Nitric Oxide Produced by Macrophages Mediates Suppression of ConA-Induced Proliferative Responses of Splenic Leukocytes in the Diabetes-Prone BB Rat

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Splenic cells from the diabetes-prone BB rat show reduced proliferative responses to concanavalin A (ConA) and other mitogens. This study was undertaken to test whether this reduced lymphoproliferation in the BB rat is mediated by an increased production of nitric oxide (NO) by macrophages. Splenic leukocytes from diabetes-prone BB rats and five strains of control rats (BB-R, Wistar-Furth, Sprague-Dawley, Wistar, and Lewis) were cultured in RPMI-1640 media containing ConA. The leukocytes from BB rats showed reduced [^{3}H]thymidine uptake and increased release of NO compared with the control rats. Partial depletion of macrophages from the culture or incubation with N^G-monomethylarginine (NGMMA), a specific NO synthase inhibitor, markedly augmented ConA-induced proliferation of the splenic leukocytes from BB but not the control rats. Enrichment of BB rat macrophages suppressed the proliferation of BB-R rat spleen cells. Excess L-arginine added to the culture reversed the NGMMA effect. These results suggest that increased production of NO by macrophages is partly responsible for the reduced proliferative responses of splenic leukocytes in the BB rat. *Diabetes* 43:1218–1220, 1994

The BB rat is a well-established model of human insulin-dependent diabetes mellitus (IDDM). As in human IDDM, diabetes in the BB rat results from the destruction of insulin-producing pancreatic β-cells by cell-mediated and/or humoral immune responses (1). Our previous studies and others have reported that macrophages play an important role in the anti-β-cell autoimmunity (2–4). It has been suggested that macrophages act either as effector cells causing β-cell destruction or as antigen-presenting cells enabling T-cell activation (2).

Macrophages occupy a pivotal position in the immune system by virtue of their capacity for augmenting or inhibiting lymphocyte activation or proliferation (5). In addition to their roles as effector or antigen-presenting cells, macrophages in the BB rat have been suggested to play a role as a suppressor, reducing the proliferative responses of splenic lymphocytes (6,7). One possible mediator of macrophage-induced suppression of lymphoproliferation in the BB rat is nitric oxide (NO). This can be suggested based on the following observations. Alloantigen-induced proliferation of rat spleen cells was augmented by inhibiting the NO synthase pathway (8). Macrophages from Coryneparvum-injected mice as well as control Fischer rats inhibited the lymphocyte proliferation by producing excessive NO (9). Wu et al. (10) recently demonstrated that NO synthesis is increased in the peritoneal macrophages from the BB rat. This study was undertaken to see whether the excessive NO production by the macrophages is responsible for the reduced mitogen-induced lymphocyte proliferation in the BB rat.

RESEARCH DESIGN AND METHODS

BB and BB-R rats were purchased from the University of Massachussetts and Wistar-Furth rats from Harlan Sprague-Dawley. Lewis, Sprague-Dawley, and Wistar rats were purchased from Clea Japan. All rats were bred and maintained in small colonies in a specific pathogen-free environment at the Asan Institute for Life Sciences in Seoul, Korea. The overall incidence of diabetes in our colony of BB rats was ~80% at the age of 120 days.

Cell culture. Spleens were obtained aseptically from 80- to 90-day-old prediabetic and acutely diabetic (within 2 days after onset) BB rats and age-matched control rats. Splenic cells were passed through a stainless steel screen by using a syringe plunger, and erythrocytes were eliminated by centrifugation over Ficoll-Paque (Pharmacia, Uppsala, Sweden). Splenic leukocytes were resuspended in RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum, 5×10^{-5} mol/l 2-mercaptoethanol, 5 mmol/l L-glutamine, 10 mmol/l HEPES, and penicillin-streptomycin. These cells were cultured at 2×10^5 cells/well in 0.2 ml in a 96-well microtiter culture plate.

Measurement of cell proliferation. Splenic cells were stimulated with ConA with or without depletion/enrichment of macrophages, and in the presence or absence of N^G-monomethylarginine (NGMMA, Calbiochem, San Diego, CA), a specific NO synthase inhibitor. Macrophages were partially depleted by a 2-h incubation of splenic leukocytes over plastic at 37°C (11). Macrophages were enriched in microtiter wells by plating 2×10^5, 1×10^6, and 2×10^6 splenic cells, respectively, in a volume of 0.2 ml for 2 h followed by repeated and thorough washing. To measure proliferation, [^{3}H]thymidine uptake was determined after 72 h of culture, following an 18-h incubation with 1 μCi of [^{3}H]thymidine (specific activity: 5 Ci/mmol, Amersham, Buckinghamshire, U.K.).

Nitrite determination. The accumulation of nitrite in the culture supernatant of splenic cells was measured using the microplate assay system described by Ding et al. (12). The concentration of nitrite in cell-free medium alone was determined in each experiment and subtracted from the value obtained with cells.

Statistical analysis. Values are presented as means ± SE. The statistical significance of differences between means was assessed by Student's t test for paired and unpaired data or by analysis of variance followed by Tukey's test when more than two groups were compared. Differences were considered significant at $P < 0.05$.

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IDDM, insulin-dependent diabetes mellitus; NO, nitric oxide; NGMMA, N^G-monomethylarginine.

TABLE 1
Strain difference in the lymphocyte proliferation in response to ConA and the nitrite production

Strain	Lymphocyte proliferation (dpm × 10^{-4})	Nitrite in media (nmol/l)
BB		
Acutely diabetic	2.0 ± 0.3*	29.9 ± 2.5*
Prediabetic	2.0 ± 0.5*	30.3 ± 1.8*
BB-R	37.5 ± 10.3	9.9 ± 2.8
Wistar-Furth	41.1 ± 5.4	8.0 ± 2.1
Wistar	37.2 ± 4.1	8.4 ± 2.3
Lewis	43.2 ± 3.3	6.3 ± 1.3
Sprague-Dawley	37.7 ± 5.3	6.6 ± 2.4

Data are means ± SE; $n = 6$ each. *$P < 0.05$ compared with other strains of rats.

RESULTS

Responses of BB spleen cells to ConA. [^{3}H]thymidine uptake measured after 72 h of culture showed very low proliferative responses in BB rats at concentrations of ConA from 0.5 to 10 μg/ml compared with BB-R rats (data not shown). Table 1 shows the strain differences in the proliferative responses of splenic cells to 5 μg/ml of ConA and the nitrite accumulation in the media. Among the six strains tested, only the diabetes-prone BB rat showed reduced proliferative responses and increased nitrite accumulation. The presence of diabetes did not influence cell proliferation or nitrite production because they did not differ between the prediabetic and the acutely diabetic BB rats.

Effects of macrophage depletion and NGMMA on lymphocyte proliferation. Figure 1 shows that depletion of macrophages from the culture or incubation with NGMMA markedly augments the ConA-induced proliferation of splenic leukocytes from BB rats. Neither the depletion of macrophages nor the presence of NGMMA resulted in the augmentation of lymphocyte proliferation in the control rats. The augmentation of lymphoproliferation by macrophage depletion or by NGMMA was associated with the reduction in nitrite accumulation in the media.

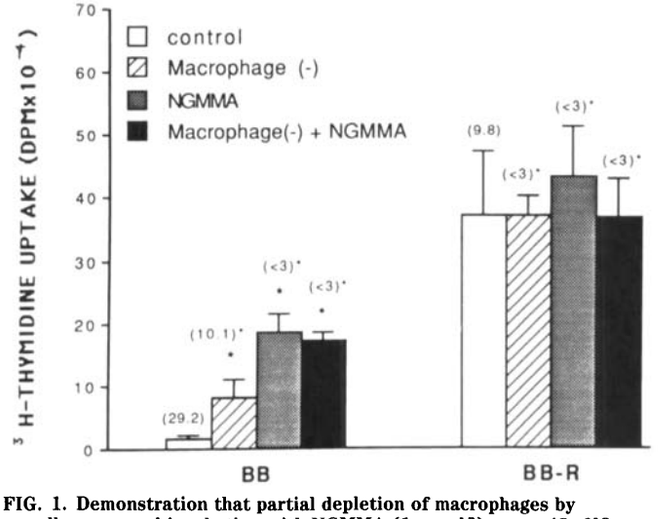

FIG. 1. Demonstration that partial depletion of macrophages by preadherence and incubation with NGMMA (1 mmol/l), a specific NO synthase inhibitor, augments ConA (5 μg/ml)-induced lymphoproliferation of BB rats ($n = 8$), but not that of BB-R rats ($n = 6$). Same pattern with BB-R rats in other strains of control rats. Numbers in parentheses are means nitrite (nmol/l) accumulated in the media (*$P < 0.05$ vs. control rats).

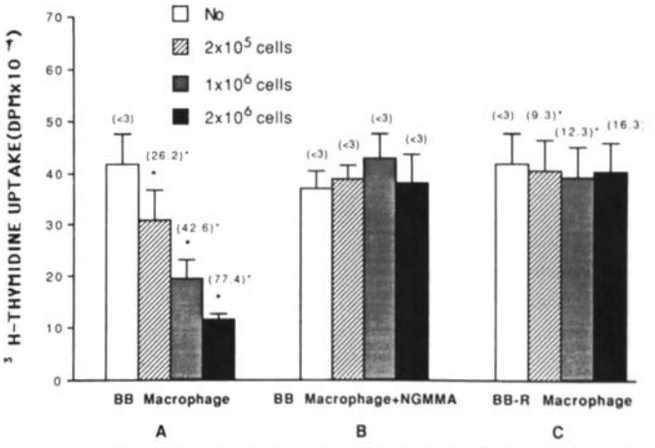

FIG. 2. Suppression of BB-R rat lymphoproliferative responses to ConA by BB rat macrophages (*A*). Macrophages were enriched by plating 2 × 10^5 (▨), 1 × 10^6, (▩) and 2 × 10^6 (■) spleen cells for 2 h followed by repeated washing. BB-R rat spleen cells (2 × 10^5) depleted of macrophages were added to wells containing macrophages. The suppression by BB rat macrophages were reversed by NGMMA (*B*). BB-R rat macrophages failed to suppress proliferation of BB-R spleen cells (*C*) (*$P < 0.05$ vs. without macrophages).

Effects of erythrocytes on lymphocyte proliferation. Erythrocytes have been shown to block suppressive activity of macrophages (13). Endogenous suppressor activity was thus examined by comparing the proliferative response of unfractionated and erythrocyte-depleted spleen cells. Unfractionated spleen cells from the BB rat showed higher proliferative response than erythrocyte-depleted spleen cells (11.0 ± 1.4 vs. 1.9 ± 0.4, × 10^{-4} dpm, $n = 6$, $P < 0.001$). In contrast, presence of erythrocytes did not stimulate proliferation in the BB-R rat (42.3 ± 3.2 vs. 38.6 ± 5.1, × 10^{-4} dpm, NS), thus suggesting a lower level of endogenous suppressor activity. No significant augmentation of ConA-induced proliferation by NGMMA was observed in unfractionated BB and BB-R rat spleen cells (data not shown).

Suppression of BB-R spleen cell proliferation by BB rat macrophages. Spleen cells from BB-R rats depleted of the macrophages by preadherence to plastic were incubated with the macrophages from the BB rat. Figure 2*A* shows that BB rat macrophages suppress proliferation of BB-R spleen cells and that the degree of suppression correlates with the number of macrophages. This suppression was completely reversed by the incubation with NGMMA (Fig. 2*B*). BB-R rat macrophages failed to suppress proliferation of BB-R spleen cells (Fig. 2*C*).

Effects of supplemental L-arginine on NGMMA-mediated augmentation of ConA-induced proliferation. If the ability of NGMMA to augment the ConA-induced proliferation results from inhibition of NO synthase, then L-arginine should be a competitive inhibitor of its action. Figure 3 shows that increasing the concentration of L-arginine partially inhibits the ability of NGMMA (0.1 mmol/l) to augment the ConA-induced proliferation. It can be seen that, as the concentration of L-arginine is increased, there is a parallel increase in the concentration of nitrite.

DISCUSSION

This study confirmed the previous findings that macrophages are responsible for the reduced lymphoproliferative responses in the BB rat in vitro (6,7). This reduced lymphoproliferation in the BB rat was associated with increased

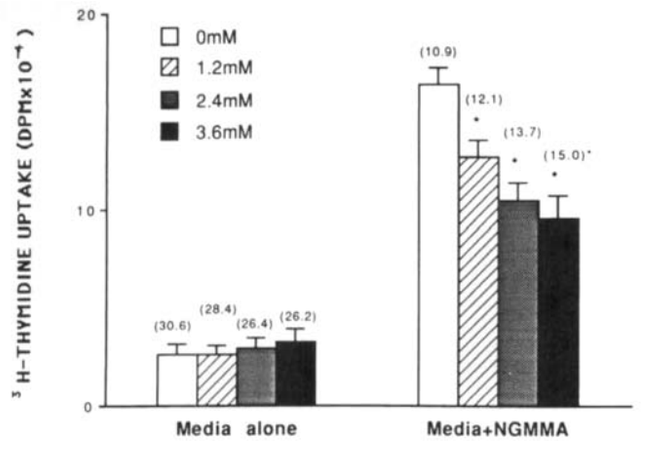

FIG. 3. Demonstration that L-arginine can partially reverse the ability of NGMMA (0.1 mmol/l) to augment ConA-induced proliferation of BB rat spleen cells. The indicated concentrations of L-arginine were added to media that contained 1.2 mmol/l L-arginine (*$P < 0.05$ vs. 0 mmol/l).

nitrite accumulation in the media. The augmentation of lymphoproliferation by macrophage depletion and by NGMMA was associated with the reduction in the nitrite accumulation. Excess L-arginine in the media resulted in the increased accumulation of nitrite and the inhibition of NGMMA-mediated stimulation. Because NO is known to be rapidly converted to nitrite and nitrate, these results provide evidence consistent with the conclusion that reduction of lymphoproliferation in the BB rat is mediated by NO or another by-product of the NO synthase pathway.

In a previous study, Mills (9) reported that macrophages from control Fischer rats, as well as Coryneparvum-injected mice, inhibit lymphoproliferation by producing excessive NO. In contrast, the present study shows that defective lymphoproliferation and increased NO production by macrophages were found only in the BB rat among the six strains of rats tested. In this study, we isolated splenic leukocytes by Ficoll-Paque separation method. This may remove some macrophages, or conversely, there may be some erythrocytes remaining that partially inhibit NO (13,14). However, this may not be the major cause of the strain difference in the lymphoproliferation, because addition of erythrocytes did not affect lymphoproliferation in the control rats. This study also showed that the macrophage-enriched culture supernatants from the BB-R rat failed to suppress lymphoproliferation, although they contained significant amounts of nitrite. These findings suggest that there is at least a quantitative, if not qualitative, difference in the capacity of NO production between the macrophages from different strains of rats.

Proliferation of the BB rat lymphocytes increased significantly by NGMMA, but was still far less than that of the BB-R rat. This suggests that chronic suppression of lymphocyte proliferation may have led to a state of unresponsiveness. However, increased NO production by macrophages may not be the sole mechanism, because Prud'homme et al. (6) reported that prostaglandin produced by macrophages acted as a suppressor of the lymphocyte proliferation. On the other hand, Weringer et al. (15) reported that there is an intrinsic defect in the BB rat lymphocytes characterized by the reduced numbers of interleukin-2 receptors.

Recently, NO has been implicated as a possible effector molecule that mediates the β-cell destruction by the macrophages (16,17). Taken together with the results of our present study, it can be suggested that increased production of NO by BB rat macrophages plays a role in the pathogenesis of IDDM. However, it remains to be determined whether the NO-mediated suppression of lymphocyte proliferation is a mechanism to limit β-cell immune destruction (18), or conversely, a mechanism to augment β-cell immune destruction by enhancing the proliferation of β-cell–specific clone in the BB rat. It is also not established whether the macrophages in the pancreas similarly produce enhanced levels of NO.

In conclusion, the increased production of NO by macrophages contributes, at least in part, to the defective proliferative responses of splenic leukocytes in the BB rat. Although the biological meaning of these phenomena remains yet to be determined, the data provide further evidence for the contention that macrophages play a central role in the development of autoimmunity in this animal model of IDDM.

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