PBS), AG and KRV/poly(I:C) (KRV + AG), or PBS alone at 3 days postinfection. A total of 1 × 10⁶ cells was stained with anti-CD45RC Ab and anti-CD4 Ab. A minimum of 16,500 cells per sample were analyzed by FACS. The percentage of labeled cells of the total cells gated is shown. B, The percentage of CD45RC+ T cells in the CD4+ T cell population was calculated using the following equation: total percentage of CD45RC+ T cells = percentage of CD4+CD45RC+ cells (percentage of CD4+CD45RC+ cells + percentage of CD4+CD45RC- T cells) × 100. *, p < 0.01, compared with the KRV + PBS group. Error bars represent the SEM (n = 2–4).
FACS analysis. KRV infection of DR-BB rats resulted in an increase of the Th1-type CD4^+CD45RC^+ T cell population compared with uninfected controls (Fig. 4). AG treatment of KRV/poly(I:C)-injected DR-BB rats prevented the amplification of the Th1-type CD4^+CD45RC^+ T cell population. These results indicate that inhibition of iNOS prevents the selective activation of Th1-type CD4^+ T cells that occurs as a result of KRV/poly(I:C) infection in DR-BB rats.

Inhibition of iNOS activity decreases the interaction between macrophages and T cells and attenuates T cell activity

To determine the effect of iNOS inhibition on the interaction between macrophages and T cells, we examined the expression of MHC class II molecules in macrophages, which are involved in Ag presentation to T cells, and the expression of IL-2Rα in T cells, an indicator of T cell activation (19), in KRV/poly(I:C)-injected DR-BB rats with or without AG treatment. We found that KRV/poly(I:C) injection significantly increased the expression of MHC class II molecules on OX-42^+ cells (macrophages) (Fig. 5A) and the expression of IL-2Rα on OX19^+ cells (T cells) (Fig. 5B) compared with uninfected controls. In contrast, AG treatment of KRV/poly(I:C)-injected rats significantly inhibited the expression of both MHC class II molecules and IL-2Rα compared with KRV/poly(I:C)-injected rats (Fig. 5). Taken together, these results suggest that inhibition of iNOS activity results in a decreased interaction between macrophages and T cells. This may lead to the prevention of T cell activation.

![Graph A](image1)

**Figure 5.** Inhibition of iNOS reduces the interactive capabilities of macrophages and T cells in KRV-infected DR-BB rats. Splenocytes were isolated from DR-BB rats (n = 3/group) treated with PBS, AG, KRV/poly(I:C) and PBS (KRV + PBS), or KRV/poly(I:C) and AG (KRV + AG) at 3 and 7 days postinfection. A, For macrophage MHC class II expression analysis at 3 days postinfection, the splenocytes were labeled with PE-conjugated OX-42 mAb and FITC-conjugated OX-6 mAb, and analyzed with a FACScan flow cytometer. B, For T cell IL-2Rα analysis at 7 days postinfection, the splenocytes were labeled with FITC-conjugated OX-39 mAb and PE-conjugated OX-19 mAb, and analyzed by FACScan flow cytometer. The percentage of positive MHC class II and IL-2Rα cells is shown on the gate of OX-42^+ cells and T cells, respectively. *, p < 0.05 compared with the KRV + PBS group.

To determine whether inhibition of iNOS activity affects the population of CD8^+ T cells, which are known to be cytotoxic effectors for KRV-induced diabetes, we examined the population of CD8^+ T cells in KRV/poly(I:C)-injected DR-BB rats with or without AG treatment at 7 days after KRV infection. We found that AG treatment resulted in a significant decrease in the CD8^+ T cell population compared with PBS-treated, KRV/poly(I:C)-injected control DR-BB rats (Fig. 6A). To examine whether inhibition of iNOS activity inhibits the cytolytic activity of CD8^+ T cells, we performed a ^51Cr release assay. We isolated CD8^+ T cells from KRV/poly(I:C)-injected DR-BB rats with or without AG treatment at 7 days after KRV infection, incubated them with ^51Cr-labeled, KRV-infected NRK cells as target cells, and measured the specific lysis. We found that target cells incubated with CD8^+ T cells from KRV/poly(I:C)-injected rats treated with AG showed 5% lysis, whereas those incubated with CD8^+ T cells from KRV/poly(I:C)-injected rats treated with PBS showed 42% lysis (Fig. 6B). These results indicate that iNOS inhibition decreases not only the CD8^+ T cell population, but also the activity of CD8^+ T cells.

Inhibition of iNOS activity prevents KRV-induced autoimmune diabetes in DR-BB rats

To determine whether inhibition of iNOS activity prevents KRV-induced autoimmune diabetes in DR-BB rats, we treated the rats with AG at 12 and 24 h before infection with KRV and every 12 h thereafter for 24 days. All PBS-treated, KRV/poly(I:C)-injected rats (six of six) developed diabetes by 16 days postinfection, whereas only two of nine (22%) of AG-treated, KRV/poly(I:C)-treated DR-BB rats became diabetic by 24 days postinfection (Fig. 7A).

To determine whether the inhibition of iNOS activity prevented the development of insulitis, the islets were examined. More than 40% of the examined islets remained intact, and ~30% of the islets showed early- or intermediate-stage insulitis in AG-treated, KRV/poly(I:C)-injected DR-BB rats. In contrast, ~50% of the examined islets from PBS-treated, KRV/poly(I:C)-injected DR-BB rats showed end-stage insulitis, and >30% of the islets showed late-stage insulitis (Figs. 7, B and C). This suggests that the inhibition of iNOS results in the prevention of diabetes and reduces the severity of lymphocyte-mediated insulitis in KRV-infected DR-BB rats.

Discussion

KRV can induce autoimmune type 1 diabetes in DR-BB rats without directly infecting pancreatic β cells (3, 8). Macrophages and T cells have been shown to be involved in the destruction of β cells in KRV-infected DR-BB rats. Inactivation of macrophages by liposomal dichloromethyl diphosphonate, which selectively induces apoptosis in macrophages, results in the near-complete prevention of insulitis and diabetes in KRV-infected DR-BB rats (5). Cytokines produced from activated macrophages were shown to create the immune environment for the activation of effector T cells that destroy β cells, resulting in the development of autoimmune diabetes in NOD mice (20) and DR-BB rats infected with KRV (5). In addition to cytokines such as IL-12, IL-1, TNF-α, and IFN-γ, activated macrophages also produce other soluble mediators such as NO.

NO is known to be an effector molecule released by islet-infiltrating, activated macrophages or monocytes that can induce the destruction of β cells (21). The production of NO within islet lesions was found to be correlated with an increase in the number of Fas molecules that are recognized by islet-infiltrating T cells expressing Fas ligand, which then allows the transfer of Fas signals, eventually resulting in β cell apoptosis (22, 23). In addition...
to the role of NO as an effector molecule in the destruction of β cells, NO has been shown to play another role as a modulator of immune responses (10, 11).

One enzyme responsible for the production of NO is iNOS, which is expressed in islet-infiltrating macrophages as well as by islet cells (24). The expression of iNOS mRNA in the islets was found to be closely correlated with mRNA expression of proinflammatory cytokines such as IL-1β and IFN-γ (24). Rats treated with iNOS inhibitor and iNOS-knockout mice showed alleviated liver injury during hemorrhagic shock, due to down-regulation of IL-6 and G-CSF mRNA levels in vivo, indicating that NO is essential for the up-regulation of the inflammatory response (11). In addition, iNOS is required for IL-12 signaling in innate immunity (10), and the production of IL-12 promotes the activation of Th1-type CD4⁺ T cells by the selective expression of IL-12Rs (25). Thus, iNOS activation is thought to play an important role in the generation of the Th1 immune response through IL-12 signaling in adaptive immunity.

In this study, we first asked whether KRV could induce the expression of iNOS and production of NO in macrophages from DR-BB rats. We infected macrophages isolated from DR-BB rats with KRV and examined the production of NO. Complete virions were not packaged and released in KRV-infected macrophages (data not shown), suggesting that KRV cannot replicate in macrophages of DR-BB rats. We found that KRV-infected macrophages clearly produced a significant amount of NO. It is known that iNOS expression can be induced by IFN-γ (26); therefore, it is possible that KRV indirectly induces iNOS expression via IFN-γ produced by KRV-infected macrophages, rather than by direct induction of iNOS. To examine this possibility, we measured NO production in KRV-infected macrophages in the presence of anti-IFN-γ Ab in vitro, and found that removal of IFN-γ did not significantly alter the level of NO production, indicating that KRV can directly induce NO production in macrophages from DR-BB rats. We further found that KRV can directly induce cytokine and chemokine gene expression in isolated macrophages.

We previously found that proinflammatory cytokines from activated macrophages play an important role in KRV-induced diabetes (5), and it was shown that NO is required for the expression of proinflammatory cytokines (11). Second, we asked whether NO is required for the induction of proinflammatory cytokines in KRV-infected DR-BB rats. We infected DR-BB rats with KRV/poly(I:C), treated them with AG, an iNOS inhibitor, and examined the expression of proinflammatory cytokine mRNA in the splenocytes. We found that the expression of IL-12, IL-1β, and TNF-α mRNA was significantly decreased by AG treatment, indicating that NO is required for the induction of these proinflammatory cytokines in KRV-infected DR-BB rats.

In our previous study, we found that KRV infection resulted in the up-regulation of Th1-type CD4⁺ T cells, which are involved in the destruction of pancreatic β cells (6). In addition, it was recently found that NO can selectively enhance the differentiation of Th1 cells, but not Th2 cells (27). Third, we asked whether NO plays a role in the regulation of the KRV-induced immune response. We injected DR-BB rats with KRV/poly(I:C), treated them with AG, and examined the expression of IFN-γ (a Th1-type cytokine) and IL-4 (a Th2-type cytokine) mRNA. We found that AG treatment significantly decreased Th1-type cytokine gene expression, but not Th2 cytokine gene expression. It is known that Th1- and Th2-type cells have different chemokine receptor profiles (28–31); therefore, we further analyzed the expression of CCR5, CXCR3, and CXCR4 in KRV-infected DR-BB rats treated with or without AG. We found that KRV/poly(I:C) injection resulted in the significant induction of Th1 chemokine receptor gene expression (CCR5, CXCR3, and CXCR4), but did not induce Th2 chemokine receptor gene expression (CCR4). However, inhibition of iNOS by AG
treatment resulted in the suppression of Th1-type chemokine receptor gene expression. We previously found that the CD4⁺/CD45RC⁺ CD45RC⁺/H11001 Th1-type cell population was significantly upregulated in KRV-infected DR-BB rats (6). Thus, to determine whether inhibition of iNOS results in a decrease in the Th1-like T cell population, we examined the proportion of Th1-like CD4⁺/CD45RC⁺ T cells in the spleen of KRV/poly(I:C)-injected, AG-treated DR-BB rats by FACS analysis. We found that AG treatment resulted in the selective decrease of Th1-like CD4⁺/CD45RC⁺ T cells. Taken together, these results indicate that NO plays a critical role in the regulation of the Th1 immune response.

Fourth, we asked whether NO affects the activation of T cells in KRV-infected DR-BB rats. The expression of MHC class II molecules was shown to be dependent on the expression of proinflammatory cytokines such as IFN-γ and TNF-α (32). Thus, we hypothesized that the decreased expression of proinflammatory cytokines by inhibition of iNOS expression may result in a decrease in the expression of MHC class II molecules. We examined the expression of MHC class II molecules by FACS analysis in KRV/poly(I:C)-injected DR-BB rats and found that KRV infection resulted in the increase of the MHC class II-expressing macrophage population, and treatment with AG inhibited this increase. We then examined the expression of IL-2Rα on T cells, a marker of T cell activation. We found that the population of IL-2Rα-expressing T cells in AG-treated, KRV/poly(I:C)-injected DR-BB rats was reduced to the level of the uninfected control group. These results suggest that inhibition of iNOS in KRV/poly(I:C)-infected DR-BB rats results in the prevention of MHC class II production, which subsequently prevents T cell activation. To further investigate whether iNOS inhibition in KRV-infected DR-BB rats truly prevents T cell activation, we examined the CD8⁺ T cell population and its cytolytic activity in AG-treated, KRV/poly(I:C)-injected DR-BB rats. We found that AG treatment significantly decreased the number of CD8⁺ T cells and their activity, confirming that iNOS inhibition prevents T cell activation.

Finally, we asked whether AG treatment can prevent the development of insulitis and diabetes in KRV/poly(I:C)-injected DR-BB rats. Our results revealed that inhibition of NO production by AG treatment resulted in a significant decrease in the incidence of diabetes and development of insulitis compared with PBS-treated, KRV/poly(I:C)-injected DR-BB rats. There are several

FIGURE 7. iNOS activity is required for the induction of diabetes in KRV-infected DR-BB rats. A, KRV (3 × 10⁵ TCID₅₀) was administered to 21-to 25-day-old DR-BB. Poly(I:C) (5 μg/g of body weight) was administered every 24 h for 3 days postinfection. AG or PBS, as a control, was administered 12 and 24 h before KRV infection and every 12 h thereafter. The development of diabetes was monitored for 4 wk by urine and blood glucose analysis. B, Animals were sacrificed 4 wk postinfection, and 25–30 islets per nondiabetic rat that was treated with KRV/poly(I:C) and PBS (KRV+pBS), KRV/poly(I:C) and AG (KRV + AG), or PBS (n = 4/group), were examined. The insulitis score was determined according to the following categories: 0, normal islets; 1, early insulitis; 2, intermediate insulitis; 3, late insulitis; and 4, end-stage insulitis. C, Photomicrographs of representative pancreatic islets from rats treated with KRV/poly(I:C) and PBS (KRV + PBS), KRV/poly(I:C) and AG (KRV + AG), or PBS.
possible mechanisms for the destruction of pancreatic β cells by NO. Proinflammatory cytokines, possibly involving NO, can induce Fas expression in β cells and generate apoptotic signals, leading to β cell death (22). NO can inhibit DNA synthesis and damage β cell mitochondria by inactivating specific iron-sulfur-containing enzymes (33, 34). In addition, NO may be involved in the regulation of the Th1 immune response, resulting in the amplification of β cell CTLs that contribute to the destruction of β cells.

In conclusion, KRV can induce the production of NO in macrophages of DR-BB rats, and KRV-induced NO plays a critical role in the up-regulation of the Th1 immune response and activation of effector T cells leading to autoimmune type 1 diabetes in KRV-infected DR-BB rats. In contrast, suppression of NO production by treatment with AG, an iNOS inhibitor, results in the down-regulation of the Th1 immune response and cytotoxic T cell activity, leading to the prevention of autoimmune diabetes in KRV-infected DR-BB rats.

Acknowledgments

We gratefully acknowledge the excellent technical assistance of Ms. L. Robertson for the FACs analysis and the editorial assistance of Dr. A. L. Kyle.

References