Effect of 5-α Dihydrotestosterone on T-Cell Proliferation of the Female Nonobese Diabetic Mouse (44060)

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Abstract. **Nonobese diabetic (NOD) mice develop type I diabetes spontaneously and have been utilized as a model for human autoimmune insulin-dependent diabetes. The disease is caused by the destruction of insulin-producing β cells in the pancreatic islet of Langerhans by infiltrating inflammatory cells, which are primarily T lymphocytes. The incidence of diabetes in NOD mice is increased in females compared with males, suggesting that sex steroid hormones play an important role in the development of the disease. We therefore investigated the effect of a male steroid, 5-α-dihydrotestosterone (5DHT), on disease development, T-cell phenotype, T-cell proliferation, and cytokine profiles in this model. None of the mice that received 5DHT for 120 days ($n = 7$) developed insulitis, whereas all control mice ($n = 8$) developed the disease. The percentage of $CD4^+$ T cells in peripheral blood mononuclear cells was markedly decreased in the 5DHT-treated females compared with those in controls (37.1 ± 4.8 vs 51.3 ± 9.3, $P < 0.02$), whereas no significant differences in the percentage of $CD8^+$ T cells were observed between treated and control female mice. Results of a syngeneic mixed lymphocyte reaction (SMLR) also suggested that T cells are major target cells of 5DHT administration. An increased expression of IL-4 mRNA, representing T helper 2 (Th2) T cells, was observed in the SMLR. On the basis of these results, a systemic administration of 5DHT appears to have direct effects on the expansion of Th2 cell populations with subsequent restoration of normal immune responses.** [P.S.E.B.M. 1996, Vol 213]

Type I diabetes is an autoimmune disease caused by destruction of insulin-producing β cells in the pancreatic islet of Langerhans (1). Studies in both human and animal models demonstrate that immune-mediated tissue injury is primarily responsible for the disease development. The nonobese diabetic (NOD) mouse model spontaneously develops type I diabetes and shares several genetic and immunopathological features with human type I diabetes (2). Therefore, the NOD mouse has been known as a model system for autoimmune type of human diabetes (3). Immunohistochemical studies show that T lymphocytes, which constitute from 26% to 97% of the total infiltrating cells, are major components of the islet inflammatory cells present in both prediabetic and diabetic NOD mice (2, 4). $CD4^+$ T cells are predominantly found in the pancreatic infiltrates and recently we and others demonstrated that a subtype of $CD4^+$ T cells, T helper 1 (Th1), is primarily responsible for the autoimmune response (2, 4, 5). These studies suggest that cytokines produced by these infiltrating T cells play a critical role in initiating the autoimmune reactions observed in the pancreatic islets (2, 4–6).

In NOD mice insulitis (islet inflammation) begins at 4–5 weeks of age and is present in 100% of females

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Received December 14, 1995. [P.S.E.B.M. 1996, Vol 213]
Accepted June 25, 1996.

This work was supported in part by the Diabetes Research & Education and the Stuart Foundations.

0037-9727/96/2133-0287$10.50/0

and more than 90% of males at 30 weeks of age (2). However, there is a sexual dimorphism in the incidence of the disease: (i) females exhibit a higher percentage of islets with more destructive lesions than observed in males, and (ii) the disease onset occurs earlier starting at 8–10 weeks of age and is more frequently observed in females with an incidence reaching 65% by the age of 36 weeks compared with males, in whom the incidence stays below 5% even at 30 weeks of age (2). The sexually biased incidence of the disease onset observed in NOD mice makes this strain an excellent model for studying the effects of steroid hormones on sexually dimorphic immune mechanisms observed in various human autoimmune diseases, such as autoimmune thyroiditis (25–50:1), systemic lupus erythematosus (SLE) (9:1) and Sjögren's disease (9:1) (7, 8). Studies have suggested an increased susceptibility to type I diabetes in females (5:1), although this increased susceptibility in females is less prominent compared with other autoimmune diseases. Evidence that male steroids might play a role in preventing the onset of Type I diabetes is provided by studies that orchidectomy increases the disease development (7), and 5-α dihydrotestosterone (5DHT) is able to prevent the disease development (9, 10), but not insulitis in sexual mature NOD females (10). However, a detailed mechanism of the effect of 5DHT on immune responses in relation to disease susceptibility is still not clear. Therefore, in the present study we examined the effect of 5DHT on specific immune parameters in female NOD mice and correlated these with disease development in the NOD mouse model.

Materials and Methods

Mice. The NOD-Sansum has been established in our facility (Sansum Medical Research Foundation) and has been maintained through brother/sister inbreeding for over 40 generations. The cumulative incidence of diabetes in female mice reaches 66%, whereas less than 5% of males becomes diabetic by 36 weeks of age. Prediabetic female NOD mice (4–6 weeks old) without insulitis were chosen for this study. The C57 mice were bred in our facility from breeding pairs purchased from Jackson Laboratories (Bar Harbor, ME). All mice were fed 50% Purma mouse diet and water *ad libitum*. Before the onset of insulitis, mice (4–6 weeks old) were implanted subcutaneously in the intracapsular region with pellets containing 5DHT (15 mg), which was released over a 60-day period (Innovative Research of America, Toledo, OH) or were implanted biodegradable carrier as control. These pellets were designed to yield plasma 5DHT level of 5–10 ng/ml (17–34 n*M*) over a 60-day period. Mice were divided into two groups: one group was implanted with pellets for 60 days, and subjected to *in vitro* studies, such as FACS, SMLR, and cytokine gene expression, and the other group received a second 60-day implantation. Both treated and age-matched control mice were sacrificed at 25 weeks for histological examination.

The pancreas was fixed in Bouin's solution, and histological analyses were carried out using hematoxylin-eosin staining. Islet mononuclear cell infiltration and islet β-cell destruction were graded.

Syngeneic Mixed Lymphocyte Reactions. Syngeneic mixed lymphocyte reactions (SMLR) were carried out according to methods described previously (11, 12). Briefly, splenocytes were isolated from mice and responder T cells were isolated after IgG panning of splenocytes as described previously (12, 13). Stimulator cell preparations were prepared by gamma irradiation of unfractionated splenocytes with 2000 R from a ^{137}Cs source. One-way SMLR responses were determined in triplicate in flat-bottomed 96-well microtiter plates (Costar, Cambridge, MA), in a final volume of 0.2 ml/well, containing 5×10^5 responder T cells/well cultured in medium RPMI 1640 (Gibco, Gaithersburg, MD) containing 25 m*M* HEPES, 2 m*M* glutamine, 5×10^{-5} *M* 2-mercaptoethanol, 100 U/ml penicillin, and 100 μg/ml streptomycin, plus 10% heat-inactivated fetal bovine serum (FBS) (Hyclone Laboratories, Logan, UT) alone or with 5×10^5 irradiated splenocytes as stimulators. Microtiter plates were incubated for 6 days at 37°C in a 5% CO_2/95% air humidified atmosphere. To assess SMLR-induced blastogenic responses, cultures were pulsed with 0.1 μCi/well [^{3}H]thymidine (^{3}H-TdR) (New England Nuclear, Boston, MA) for the final 12 hr of culture, and the incorporation was measured as described previously (12). Data were presented as the mean cpm of triplicate T-cell cultures in the presence of stimulators minus the mean cpm of T-cell cultures in medium alone. In controls cpm uptake by irradiated splenocytes (stimulators) in response to ConA (2.5 μg/ml) was less than 100.

Cytofluorometric Analysis. To isolate peripheral blood mononuclear cells (PBMNC), mice were anesthetized with isoflurane (AErrane; Anaquest, Madison, WI) and 65 μl of blood withdrawn from the retroorbital sinus using a heparinized capillary. The blood was immediately diluted with 600 μl phosphate-buffered saline (PBS) in a microfuge tube at room temperature, layered on top with a 250-μl cushion of Lymphocyte-M (Accurate Chemicals & Scientific Corp., Westbury, NY) and centrifuges at 700*g* for 10 min. The lymphocyte layer was harvested and washed once in 1.4 ml PBS, centrifuged for 5 min at 4000*g* and the cell pellet was resuspended in 50 μl PBS. For immunofluorescent staining, 50 μl PBS containing 3% FBS and fluorochrome-conjugated monoclonal antibody was added to the 50 μl aliquot containing the harvested PBMNC or approximately 2×10^5 of SMLR cultured

cells and incubated at 4°C in the dark for 20 min. FITC-conjugated anti-CD8α and phycoerythrin-conjugated anti-CD4 monoclonal antibodies (clones 53–6.7 and RM4-5; Pharmingen, San Diego, CA) were diluted 1:199 and used to determine the percentage of $CD4^+$ and $CD8^+$ PBMNC. Anti-IL-1R (rat anti-mouse Ig2a, Genzyme), FITC-conjugated mouse anti-rat Ig2a (clone G28-05, Pharmingen) and phycoerythrin-conjugated anti-CD3ε (clone 145-2C11, Pharmingen) monoclonal antibodies were diluted 1:99 prior to use. Fluorescence intensity was measured by argon flow cytometry (FACscan; Becton Dickinson, Mountain View, CA). Analysis was restricted to lymphocytes ($3–9 \times 10^3$ cells per run) by applying an electronic gate that excluded residual granulocytes, monocytes, and erythrocytes. These data are expressed as percent of total fluorescent events. All data are presented as the mean of triplicate ± SD.

Nucleic Acid Isolation and Polymerase Chain Reaction. Total RNA was prepared by guanidinium-thiocyanate method (14) from SMLR cultures. The reverse transcriptase–mediated polymerase chain reaction (RT-PCR) was performed according to methods described previously (15). In brief, 20 μl of reaction mixture contained 100 ng of RNA, 0.75 μ*M* downstream primer, 1 m*M* dNTPs, and 5 m*M* $MgCl_2$. DNA-PCR was then carried out in 100 μl of reaction solution containing 10 m*M* Tris-HCl (pH 8.3), 50 m*M* KCl, 2 m*M* $MgCl_2$, 0.15 μ*M* of both upstream and downstream primers, and 2.5 units of *Taq* polymerase using a Perkin Elmer Cetus thermocycler 480 (Perkin Elmer Cetus). Conditions of the DNA-PCR were; 1 cycle of 4 min at 94°C, followed by 25 to 40 cycles of 30 sec at 94°C, 1 min at 60°C, and 1 min at 72°C. After PCR, an aliquot was electrophoresed on 6% PAGE, and bands were detected by staining with ethidium bromide. IL-2 and IL-4 genes were amplified using following primers (16): IL-2 was amplified by upstream (5′-AACA-GCGCACCCACTTCAA-3′) and downstream (5′-TTGAGATGATGCTTTGACA-3′) primers with a product size of 442 bp, and IL-4 was amplified by upstream (5′-TAGTTGTCATCCTGCTCTT-3′) and downstream (5′-CTACGAGTAATCCATTTGC-3′) with a product size of 404 bp. The same RNA was amplified using β-actin primers (5′-GTGGGGCGC-CCCAGGCACCA-3′) for upstream and (5′-CTCCTTAATGTCACGCACGATTTC-3′) for downstream which gave 550 bp product for normalization. The level of gene expression was then determined by densitometer using β-actin genes as an internal control.

Results

Effects of 5DHT on the Development of Diabetes. NOD/Sansum female mice, age 4–6 weeks, that received two consecutive implants of 5DHT for 60 days each did not develop insulitis or diabetes. Histological examination of 25-week old 5DHT-treated mice showed typical normal islet structure with no signs of periductal and perivascular infiltrates (Fig. 1B), whereas the islet from vehicle controls revealed insulitis with some structures completely degranulated (Fig. 1A). By 37 weeks of age none of the remaining 5DHT-treated mice ($n = 7$) had developed diabetes. In contrast all mice ($n = 8$) that received placebo pellets developed diabetes, although this number is higher than normal range in our colony where the incidence of the disease at this age is approximately 75%.

Phenotypic T-Cell Distribution in 5DHT-Treated Mice. We examined the effect of 5DHT treatment on the distribution of $CD4^+$ and $CD8^+$ T cells by FACS analyses. Both splenocytes and peripheral blood mononuclear cells (PBMNC) isolated from treated (60 days) and age-matched NOD female mice were examined. No significant differences in the percentage of $CD8^+$ T cells between treated (16.9 ± 0.8 [$n = 8$]) and controls (18.0 ± 3.0 [$n = 8$]) were observed in

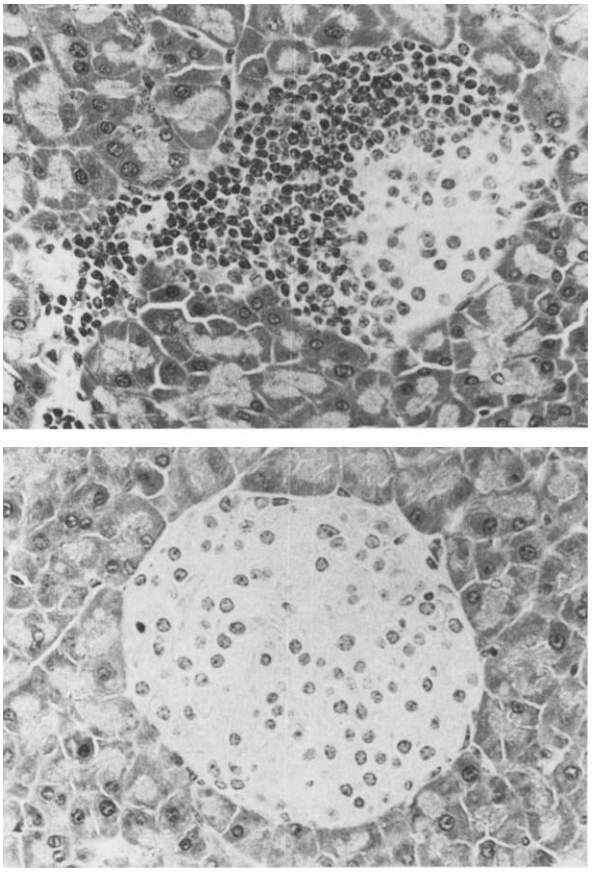

Figure 1. Histopathological examination of control and 5DHT-treated female NOD mouse pancreata. Histological analyses of pancreata both from 25-week-old 5DHT-treated (120 days) and age-matched nontreated female NOD mice. No lymphocytic infiltration was observed in the islet of 5DHT-treated mouse (B), whereas the islet of nontreated mouse showed severe periinsulitis as well as intraislet insulitis (A).

PBMNC. In contrast, the percentage of $CD4^+$ T-cells were markedly decreased in the 5DHT-treated mice (37.1 ± 4.8 vs 51.3 ± 9.3, $P < 0.02$) (Table I). Results of the splenocyte were not different from those of the PBMNC (data not shown). It should be noted that the percentage of $CD4^+$ T cells in the treated NOD females was not statistically different from that of age-matched male NOD mice (41.6 ± 3.4 [$n = 6$]).

Effect of 5DHT on SMLR. The SMLR was carried out using splenocyte T cells from treated (R_T) and nontreated (R_N) female mice as responder cells and whole irradiated splenocytes from treated (S_T) and nontreated (S_N) as stimulator cells. A representative result from two separate experiments is shown in Table II. Relative uptake of ^{3}H-TdR of $R_T - S_T$ in SMLR was approximately four to five times higher than that of nontreated combination ($R_N - S_N$), and significantly different (Experiment I, $P < 0.0001$). It is important to note that ^{3}H uptake of male NOD mice ($R_M - S_M$) was similar to that of $R_T - N_T$ (Experiments I and II). Restoration of T-cell proliferation was also observed when treated stimulator cells (S_T) was used (Experiment III), although to a lesser extent compared with that of $R_T - S_T$, suggesting that 5DHT treatment affects non–T stimulator cells, such as macrophages and B lymphocytes, as well as responder T cells. This is further supported by results from a different responder-stimulator combination in mixed lymphocyte reaction (MLR) (Experiments IV and V). The MLR clearly demonstrated that stimulator cells from nontreated female NOD were poor stimulator for T-cell proliferation compared with those from treated (Experiment IV, $P < 0.0001$). However, T-cell proliferation was greatly augmented when responder T-cell from 5DHT-treated female NOD were utilized (Experiment V, $P < 0.0001$).

Th2 T-Cell Expansion during SMLR. We then examined whether 5DHT had any effect on $CD4^+$ T-cell subsets Th1 and Th2. Dual-staining FACS analyses revealed that the number of IL-1R molecules in $CD4^+$ cells increased during SMLR (Fig. 2). The results clearly demonstrated that the increase of $IL\text{-}1R^+$ (16.8% vs 7.8%) cells was due to an increase of $IL\text{-}1R^+$ among $CD4^+$ cells, since the number of $CD4^+$ cells increased in 5DHT-treated mice compared with those in nontreated mice (12.5% vs 5.2%).

Profile of Cytokine Gene Expression in SMLR. We examined the cytokine gene expression to determine whether 5DHT treatment was able to expand Th2-type cells. The expression of two cytokine genes representing Th1 and Th2 T cells (IL-2 [Th1] and IL-4 [Th2]), was examined by RNA reverse RT-PCR. RNA was prepared from different time points in SMLR reaction. As shown in Figure 3, the expression of IL-2 in $R_T - S_T$ was reduced when compared with that of $R_N - S_N$. The level of spontaneous expression of IL-2 in $R_T - S_T$ was also lower than that of $R_N - S_N$. The maximum level of IL-2 expression in $R_N - S_N$ was achieved at 24 hr after SMLR was initiated.

Table I. Effect of 5DHT on Circulating PBMNC $CD4^+$ T Cells in Female NOD Mice

	$CD4^+$	$CD8^+$
Control ($n = 8$)	51.3 ± 9.3	18.0 ± 3.0
5DHT ($n = 8$)	37.1 ± 4.8	16.9 ± 0.8

Note. Number of $CD4^+$ of male NOD mice: 41.6 ± 3.4 ($n = 6$).

Discussion

Results presented here clearly demonstrated that the systemic administration of 5DHT to female NOD mice can block the development of diabetes (Fig. 1). Our results agreed well with the data reported by Fox (10). However, in his study 5DHT was administered to mice ($n = 8$) at 8 weeks of age, a time at which all signs of inflammatory infiltration of the islets (i.e., mild insulitis). Therefore, it is possible to speculate that the timing of the steroid administration is very critical to preventing the development of insulitis, since our results clearly demonstrated that the onset of insulitis can be suppressed by 5DHT treatment when it is administered prior to the age of 6 weeks, when the first sign of insulitis can be observed. Unexpectedly, all treated mice survived without developing the disease for almost 1 year after the administration was stopped at 120 days. Hence, administration of the steroid for 120 days from an age prior to the onset of insulitis may be sufficient to alter immunoregulatory thymic and/or peripheral pathways in order to prevent the disease development in NOD mice. Furthermore, the percentage of $CD4^+$ T-cells in the treated NOD females was not statistically different from that of age-matched male NOD mice (Table I), which may explain a low incidence of diabetes development in male NOD mice.

Much attention has focused on whether two functionally different T-cell subsets, Th1 and Th2 (17), can be discriminated based on the expression of certain cell-surface markers including CD45 isoforms and immunoglobulin Fc (18–20). Another molecule which has gained more attention is IL-1 receptor (IL-1R) (21–23). A study demonstrated that only Th2 T cells depend on the presence of IL-1 for proliferation, particularly IL-1α (21). Importantly, IL-1α was produced only by Th2 cells within T-cell populations which correlates with the increase the number of IL-1R (24, 25). Furthermore, exogenous administration of IL-1α has the potential to prevent the development of insulitis and the onset of diabetes in our previous study (26). On the basis of our present result (Fig. 2) together with these previous studies, it is hypothesized that 5DHT treatment may increase Th2-cell populations, which in turn lead to the shift from Th1 > Th2 to Th2 > Th1 in NOD

Table II. Effects of 5DHT on SMLR

Experiment	Responder	Stimulator	Reaction	^{3}H-TdR uptake (cpm)	
I	NOD (R_T)	NOD (S_T)	SMLR	6,409 ± 685	
	NOD (R_N)	NOD (S_N)	SMLR	2,279 ± 476	$P < 0.0001$
II	NOD (R_M)	NOD (S_M)	SMLR	6,882 ± 698	
	NOD (R_N)	NOD (S_N)	SMLR	1,928 ± 862	$P < 0.0001$
III	NOD (R_T)	NOD (S_N)	SMLR	4,408 ± 826	
	NOD (R_N)	NOD (S_T)	SMLR	2,750 ± 386	$P < 0.035$
IV	C57 (R_{C57})	NOD (S_T)	MLR	14,468 ± 1,011	
	C57 (R_{C57})	NOD (S_N)	MLR	6,270 ± 1,187	$P < 0.0001$
V	NOD (R_N)	C57 (S_{C57})	MLR	9,758 ± 787	
	NOD (R_T)	C57 (S_{C57})	MLR	27,094 ± 1,618	$P < 0.0001$

Note. R_T, responder T cells from 5DHT-treated female NOD splenocytes; R_M, responder T cells from male NOD splenocytes; R_N, responder T cells from nontreated female NOD splenocytes; S_M, stimulator cells from male NOD splenocytes; S_T, stimulator cells from 5DHT treated female NOD splenocytes; S_N, stimulator cells from nontreated female NOD splenocytes.

splenocytes. The number of $CD8^+$ T cells in SMLR did not change during the course of the reaction and was not affected by 5DHT treatment.

It was first demonstrated that T-cell proliferation in the autologous mixed lymphocyte culture from patients with systemic lupus erythematosus was reduced (27). Similar findings are observed in other human autoimmune diseases (28, 29) including human type I diabetes (30). Studies using several autoimmune rodent models also demonstrated reduced proliferation in syngeneic mixed lymphocyte reaction (SMLR) (31, 32). The SMLR is the result of T-cell proliferation, presumably $CD4^+$ T cells, that occurs when T lymphocytes (responder) are co-cultured with autologous or syngeneic non-T cells (stimulator). Both helper and suppressor functions are required for autoreactive T-cell proliferation (33). Our results (see Tables I and II) suggest that the increased proliferative response of T-cells from 5DHT-treated female NOD mice resulted from restoration of regulatory (helper/suppressor) $CD4^+$ T cells. This hypothesis is also supported by an increased T-cell responsiveness in male NOD mice (Experience II in Table II), which may explain a low incidence of diabetes development. Although results suggest that T cells appeared to be primary cells responding to 5DHT treatment, stimulator cells, particularly macrophages, may also be affected by the treatment. This hypothesis is supported by recent findings that low proliferative T-cell responses observed in female NOD mice are due to defective functions of antigen-presenting cells (11, 32). Results from kinetics of IL-2 gene expression during SMLR also suggest that the IL-2 message was less stable in $R_T - S_T$ than that of $R_N - S_N$, since the level of IL-2 message of $R_N - S_N$ at 24 hr after SMLR is much higher than that of $R_T - S_T$ (Fig. 3).

The mechanisms by which sex hormones alter immune responses are complex, but it is possible that sex hormones affect immune responses by acting on a wide range of nonlymphoid target tissues, which then affect the immune system. This effect may be prominent for global immune regulation. Alternatively, sex hormones may affect immune cells directly, which produce effector molecules, such as cytokines, followed by alteration of immune systems. This hypothesis is supported by several lines of evidence demonstrating the presence of a steroid receptor in mononuclear cells, splenocytes, and thymocytes (7, 8, 34).

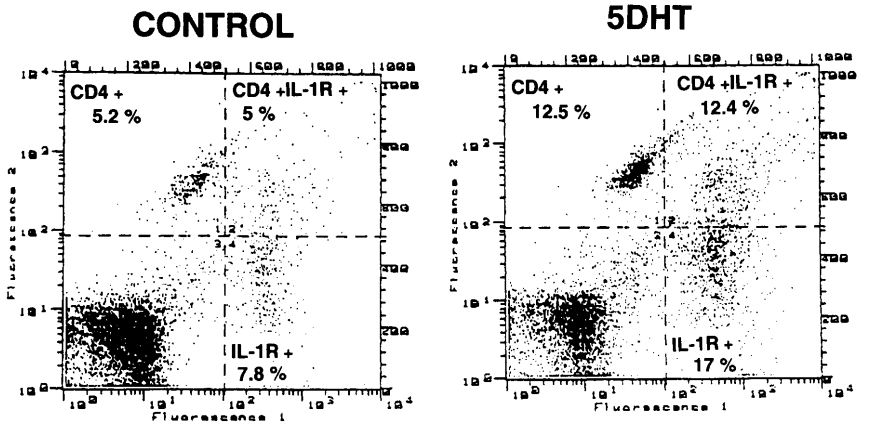

Figure 2. Expression of IL-1 receptor molecule by $CD4^+$ cells during SMLR. After 6 days incubation in SMLR, the expression of IL-1 receptor molecule was determined by FACS analyses as described in the text. A representative result from three independent experiments is shown here.

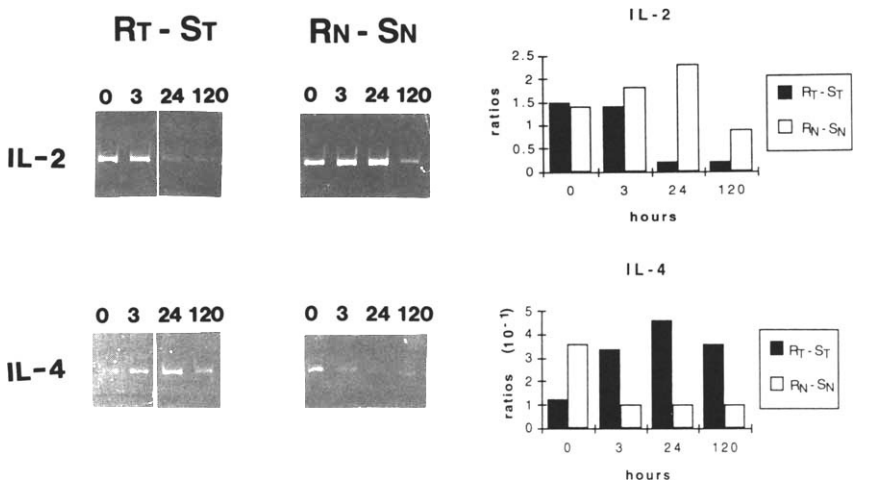

Figure 3. Cytokine gene expression in SMLR. RNA was isolated from each set of reactions (1–2 × 10^6 cells) using TRIZOL RNA isolation reagent (GIBCO-BRL), followed by RNA-PCR using 100 ng of RNA and 2.5 m*M* random-hexamer as a downstream primer for the first cDNA synthesis. DNA-PCR was carried out in the presence of primer sets specific for IL-2 and IL-4 genes using our standard PCR conditions in 50 μl reaction volume. After 40 cycles for the IL-2 gene and 45 cycles for the IL-4 gene, an aliquot of the PCR reaction mixture (10 μl) was electrophoresed on 6% PAGE and stained. Densitometric analysis were performed to quantitate relative ratios between cytokine genes and β-actin gene. $R_N - S_N$ and $R_T - S_T$ represent SMLR between responder T cells and stimulator cells from nontreated female NOD splenocytes and 5DHT-treated female NOD splenocytes, respectively.

This effect may be responsible for local and compartmentalized immune reactions. Although these two possibilities are not mutually exclusive and further studies are needed, the results presented here appear to support the latter mechanism. Furthermore, a recent study demonstrated that expression of γ-interferon (IFN-γ) gene was under direct regulation of estrogen (35). This observation is of particular interest, because Type I diabetes is believed to be mediated by Th1 T cells, which are the primary cells responsible for producing IFN-γ (2, 4, 5). A recent study also demonstrated that $CD4^+$ T cells were more responsive to 5DHT treatment (36). In this study, 5DHT suppressed the production of IL-4 and IFN-γ but did not affect the production of IL-2 using lymph nodes from normal mice, suggesting that 5DHT treatment suppresses the cytokine production both from Th1 and Th2 cell types. In contrast, our results suggest that 5DHT enhances the development of Th2 T-cell populations and the expression of cytokines produced by Th2 cells. This may be due to strain differences (e.g., autoimmune strains versus normal strains).

In conclusion, administration of 5DHT to female NOD mice can prevent the development of insulitis as well as disease development when the hormone was administered prior to the onset of insulitis. The results clearly indicate that sex steroid hormones may affect immune system and hence modulation of the immune response by the hormone may lead to develop a new immunotherapy for various autoimmune diseases.

The authors thank Drs. D. Cramer, R. Tun, and S. Jordan for reviewing and Ms. Andreea Redford for editing of this manuscript, and Drs. D. L. Rimoin and J. I. Rotter for their encouragement.

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