Brief Definitive Report

Androgen Treatment Prevents Diabetes in Nonobese Diabetic Mice

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Summary

The nonobese diabetic (NOD) mouse strain provides a model system for human autoimmune diabetes. This disease model is extensively used not only to examine the etiology and pathogenesis of diabetes, but also as a means to evaluate therapies. In NOD mice, the disease progresses from insulitis to islet destruction and clinical diabetes in a high percentage of female mice. In this study, androgen therapy, begun after the onset of insulitis, was found to prevent islet destruction and diabetes without eliminating the islet inflammation in female NOD mice. However, diabetes can be adoptively transferred into such hormone-treated recipients. The prevention of disease onset by androgen is likely due to the hormonal alteration of the development or function of the immune cells necessary for islet destruction.

The alleviation of pathogenic autoimmune responses is an objective not only in the quest to relieve the suffering of those afflicted by the range of autoimmune diseases, but also to help understand the basic pathogenic mechanisms leading to autoimmunity. In experimental animal models of autoimmunity, a great variety of exogenous substances have been used therapeutically to alter autoimmune interactions, including antibodies, immunosuppressive drugs and viruses. One of the most extensively utilized models for the study of autoimmunity is the nonobese diabetic (NOD) mouse strain.

NOD mice provide an animal model of human type I (juvenile-type or insulin-dependent) diabetes mellitus (1). In humans, insulitis (an inflammatory infiltrate involving the pancreatic islets of Langerhans) is found in recent-onset diabetics. In general, most NOD mice have insulitis by 5–6 wk of age. The inflammation is at first periductal, and then surrounds and infiltrates the islets, after which clinical diabetes develops. The spontaneous inflammatory diabetes that develops in NOD mice appears to be due to cell-mediated immunity. Disease can be prevented by genetic or physical removal of the thymus (2, 3), by treatment with anti-Thy-1 (4) or anti-CD4 antibodies (5), or by therapy with cyclosporin A (6). The inflammatory infiltrate is pleomorphic in nature (7). Adoptive transfer experiments have revealed that T cells can transfer disease (8) and that macrophages are essential to pathogenesis (9), but that islet-cell destruction does not require B cells (10).

Overt diabetes occurs in most female mice beginning at 3 mo of age. Although insulitis is present in male NOD mice, less than half of these mice progress to diabetes. A role for sex steroids in this sexual dimorphism is likely. Castration of male mice raises their incidence of diabetes, and oopherectomy of females decreases the progression to diabetes (11, 12). However, in the NOD strain, direct effects of steroid hormones on disease have not been directly examined. This is in contrast with another extensively studied animal model of autoimmunity, the F_1 hybrid between NZB and NZW mice. Such B × W mice develop a disease resembling human systemic lupus erythematosus, and in this model, female mice suffer from a more severe disease than do male mice. In these mice, estrogen has an accelerating effect on the humoral-based autoimmunity, whereas testosterone improves survival from the disease (13).

Androgens (testosterone and its more potent metabolite, 5α-dihydrotestosterone [dhT]) have a beneficial effect not only on the B × W autoimmune disease, but on other unrelated models, such as autoimmune thyroiditis in chickens (14). Despite their beneficial actions on autoimmunity, little is known about the actions of androgen on the immune system. Only immature cells types in the immune system have been shown to express androgen receptors and therefore be candidates for androgen effects (15, 16), suggesting that androgens affect the precursors of the effector cells.

The present study was undertaken to examine whether androgens may have a protective role in the autoimmune diabetes of NOD mice. These studies demonstrate that the hormone indeed has a profound effect: dhT prevents the development of diabetes. Adoptive transfer experiments reveal that this sex steroid likely acts to prevent the development or perpetuation of a destruction immune attack on the pancreatic β cells.

Materials and Methods

Animals. Female NOD/Shi mice were obtained from the rodent breeding facility at The Scripps Research Institute. Mice were

 J. Exp. Med. © The Rockefeller University Press • 0022-1007/92/05/1409/04 $2.00
Volume 175 May 1992 1409–1412

maintained in microisolator cages under specific pathogen-free conditions. Glucose determinations were made using a drop of retroorbital sinus blood obtained from mice under methoxyflurane anesthesia. The blood was reacted with Chemstrip bG and glucose concentrations read on an AccuChek II monitor following manufacturer's (Boehringer Mannheim Diagnostics, Indianapolis, IN) instructions. Diabetes was defined as a blood glucose reading of >400 mg/dl.

Sex Steroid Therapy. Hormone treatment consisted of subcutaneous implantation of pellets (Innovative Research of America, Toledo, OH) containing 15 mg of dhT released over a 60-d period, designed to yield plasma dhT levels of 5–10 ng/ml (17–34 nM). Control mice received pellets containing the carrier-binder alone. Pellets were placed in mice receiving methoxyflurane anesthesia, and were first implanted when mice were 51–61 d old, and repeated at 60-d intervals.

Adoptive Transfer. Eight recipient mice each received a hormone pellet a 51–57 d of age, and were prepared for adoptive transfer of cells at 61–68 d of age (8–11 days after the onset of hormone treatment) by whole body irradiation from a cesium source using 775 rad. The mice were then given an intravenous injection containing 1–1.5 × 10^7 spleen cells, prepared from untreated diabetic NOD female mice. Blood glucose levels were measured at least twice in the next 4 wk.

Histology. Pancreata were removed from killed animals, fixed for 4 h in Bouin's fixative, transferred to 70% ethanol, and processed for paraffin embedding. Tissue sections were stained with hematoxylin and eosin, and three different levels sections were examined from each specimen. For immunohistology, unstained sections were deparaffinized, incubated in 0.1 M glycine to quench aldehydes, blocked in 2% goat serum, then reacted sequentially with guinea pig antiinsulin (1/300 dilution) and biotinylated goat anti-guinea pig (1/200 dilution) antibodies. Sections were then reacted with hydrogen peroxide/methanol to block endogenous peroxidase activity, and then incubated with avidin-biotin-horseradish peroxidase complex. Antibodies and avidin-biotin-horseradish peroxidase complex were obtained from Vector Laboratories, Inc. (Burlingame, CA). The peroxidase was developed using 3,3'-diaminobenzidine as a chromogen in the presence of hydrogen peroxide and nickel chloride, and sections were lightly counterstained with methyl green.

Results and Discussion

Both male and female NOD mice develop insulitis, but only a high percent of female mice progress to clinical diabetes. To determine whether part of this effect may be due to androgenic hormones, dhT treatment of female NOD mice was begun. Therapy with dhT was chosen to insure the presence of this more potent form of androgen in the tissues, and to prevent the possible peripheral aromitization of testosterone to estrogen.

Beginning at ~8 wk of age, pellets containing dhT, or control pellets lacking the hormone, were implanted subcutaneously into NOD mice. Most control mice, as expected, developed clinical diabetes (Fig. 1), yet none of the dhT-treated animals, followed to 31 wk of age, became diabetic. Average blood glucose values (± SD) from this treated group ranged from 144 (± 14) mg/dl to 162 (± 38) mg/dl, only slightly higher than those found in C57BL/6J mice maintained under similar conditions (138 [± 20] mg/dl).

Since insulitis precedes the onset of diabetes, one mechanism by which dhT may prevent diabetes is by eliminating

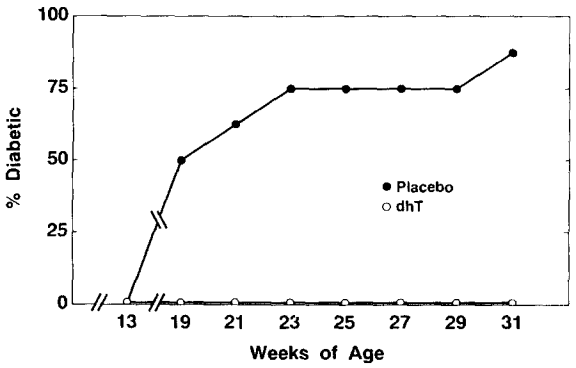

Figure 1. Androgen treatment prevents the onset of diabetes in NOD mice. The percentage of mice with clinical diabetes is indicated for the two treatment groups: Placebo treated (●) and dhT treated (○). Blood glucoses were measured biweekly; values >400 mg/dl were defined as diabetic since animals with such blood glucose readings had clinical signs of diabetes, such as polydipsia, polyuria, and weight loss. Eight mice received 60-d release control or dhT-containing pellets at the onset of the study and new pellets were given twice at the end of the 60-d interval. One dhT-treated mouse lost its hormone pellet and was killed before the conclusion of the study.

or reducing the inflammatory infiltrate in the islets. Histological examination of the pancreata from female NOD mice at 8 wk of age, when the hormone treatment began, revealed the presence of islets with inflammatory infiltrates of varying degree, and evidence of destructive lesions (Fig. 2, *A* and *B*). Analysis of the pancreata from 31-wk-old dhT-treated mice revealed the continued presence of insulitis, ranging from lesions with minimal inflammation to islets with destructive lesions (Fig. 2, *C* and *D*). In these mice, the islets of Langerhans were easily identifiable. This is in contrast to the control group, killed after the onset of diabetes, in which only atrophic islets can be identified, with some showing residual inflammation (Fig. 2 *E*). Immunohistochemical analysis confirmed the presence of islets with abundant insulin-containing β cells in the pancreata of hormone-treated animals (Fig. 2 *F*) and their absence in control mice.

There are two distinct classes of mechanisms by which dhT may be preventing the β cell death. First, the effect may be on the immune system, preventing the killing of the β cells by eliminating, inactivating, or altering the functional properties of the cells necessary for the self destruction. Second, the effect may be on nonimmune cell types, such as on the endothelium to prevent trafficking of certain immune cells into the islet, or on the β cell itself to protect it from immune attack.

Adoptive transfer experiments were performed to attempt to distinguish between these possibilities. In NOD mice, transfer of spleen cells from diabetic animals into young, irradiated recipients leads to an acceleration of disease in the recipients (17). Young dhT-treated female mice received spleen cells from older, diabetic female mice, and the recipients were monitored for the onset of diabetes. As shown above, dhT-treated mice did not become diabetic by at least 31 wk of age. In contrast, all dhT-treated mice receiving diabetic spleen cells became diabetic by 13 wk of age (4 wk after transfer).

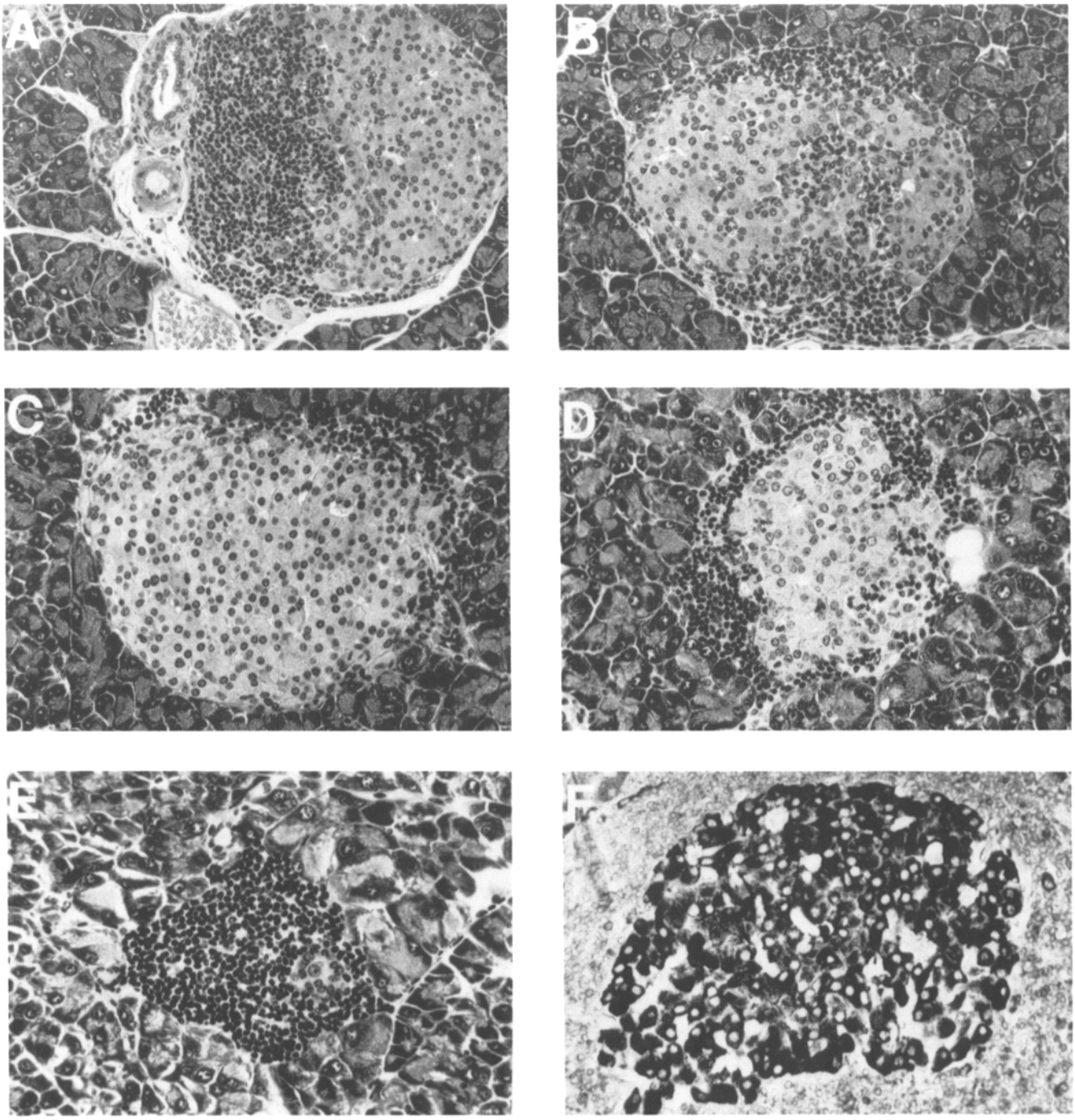

Figure 2. Histopathology of control and dhT-treated NOD mouse pancreata. (*A* and *B*) Hematoxylin and eosin–stained pancreatic sections from 8-wk-old mice (×77 and ×83). Inflammatory infiltration of the islets and islet cell destruction is already present in mice of this age. (*C* and *D*) Hematoxylin and eosin–stained pancreatic sections from 31-wk-old, dhT-treated mice (×83). Varying degrees of inflammation are present in these mice. (*E*) Hematoxylin and eosin–stained pancreatic section from a 22-wk-old placebo-treated mouse (×128). Remaining islets contain a paucity of cells, and some still show inflammation. (*F*) Immunoperoxidase staining for insulin in islet of a 31-wk-old, dhT-treated female mouse (×95). Islets in dhT-treated mice contain abundant immunoreactive insulin in β cells.

Pretransfer blood levels averaged 141 (± 19) mg/dl, whereas after transfer blood glucose levels were diagnostic of diabetes, 445 (± 17) mg/dl. Similarly manipulated dhT-treated mice receiving control spleen cells (from young, nondiabetic female NOD mice) did not become diabetic in this period.

These adoptive transfer experiments reveal that spleen cells from a diabetic animal are capable of inducing disease in dhT-treated recipients. Therefore, dhT cannot be acting to protect the β cell itself from damage, by preventing the infiltration of specific pathogenic cells into the islets, or by inhibiting

effector cells that have developed in nontreated animals. In dhT-treated mice, the progression of the immune cell attack is blocked.

The identity of the cell type(s) affected by androgen therapy remains unknown. However, a synergistic effect of thymectomy with castration was found to increase diabetes in male NOD mice (12). Given the presence of androgen receptors in thymocytes (15), the thymus is a potential target for dhT, affecting T cell differentiation and function. Since the molecular mechanisms by which steroid hormones exert their effects is becoming increasingly elucidated, this system should be fruitful to unravel the pathway responsible for the protective effect of androgens on autoimmunity.

I thank Dr. Judy Shizuru for help with the adoptive transfers, and Drs. Michel Duchosal and Nora Sarvetnick for comments on the manuscript.

This is publication number 7181-IMM from the Department of Immunology, The Scripps Research Institute.

This work was supported by National Institutes of Health grant AR-40981-01; H. Fox is the recipient of a fellowship from the Terri Gotthelf Lupus Research Institute and an Arthritis Foundation Investigator Award.

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Received for publication 5 February 1992.

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