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AN ABSTRACT OF THE THESIS OF Debra Ann Weyant for the Master of Science in Biology presented May 18, 1979.

Title: Androgen-Induced Immunosuppression.

APPROVED BY MEMBERS OF THE THESIS COMMITTEE:

David T. Clark, Chairman

Trygve P. Steen

Stanley S. Hillman

Jane I. Morton

It is well established that females are more immunocompetent than males as evidenced by higher humoral antibody titers, lowered susceptibility to infection, and more efficient graft rejection. Furthermore, females also exhibit a much higher incidence of autoimmune disease. These observations have led investigators to believe that the male hormonal environment may play a key role in the regulation of immune response. For this reason, this study is concerned with the expression of autoimmunity and of immune function in the mouse.

This study included the New Zealand Black (NZB) mouse strain, as an animal model for human SLE, as well as normal DBA/2 and Balb/c strains. Animals were administered testosterone via subcutaneous implants in silastic tubing or by injection. Mice used were intact females, intact males and castrated males. Animals were otherwise untreated or had been exposed to a sublethal dose (400-550 rads) of irradiation. Target organ weight changes, immune capacity and peripheral blood picture changes were measured.

In all experiments, marked changes in seminal vesicle, kidney and thymus weights were observed following castration or testosterone treatment. However, numbers of spleen plaque-forming cells following SRBC immunization were not significantly changed in normal or autoimmune strain mice not receiving irradiation.

In order to amplify the sensitivity of the responding system, we then elected to study testosterone immunosuppression in a regenerating hemopoietic system which was recovering from the ablative effects of sublethal irradiation.

It was observed, using irradiated mice, that orchidectomy resulted in striking immunoenhancement, where as testosterone treatment produced suppression of the antibody response to SRBC immunization.

A possible mechanism for the testosterone effect consisting of a preferential differentiation of the regenerating stem cell population along the myeloid pathway was explored. Differential white blood cell counts indicated that testosterone administration can produce a reversal of the granulocyte:lymphocyte ratio resulting in a marked granulocytosis with accompanying lymphopenia. Interestingly, in one experiment

where testosterone had been observed to induce this reversal of the granulocyte:lymphocyte ratio at eight weeks following irradiation, by 12 weeks the blood picture had returned to normal. However, marked immunosuppression was still evident in these animals.

These data have suggested that the immunosuppression produced by testosterone may be the result of a preferential differentiation of the regenerating stem cell population along the myeloid pathway, with a concurrent suppression of lymphocyte production. Furthermore, evidence suggests that there may be a selective sensitivity of one immunocyte subpopulation to testosterone modulation of differentiation.

ANDROGEN-INDUCED IMMUNOSUPPRESSION

by

DEBRA ANN WEYANT

A thesis submitted in partial fulfillment of the
requirements for the degree of

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in
BIOLOGY

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1979

TO THE OFFICE OF GRADUATE STUDIES AND RESEARCH:

The members of the Committee approve the thesis of
Debra Ann Weyant presented May 5, 1979.

David T. Clark, Chairman

Trygve P. Steen

Stanley S. Hillman

Jane I. Morton

APPROVED:

Herman Taylor, Head, Department of Biology

Stanley E. Rauch, Dean of Graduate Studies and Research

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CHAPTER I

INTRODUCTION

Hormonal stimulation and modulation of lymphatic tissue has been recognized for some time as a normal mechanism in development (1). Growth hormone and thyroxine are important hormones in the maturation of the immune system and greatly affect the immunological capability of the adult (1). The effects of the steroid hormones, and, in particular, the sex hormones, are clearly evident in the marked differences reported between male and female immunological responses. Females are more immunocompetent than males as evidenced by higher humoral antibody production (2,3), lowered susceptibility to infection (4,5), and more efficient graft rejection (6). In addition, the incidence of autoimmune disease, for example, systemic lupus erythematosus (SLE), has long been noted to be higher in females than males during the reproductive years (7,8,9); the female:male ratio of SLE in humans being approximately 9:1 (10). These observations have led investigators to believe that the male hormonal environment may play a key role in the regulation of immune response. For this reason, this study is concerned with evaluating the possible role and mechanism of androgen modulation of the expression of autoimmunity and of immune function in the mouse.

The animal model for human systemic lupus erythematosus used in this study is the New Zealand Black (NZB) mouse strain. The NZB develops an autoimmune disease characterized by the production of anti-

DNA autoantibodies with a resultant glomerular nephritis of the kidneys, and a general immune function imbalance, i.e., hyperactive humoral and cellular immune responses when young; impaired function when old (11). In addition, the sex differences noted in lupus are present in NZB mice where females manifest an earlier onset of disease and usually die much earlier than the males (12). Therefore, the NZB provides a valuable model for experimentation in this field of research.

In order to modify the hormonal environment in several strains of normal and autoimmune mice, intact and castrated males as well as intact females were administered testosterone either by injection or by subcutaneous implantation of testosterone-contained silastic tubing.

CHAPTER II

REVIEW OF THE LITERATURE

STEROID HORMONES AND LYMPHOID TISSUES

Early work had shown that the exogenous administration of androsterone, 5-androstenediol, testosterone and testosterone propionate resulted in thymic atrophy in intact or castrated animals (13,14,15). Androsterone and testosterone propionate were found to be the most effective (16). Estrogenic compounds also produce thymic involution in puppies (17) and rats (18,19) as does progesterone (20), but only in large amounts. Money *et al* (18) tested the activity of various steroids, i.e., ACTH, cortisone, 11-dehydrocorticosterone, 17 α -hydroxy-11,21-acetoxy-desoxycorticosterone, 17 β -hydroxyprogesterone, desoxycorticosterone, dehydrocortisone, pregnenolone, testosterone, progesterone, and various estrogens. All were found to produce thymic involution. ACTH, cortisone and 11-dehydrocorticosterone were shown to effect lymph nodes as well.

Gonadotrophins will also produce acute involution of the thymus, but only in intact animals. Castration blocked this thymolytic effect in the rat (21), suggesting that the gonadotrophic-induced involution was mediated through the release of the sex hormones from stimulated gonadal organs. In addition, the action of the sex hormones on the thymus was not reversed by adrenalectomy in rats. However, those

compounds related chemically to the sex hormones, but not hormonally active, had no thymolytic activity in adrenalectomized rats (19).

These data indicate a direct link between hormonally active sex hormones and thymic integrity.

The mechanism of action of adrenocortical hormones on lymphatic tissues of mice, rats and rabbits was also investigated (22,23). It was reported that ACTH or adrenocortical extracts produced a marked decrease in lymphocyte numbers and a cessation of mitosis in small and medium-sized lymphocytes. In addition, severe degenerative changes in the cells were noted. Lymphocytolysis could also be produced in vitro (24,25,26). However, lymphocytolysis could not entirely explain prolonged or sustained atrophy. Baker et al (27) reported the absence of many mitotic figures on prolonged ACTH treatment and concluded that suppression of lymphocytopoiesis was the more likely mechanism of prolonged thymic involution. Dougherty (28) confirmed these conclusions with histological observations. Conversely, gonadectomy of either the male or female mouse produced thymic enlargement (3,29). It was suggested that gonadal hormones exert a constant moderating influence on the growth and maintenance of lymphatic organs.

Hormonal effects on blood or peripheral lymphocytes were also investigated. Daily injections of ACTH in mice (30), rats and rabbits (31) produced a significant decrease in the numbers of circulating lymphocytes. These early reports showed no significant changes in blood lymphocyte levels with either gonadectomy or administration of exogenous testosterone (32).

In the chicken, treatment with androgens induced bursal involution (33). The bursa of Fabricius is a major immunological organ in the chicken where it serves as the site of B-cell maturation. Also, it was found that 19-nortestosterone could also prevent the development of the bursa of Fabricius in chick embryos (34). Chicks treated with testosterone as embryos are unable to produce a detectable antibody response (35). Thymus development was also shown to be suppressed in about 40 per cent of these treated embryos (36).

More recently, Eidinger and Garrett (3), looking at the effects of castration on lymphoid tissue, substantiated the earlier reports of thymic enlargement. They also reported an increase in the spleen weight of castrated male mice. In comparing females with intact and castrated males, the intact males exhibited the smallest spleens and thymuses, the female organs were significantly larger, but the largest organs were found in the castrated males. They were also able to show that it was an increase in the number of cortisone-sensitive or immature thymic lymphocytes that resulted in thymic and splenic enlargement.

This observed thymic hypertrophy with castration could be reversed by the administration of replacement dose of testosterone, and hormone supplements to normal mice produced an accelerated rate of thymic involution. Similar hypertrophy of the lymph nodes was also noted with orchidectomy, but not in combined thymectomy and orchidectomy. Histological examination of the enlarged nodes showed an increase in thickness of the paracortical or T-dependent region, suggesting the importance of the thymus in castration-induced nodal enlargement (29).

In studies in which mice were given a single injection of testosterone immediately following irradiation and marrow reconstitution, severe depletion of the thymus-independent (B-cell) regions of the spleen, lymph nodes and Peyer's patches were noted. Also the size of the lymph follicles in these organs were decreased with testosterone treatment as compared to irradiated controls. Mature plasma cells were unaffected. Myelopoiesis and particularly erythropoiesis was stimulated in these testosterone-treated mice (37).

INFLUENCE OF TESTOSTERONE ON ANTIBODY PRODUCTION

As previously mentioned, males tend to be less responsive immunologically than females. Females respond to antigenic stimulation with higher humoral antibody production (2,3), and are less susceptible to infection (4,5). Investigations have indicated that testosterone plays a primary role in regulating antibody response.

In experiments where castration of the males had been performed, Eldinger and Garrett (3) found that the primary immune response to polyvinylpyrrolidone (PVP), a T-independent antigen, was significantly higher than in intact males and even exceeded that of the females. When a T-dependent antigen was used, a significantly large difference was apparent in the early 19 S phase of the response to horse erythrocytes. The gonadectomized males exhibited early antibody titers comparable to the female which far exceeded that of intact males. There was no difference between gonadectomized and intact males by 20 days post immunization. Secondary antibody responses to erythrocytes were similar for castrated males and normal females, but both responses

exceeded those of intact males. Thus, when T-dependent antigens were used for immunization, castration appeared to convert the male response to that of the female. However, castration induced a responsiveness to PVP, the T-independent antigen, which exceeded even that of the female. When thymectomy accompanied castration, the enhanced antibody response to PVP was abolished suggesting a role of the thymus in this phenomenon. It was suggested that the enhancement could be mediated through a thymic enhancing factor or hormone.

However, in a similar experiment looking at the late phase of the oxazolone reaction and response to pneumococcal polysaccharide (SIII) immunization, both T-independent responses, no significant differences between the orchidectomized and intact male mice were detected. They did, however, show a significant increase in antibody response to sheep erythrocytes with castration (29).

While castration has an enhancing effect on antibody production, Fujii et al (37) showed that the administration of a single intraperitoneal injection of 10 milligrams of testosterone could significantly suppress the plaque-forming cell response of normal intact female C3H/He mice to sheep erythrocyte immunization for up to 30 days. Histologically, no differences in cellularity of lymphoid tissues were observed between treated and untreated groups. Interestingly, this same antibody response could be suppressed for as long as 60 days by a single testosterone injection immediately following lethal irradiation and bone marrow cell reconstitution. Histological examination of lymphoid tissues at 30 days revealed severe depletion of thymic-independent, B-cell, areas of spleen, lymph nodes and Peyer's patches in the testosterone-treated

group, but these areas had fully recovered by 60 days. By 90 days, antibody suppression was also no longer evident.

In autoimmune NZB mice, the production of autoantibodies, as well as antibodies to foreign antigens, is much more pronounced in the female (11,12). A naturally occurring thymocytotoxic autoantibody (NTA) has also been found to be abnormally high in young NZB's (38). Raveche et al (39) determined that (NZB X DBA/2) F_1 females showed a significantly higher incidence of a positive test for this antibody as compared to the hybrid males and a higher titer in NTA-positive females than in NTA-positive males. Castration of the females had no effect on either parameter of NTA measurement, but castration of the male increased the incidence and titer of antibody to equal those seen in the female. Failure of the NZB-mothered F_1 males to produce NTA did not substantiate an x-linked immune response hypothesis. These data lend further support to the important role of male sex hormones in the expression of autoimmunity.

Roubinian and coworkers (40) asked the question: Can survivals in murine lupus be improved with androgenic hormones? They found that prepuberal castration of the male NZB accelerated the rate of death from disease in 60 per cent of these mice. Castration of the female had no effect on survival. However, treatment of NZB females with nafoxidine, an anti-estrogenic agent, led to a decrease in anti-DNA antibodies and an increase in survival (41). When the administration of androgen followed castration of the female, a dramatic decrease in mortality was observed to 14 from 94 per cent for the intact female. Furthermore, the titer of anti-DNA antibodies was significantly less in androgen-

compared to estrogen-treated females. Male castration accelerated the age of appearance of anti-DNA autoantibodies in the male NZB and facilitated an earlier switch from IgM to IgG immunoglobulin. The earlier appearance of 7S anti-DNA antibody has been correlated with the more severe disease of the female (40). While sham-operated males developed 7S IgG anti-DNA antibodies at seven months of age, these antibodies made a much earlier, four-month, appearance in the castrated male, which was the age of their development in the normal NZB female. Interestingly, results similar to the effects of orchidectomy could be achieved with neonatal thymectomy. Neonatal thymectomy was found to produce initial increases in anti-DNA and poly-A antibody formation and prevent the later switch to 7S antibodies observed in the NZB males. This led these investigators to propose that the thymus is the target for, or mediates, the androgen effect (42).

Other parameters of murine systemic lupus were also alleviated by androgen treatment of castrated females. Proteinuria was significantly decreased and the degree of lymphocytic infiltration and glomerular inflammation of the kidneys was lessened. Other investigators have obtained similar results (43).

With the protective effects of androgen in murine lupus in mind, Stahl and Decker (44) looked at the androgenic status of 12 men with human systemic lupus. They could find no deficiency in serum levels of testosterone, luteinizing hormone, or follicle-stimulating hormone. They concluded that gonadal function in these men was not a primary factor in their disease.

EFFECTS OF TESTOSTERONE ON STEM CELL DIFFERENTIATION

Currently, work by Abramson et al (45) supports the monophyletic theory that the pluripotential hemopoietic stem cell is the precursor of all myeloid and lymphoid components of blood and lymph. From this cell is derived the restricted or committed precursor of these elements. A single precursor cell is thought to be the parent of the erythrocyte, granulocyte and megakaryocyte populations. A separate precursor cell arising from the stem cell was shown to be committed to the T-lymphocyte pathway of differentiation, and B-cells, the antibody producers, apparently arise directly from the stem cell without a committed progenitor as an intermediate. Most importantly, they provide evidence which suggests that there is not a separate lymphocytic stem cell, or progenitor cell, giving rise to both T and B cells. This differentiation scheme is presented in Figure 1. In the mouse, these pluripotential stem cells are located in the hemopoietic tissue of the embryonic yolk sac, fetal liver, spleen and bone marrow (46).

Factors influencing the pathways of differentiation of this pluripotential cell could play an important role in the establishment of populations of these various cells. One such factor to have a known modulating influence is testosterone. Its potent erythrogenic capabilities have been known for many years. Testosterone has been used in treatment of patients with aplastic anemia (47), multiple myeloma (48), pure red cell aplasia (49), chronic lymphocytic leukemia (50), namely, those diseases in which anemia was a major symptom. The mechanism of this erythropoietic action has been proposed to be, at

Figure 1. Model for the differentiation of bone marrow stem cells along myeloid and lymphoid pathways.

least in part, through stimulation of erythropoietin production by the kidney (51). Elevated levels of serum erythropoietin following testosterone have been reported (52). Interestingly, Jepson (53) has postulated that testosterone may first influence bone marrow stem cells towards the erythropoietic pathway and increase the number of erythropoietin-sensitive precursors. This would propose a combined or concerted activity of two potent erythropoietic agents, testosterone and erythropoietin, acting at different stages of red cell formation.

In seemingly conflicting results, removal of endogenous testosterone by orchidectomy followed by lethal irradiation and bone marrow reconstitution has been shown to increase the uptake of ^{59}Fe by the spleen (3). The role testosterone plays in erythropoiesis is clearly a very complex one and not well defined, but Morton (personal communication) has speculated that the sex hormones have differing effects on marrow and splenic pluripotential stem cells.

In 1970, Byron (54) studied the effect of single doses of various steroids on the cycling of bone marrow stem cells. Using high specific activity ^3H -thymidine lethal to replicating cells, he determined the per cent cell loss of femur marrow cells following steroid treatment. Cell loss was assayed by comparing spleen colony numbers in irradiated recipient mice. He found that the greatest sensitivity to killing was in those cultures which had received testosterone propionate, etiocholan-17 β -ol-3-one, and to a lesser extent 19-nortestosterone phenylpropionate. Consequently, he proposed that these three steroids can trigger bone marrow cells from the resting G_0 phase into the S phase of DNA replication, an initial event in cellular division.

As previously mentioned, Fujii (37) was able to show a depletion of the lymphocytic areas of thymus, spleen and lymph nodes, and a decrease in the numbers of antibody-producing cells to sheep erythrocytes in testosterone-treated, irradiated, and marrow reconstituted mice, effects not seen in irradiated, steroid untreated, controls. Furthermore, the most pronounced suppression of the plaque-forming cell response produced by testosterone occurred when the regimen of hormone injection followed a daily pattern up to seven days after irradiation (55).

This period immediately following irradiation is the time in which a rapid regeneration of the cellular components is taking place. They concluded that testosterone may act to alter the differentiation of stem cells at the expense of the lymphocytic series. Thus, histologically, impaired lymphocytic regeneration would be expressed as depletion of lymphocytes in those areas normally populated by this cell. A decrease in B-lymphocyte and plasma cell precursors could be manifest in a decrease in the plaque-forming cell response.

In a study done by Porcellini (56), the in vivo effect of testosterone enanthate on bone marrow cells enclosed in intraperitoneally-implanted diffusion chambers was followed. Female recipients previously received a single intramuscular injection of 2.5 milligrams testosterone enanthate in sesame oil. By day three following implantation of the chambers, an increase in the total cellularity and, differentially, an increase in granulocytes and macrophages was observed as compared to implanted, steroid-untreated, controls.

However, conflicting evidence has also appeared in the literature. Gonadectomized males, lethally irradiated and marrow reconstituted, have been shown to afford an environment of enhanced spleen colony formation significantly higher than intact males and females (3).

In an in vitro study, Francis (57) showed that human peripheral blood leucocytes can be stimulated by testosterone to release some factor into the medium. This conditioned medium, when added to cultures of bone marrow cells, stimulated an increase in the numbers of aggregate cell colonies in the culture.

The mechanism by which this hormone may stimulate or modify the maturational direction of stem cells has been the purpose of investigations seeking a receptor for testosterone in bone marrow cells. Minguell and Valladares (58) were unable to isolate a cytoplasmic receptor for testosterone in marrow cells. They did, however, confirm the existence of a nuclear receptor. Minguell and Grant (59) then showed that testosterone itself and not 5 α -dihydrotestosterone (5 α DHT), its major metabolite in androgen-dependent tissues, will stimulate RNA synthesis as evidenced by incorporation of labelled formate into RNA-adenine. This was confirmed by Byron (60) in a study of the Tfm mouse mutant. This mutant mouse strain lacks the receptor for 5 α DHT. When multiple doses of this hormone were administered, incorporation of ⁵⁹Fe was not stimulated, but the expected increase in committed granulocyte and macrophage precursors was not blocked. Therefore, while erythropoietic stimulation seems to be mediated through the metabolite 5 α DHT, the selective differentiation of marrow stem cells to granulocyte/macrophage precursors is a result of testosterone itself.

In summary, evidence seems to indicate that testosterone may have its greatest effect in modulating the differentiation of bone marrow stem cells along the myeloid pathway with a competitive decrease in cells of the lymphocytic lineage. The mechanism involved may include a nuclear receptor for testosterone which influences DNA and RNA synthesis.

Enhanced red cell maturation seems to be mediated by the testosterone metabolite, 5 α DHT. A serum factor liberated by steroid-activated peripheral leucocytes may also be involved in directing stem cell replication and differentiation along the myeloid pathway.

The present study was designed to test the hypothesis that androgen-induced immunosuppression is mediated by the preferential differentiation of stem cells along the myeloid pathway resulting in a loss of antibody-producing cells.

CHAPTER III

MATERIALS AND METHODS

EXPERIMENTAL ANIMALS

The animals used in these experiments were male and female mice of the Balb/c, DBA/2 and autoimmune NZB strains, and (NZB X DBA/2)F₁ and (Balb/c X DBA/2)F₁ hybrids. NZB breeders were obtained in 1966 from Otago University Medical School, Dunedin, New Zealand where they were in their fiftieth generation. Approximately 15 generations have been bred in Dr. Siegel's laboratory since that time. DBA/2 and Balb/c breeders were obtained from Jackson Laboratories in Bar Harbor, Maine. All mice were allowed to feed ad libitum on Purina Lab Chow except where x-irradiation had been used. In that case mice were fed Purina Mouse Chow and drank water adjusted to pH 2.4 to minimize pseudomonas infection.

TESTOSTERONE ADMINISTRATION

In initial experiments, testosterone propionate, 250 micrograms, (United States Biochemical Corporation) was incorporated in 0.2 milliliters corn oil and injected subcutaneously daily for a period of several weeks depending on the experiment. Corn oil alone was used as a control.

Later, testosterone propionate was delivered through silastic implants (61,62). These implants were prepared as follows: Medical

grade silastic tubing, outer diameter 0.125 centimeters (Dow Corning Corporation), was cut into two centimeter lengths, filled with 20 milligrams testosterone propionate powder and then sealed with soft silicone rubber medical adhesive, Silicone Type A. Overnight air drying was sufficient for proper hardening of the soft compound.

CASTRATION AND IMPLANTATION

Castration was performed through a scrotal incision. The testes were removed and blood vessels sealed with applied pressure. A stitch with 5-0 silk suture closed the wound.

The silastic tubes were implanted subcutaneously via a small incision in the lower back. The incision was closed with one stitch of 5-0 silk suture. In the initial experiments the implantation was delayed one week after castration. However, in later experiments both were performed on the same day.

The anesthetic used for all operations was ketamine hydrochloride (Bristol Laboratories). This was diluted 1:10 with 0.85 per cent saline and given at a dose of approximately 0.25 cc per 20 grams body weight. The drug was administered intraperitoneally using a one milliliter syringe with a 25 gauge needle. After approximately 10 minutes, mice became limp and were taped with masking tape on an operating board for surgical procedures.

X-RAY TREATMENT

One week prior to irradiation, all animals were switched from tap water to acid water, 0.006 molar HCl, pH 2.4. Irradiations were

performed with an x-ray unit (PickerVanguard) operated at 277 kV peak and 15 MA with added filtration yielding a half-value layer equivalent to 1.9 mmCu. The exposure rate averages 71 R per minute. Mice are exposed in plastic containers mounted on a turntable which is rotated during exposure.

Immediately following irradiation each mouse was given a 0.1 cc subcutaneous injection of the antibiotic Garamycin (Schering Pharmaceutical Corporation), at a dilution of 1:10 in 0.85 per cent saline. The antibiotic was continued daily for two weeks. At the end of the two week period, the animals were also taken off the acid water and returned to tap water.

IMMUNIZATION

Antibody-producing capability was assayed following immunization with sheep erythrocytes. The erythrocytes, preserved in Alsevers' anticoagulant, were obtained from Prepared Media Laboratory, Tigard, Oregon. For each immunization an aliquot of the cells was washed three times in phosphate buffered saline, pH 7.5, by centrifuging at 1600 rpm for 10 minutes. The buffered saline was then aspirated and the remaining cells diluted 1:10 with 0.85 per cent saline. Each mouse received 0.2 cc intraperitoneally. This has been calculated to represent a red cell dose of approximately 5×10^8 cells. The hemolytic plaque assay was performed on the fourth day following immunization on all strains except the NZB and (NZB X DBA/2) F_1 hybrid. Assay of the NZB mice was performed on day five since this had been previously determined to be the day of peak antibody response.

HEMOLYTIC PLAQUE ASSAY

Enumeration of antibody-producing cells was determined by the hemolytic plaque assay of Cunningham and Szenberg (63). Mice were sacrificed by cervical dislocation, spleens removed and weighed, and placed on ice. They were then crushed through sterile wire screens of 0.2 millimeter mesh to obtain single cell suspensions. Physiosol irrigation solution (Abbott Laboratories), pH 7.4, was used to flush the screens and suspend the cells. The cells were then centrifuged at 1700 rpm for 20 minutes. The supernatant was removed by aspiration and the spleen cell pellet resuspended in one or two milliliters of the physiosol irrigation solution depending on anticipated response. Clean and dry microculture plates were used to combine medium, complement, antigen and spleen cells. To each well was added: 3 drops (each drop was standardized to approximately 0.15 ml) of 10X Medium 199 diluted 1:10 with distilled water and adjusted to neutral pH with 7.5 per cent sodium bicarbonate, one drop of guinea pig serum (complement) diluted 1:3 with diluted medium, one drop of washed sheep erythrocytes diluted 1:5 in phosphate buffered saline, and 0.1 ml of the spleen cell suspension. Each well was mixed and added to individual culture chambers. Chambers were prepared by pressing two microscope slides back to back with double-coated tape (Scotch Brand) affixed to the center and at each end. This created two chambers holding a total volume of 0.85 ml. After samples were introduced into the chamber with a pasteur pipet, the edges were sealed by dipping in hot paraffin. Each sample was then placed in a 37 degree centigrade incubator for 30

minutes. The plaques, consisting of round clear areas resulting from red cell lysis, were counted by hand lens magnification if the total number per slide was between 50 and 200. If counts were greater than 200, further dilutions of the spleen cell suspensions were prepared, the samples replated and incubated, and the plaques recounted. Each sample at the final dilution was plated in triplicate, the average of the three counts used in computation of total plaque-forming cells per spleen and plaque-forming cells per milligram spleen weight.

ORGAN WEIGHTS

In addition to the spleen, the thymus, both kidneys and seminal vesicles (uteri in females) were weighed on a Mettler Type H6 balance in order to assess the androgenic effects on these tissues. This assessment also served to confirm that the animal had absorbed the hormone from the implant since androgen is known to cause lymphoid tissue involution, and seminal vesicle and renal enlargement.

BLOOD

Blood samples were collected in heparinized microcapillary tubes (Scientific Products) by puncturing the retroorbital plexus. The samples were spun in an International microcapillary centrifuge for three and one half minutes and packed cell hematocrits read on an International microcapillary reader. When appropriate, the plasma from these samples was collected and assayed for antinuclear antibodies as described below.

In some experiments, blood was also obtained for hematological study. Total white cell numbers were counted using a hemocytometer

and slides stained with Giemsa for differential readings.

ANTINUCLEAR ANTIBODY ASSAY

Antinuclear antibodies in the serum were detected by an indirect immunofluorescence assay involving a fluorescein-conjugated goat anti-mouse immunoglobulin antibody following incubation of test mouse plasma samples with chicken erythrocyte nuclei as antigen.

The chicken cell nuclei had been isolated as described by Ten Veen and Feltkamp (64) and were kept in the laboratory as a stock suspension in 10 per cent neutral buffered formalin, pH 7.0. An aliquot of this stock solution of nuclei was washed three times with phosphate buffered saline and diluted 1:30 for use.

On a clean, dry microscope slide single drops of the nuclei were placed at four sites along the length of the slide within approximately one centimeter of each other. The slides were then air dried for at least 45 minutes to fix the nuclei to the slide.

Test mouse plasma samples were diluted 1:20 by adding five microliters of plasma to 0.1 milliliters of warm phosphate buffered saline (PBS). The chicken cell nuclei fixed to the slides were rehydrated by placing the slide in warm PBS for 10 seconds. After wiping the slide dry around the nuclei, a drop of a diluted plasma sample was added to each area of nuclei by a nine inch pasteur pipet. The slides were then placed in closed plastic containers containing moistened paper towels and incubated at 37 degrees for 30 minutes. Following incubation, the slides were placed in Wheaton jars with slide racks containing warm PBS for five minutes. Three serial transfers to fresh PBS were made in

order to wash the slides. After wiping the slides dry around the test spots, one drop of fluorescein-conjugated anti-mouse immunoglobulin antibody, at a 1:30 dilution with PBS, was added to each sample. Slides were then incubated again for 30 minutes. The slides were washed three times again as previously described, followed by a final washing in distilled water. The slides were wiped dry around the spots and allowed to completely air dry. Cover slips were added over a thin layer of buffered glycerol, pH 8.5. Slides were examined with a Leitz fluorescent microscope. The light source was an HB200 (Osram, Germany) high pressure mercury lamp and a primary B612 and an OG1 barrier filter. The intensity of fluorescence was rated on a 0 - 4+ scale with reactions of 2 or greater considered positive.

STATISTICAL EVALUATION OF RESULTS

Most of the data were evaluated using the student t test for significance. Statistically significant results were those with p values less than 0.05. However, in cases where more than two groups were compared, analysis of variance was performed followed by Scheffe's test for significance. The p values are recorded in the bottom of the corresponding table. Statistically significant results were those with p values less than 0.05.

CHAPTER IV

RESULTS

ORGAN WEIGHTS AND ANTIBODY PRODUCTION

Tables I and II present the effects on selected organ weights and antibody formation in the male DBA/2 and NZB mouse strains seven weeks following sham or testosterone implantation and castration. As might be expected, the organ most dependent on testosterone for its integrity is the seminal vesicle. Castration plus the sham implant resulted in a marked decrease in the weight of this organ. Those castrated mice that had received the testosterone implant showed a slight increase in seminal vesicle weight but was not significant compared to normal, intact mice.

Kidney weights also responded to treatment in both strains. The combined weight of both kidneys decreased with castration and increased with the testosterone implant. The thymus responded to treatment as anticipated; hypertrophy resulted from castration alone, while marked involution occurred with testosterone treatment. While castration alone produced a significant increase in spleen weight compared to both intact and testosterone-treated groups of the DBA/2 strain, testosterone administration had no significant effect compared to intact males. However, manipulation of testosterone levels had no significant effect on spleen weights of the NZB strain. DBA/2 mice did not show any

TABLE I

ORGAN WEIGHTS AND ANTIBODY FORMATION IN DBA/2 MALE MICE SEVEN WEEKS
AFTER CASTRATION AND IMPLANTATION OF TESTOSTERONE^a

Treatment	Sem. Ves.(mg) ± S.D.	Kidney wt(mg) ± S.D.	Thymus wt(mg) ± S.D.	Spleen wt(mg) ± S.D.	PFC/Spleen ± S.D.	PFC/mg Spleen ± S.D.
DBA/2-Control	152.5 ± 24.2	472.4 ± 18.4	29.8 ± 7.1	111.9 ± 6.7	105,258 ± 46,562	955 ± 461
Castrated + Sham	7.5 ± 4.5	303.1 ± 23.2	36.6 ± 6.4	142.2 ± 14.9	96,257 ± 32,261	666 ± 152
Castrated + Test.	187.2 ± 42.1	594.2 ± 28.4	13.3 ± 9.4	119.4 ± 11.4	104,181 ± 41,070	861 ± 282
Statistical Evaluation						
Control vs. Castrated Sham	p < .001	p < .001	p < .2	p < .005	p > .5	p < .2
Control vs. Castrated Test.	p < .2	p < .001	p < .025	p < .2	p > .5	p > .5
Castrated Sham vs. Castrated Test.	p < .001	p < .001	p < .005	p < .025	p > .5	p < .2

^a All mice were two months of age at the time of castration. Two months later they were implanted with either the testosterone or sham implant. After seven weeks the animals were sacrificed and the hemolytic plaque assay performed. Data represent 3-6 mice per point.

TABLE II

ORGAN WEIGHTS AND ANTIBODY FORMATION IN NZB MALE MICE SEVEN WEEKS
AFTER CASTRATION AND IMPLANTATION OF TESTOSTERONE^a

Treatment	Sem. Ves.(mg) ± S.D.	Kidney wt(mg) ± S.D.	Thymus wt(mg) ± S.D.	Spleen wt(mg) ± S.D.	PFC/Spleen ± S.D.	PFC/mg Spleen ± S.D.
NZB- Control	104.6 ± 19.7	426.4 ± 29.9	24.6 ± 5.7	192.4 ± 62.2	623,343 ± 58,376	3399 ± 690
Castrated + Sham	7.4 ± 2.3	349.6 ± 40.4	51.0 ± 8.9	143.7 ± 10.4	490,675 ± 274,401	3363 ± 1849
Castrated + Test.	244.9 ± 129.6	587.6 ± 83.9	8.6 ± 2.3	154.8 ± 36.9	326,435 ± 60,087	2429 ± 715
Statistical Evaluation						
Control vs. Castrated Sham	p < .001	p < .025	p < .005	p < .2	p < .4	p > .5
Control vs. Castrated Test.	p < .1	p < .025	p < .01	p < .4	p < .005	p < .2
Castrated Sham vs. Castrated Test.	p < .025	p < .005	p < .001	p > .5	p < .4	p < .5

^a All mice were two months of age at the time of castration. Two months later they were implanted with either the testosterone or sham implant. After seven weeks the animals were sacrificed and the hemolytic plaque assay performed. Data represent 3-6 mice per point.

significant differences in the numbers of antibody-producing cells per spleen or per milligram spleen as determined by the hemolytic plaque assay following either castration or castration plus testosterone supplementation. The castrated, testosterone-treated NZB male did, however, show a significant suppression of antibody response per spleen compared to intact controls.

A group of intact NZB females which had been injected daily with testosterone dissolved in corn oil or corn oil alone as controls for 10 weeks displayed marked differences in all parameters (Table III). The kidneys increased in size with testosterone treatment as, remarkably, did the uteri. Although thymic involution, as in the males, was evident with testosterone treatment of females, the female spleen weights also decreased with hormone treatment, an effect not observed in the castrated, testosterone-treated males. The spleens of these animals also showed a small, but significant, decrease in antibody response compared to females receiving corn oil injections alone.

Because the mice in these experiments did not show striking differences in spleen weights or antibody formation, it was decided to evaluate these parameters in treated mice undergoing hemopoietic regeneration following sublethal irradiation. Table IV compiles the results of such an experiment using NZB females, sham or testosterone implanted, followed three weeks later by exposure to 550 rads of whole body x-irradiation. These data were collected six weeks after irradiation. Kidney weights were increased with testosterone treatment, while thymic weights were markedly decreased. No significant differences in uterine or splenic weights were observed. However, both the numbers of plaque-

TABLE III

ORGAN WEIGHTS AND ANTIBODY FORMATION IN NZB FEMALES
AFTER 10 WEEKS OF TESTOSTERONE INJECTIONS^a

Treatment	Uterus wt(mg) ± S.D.	Kidney wt(mg) ± S.D.	Thymus wt(mg) ± S.D.	Spleen wt(mg) ± S.D.	PFC/Spleen ± S.D.	PFC/mg Spleen ± S.D.
Corn Oil	150.1 ± 17.7 p < .025	320.5 ± 18.2 p < .001	54.6 ± 16.7 p < .005	158.8 ± 18.3 p < .005	128,462 ± 29,198 p < .01	813 ± 186 p < .1
Testosterone	210.5 ± 37.9	492.4 ± 36.9	16.4 ± 6.8	118.4 ± 10.8	75,696 ± 8,999	639 ± 42

^aThe first injection of 250 micrograms was given at two months of age and continued for 10 weeks. Data represent five mice per point.

TABLE IV
EFFECTS OF TESTOSTERONE ON ORGAN WEIGHTS AND ANTIBODY FORMATION IN
SUBLETHALLY IRRADIATED NZB FEMALES^a

Treatment	Uterus wt(mg) ± S.D.	Kidney wt(mg) ± S.D.	Thymus wt(mg) ± S.D.	Spleen wt(mg) ± S.D.	PFC/Spleen ± S.D.	PFC/mg Spleen ± S.D.
Sham- Implanted	92.2 ± 21.4	300.4 ± 46.1	63.1 ± 6.6	143.4 ± 4.9	90,000 ± 17,534	626 ± 109
	p < .4	p < .001	p < .01	p < .1	p < .005	p < .005
Testosterone- Implanted	106.5 ± 4.7	466.7 ± 37.2	18.0 ± 4.7	99.9 ± 27.9	17,778 ± 5747	177 ± 7

^aAll mice were two months of age at the time of implantation. Three weeks later both groups received 550 rads of x-irradiation and were sacrificed six weeks later. Data represent three mice per point.

forming cells per spleen and per milligram spleen were significantly suppressed in the testosterone-treated groups, by as much as four-fold.

Balb/c males and females were also examined using the experimental system involving hormone treatment and irradiation. Lymphoid organ weights and antibody formation in Balb/c males were examined five (Table V) and nine (Table VI) weeks following irradiation. At five weeks, the thymus weight changes followed the same pattern previously described for the mice in Tables I-IV; testosterone produced thymic involution, while castration resulted in hypertrophy. While there was no difference in splenic weights between the intact group and the castrated plus testosterone-treated group, the castrated, sham-treated males showed a significant increase in splenic weight compared to both. The number of plaques per spleen from the castrated, sham-treated males were significantly higher from both the intact and castrated plus testosterone groups. There was no difference between intact and testosterone-treated males. After adjusting for differences in spleen weight, the PFC's per milligram spleen were not significantly different between any of the three groups. At nine weeks, Table VI, the organ weights and antibody responses exhibited the same patterns evident at five weeks. Antibody responses per spleen were greatest in the castrated, sham-implanted males, while no significant difference was observed between intact and castrated, testosterone-treated mice. However, the number of PFC's per milligram spleen were significantly different when comparing castrated, sham- and testosterone-treated groups.

Balb/c females, either sham- or testosterone-implanted one day before radiation exposure, also showed significant differences in organ

TABLE V

EFFECTS OF TESTOSTERONE ON LYMPHOID ORGAN WEIGHTS AND ANTIBODY FORMATION
FIVE WEEKS AFTER SUBLETHAL IRRADIATION OF Balb/c MALES^a

Treatment	Thymus wt(mg)±S.D.	Spleen wt(mg)±S.D.	PFC/Spleen±S.D.	PFC/mg Spleen±S.D.
Intact	33.1±4.6	95.9±7.5	4822±4144	51±42
Castrated + Sham	69.8±3.9	158.5±11.1	15,278±3505	96±18
Castrated + Test.	10.5±3.9	95.0±7.2	2652±3187	26±30
Statistical Evaluation				
Intact vs. Castrated Sham	p<.001	p<.005	p<.05	p<.2
Intact vs. Castrated Test.	p<.005	p<.5	p<.5	p<.5
Castrated Sham vs. Castrated Test.	p<.001	p<.005	p<.01	p<.1

^a Castration and implantation was performed simultaneously when the mice were seven weeks of age. The following day all mice received 400 rads of x-irradiation. Data represent 3-4 mice per point.

TABLE VI

EFFECTS OF TESTOSTERONE ON LYMPHOID ORGAN WEIGHTS AND ANTIBODY FORMATION
NINE WEEKS AFTER SUBLETHAL IRRADIATION OF Balb/c MALES^a

Treatment	Thymus wt(mg)±S.D.	Spleen wt(mg)±S.D.	PFC/Spleen±S.D.	PFC/mg Spleen±S.D.
Intact	19.4±6.6	113.9±17.8	55,017±30,897	505±288
Castrated Sham	33.4±10.7	182.6±16.4	164,250±48,855	896±226
Castrated Test.	13.7±4.4	98.9±9.2	13,096±12,293	132±125
Statistical Evaluation				
Intact vs. Castrated Sham	p<.025	p<.005	p<.025	p<.1
Intact vs. Castrated Test.	p<.4	p<.2	p>.1	p<.1
Castrated Sham vs. Castrated Test.	p<.025	p<.005	p<.001	p<.005

^a Castration and implantation were performed simultaneously when the mice were seven weeks of age. The following day all mice received 400 rads of x-irradiation. Data represent 3-4 mice per point.

weights and antibody response (Table VII). At both five and eight weeks following irradiation, thymic and splenic weights were decreased with testosterone treatment. Significant suppression of PFC's per spleen was produced by the hormone at both five and eight weeks, however, the differences in PFC's per milligram spleen were not statistically significant.

In yet another experiment using (Balb/c X DBA/2) F_1 hybrid males and females, similar responses were observed (Tables VIII and IX). For the males (Table VIII), 10 weeks after irradiation, organ weights were modified as previously described for Balb/c strain mice (Tables V and VI). Here, numbers of PFC's per milligram spleen were significantly different in all experimental groups. Testosterone produced marked suppression of responses to sheep erythrocyte immunization, while castrated, sham-implanted males exhibited the greatest number of antibody-producing cells per milligram spleen.

Table IX demonstrates that numbers of PFC's per milligram spleen in the (Balb/c X DBA/2) F_1 females at both eight and 12 weeks after irradiation are significantly suppressed by testosterone treatment. Recovery from irradiation has continued to progress between eight and 12 weeks as evidenced by the increase in numbers of PFC's of both groups with time.

HEMATOLOGY

Total peripheral white cell counts and differentiation of blood leucocytes were also performed on castrated and testosterone-treated groups in order to determine whether changes occurred that could be

TABLE VII

EFFECTS OF TESTOSTERONE ON LYMPHOID ORGAN WEIGHTS AND ANTIBODY FORMATION IN SUBLETHALLY IRRADIATED Balb/c FEMALES^a

Treatment	Time after Irradiation	Thymus wt(mg) ± S.D.	Spleen wt(mg) ± S.D.	PFC/Spleen ± S.D.	PFC/mg Spleen ± S.D.
Sham-implanted		60.1 ± 1.2	130.0 ± 27.0	30,947 ± 12,147	235 ± 75
	5 weeks	p < .001	p < .05	p < .025	p < .1
Test.-implanted		20.8 ± 7.1	67.2 ± 27.6	3607 ± 2517	78 ± 91
Sham-implanted		35.3 ± 11.5	164.9 ± 20.3	17,708 ± 12,108	106 ± 68
	8 weeks	p < .001	p < .001	p < .05	p < .2
Test.-implanted		5.5 ± 1.8	82.8 ± 12.7	3946 ± 2511	50 ± 39

^a Implantation of sham and testosterone implants was performed when mice were seven weeks of age. Irradiation was given the next day at a dose of 400 rads. Data represent 5-8 mice per point.

TABLE VIII

EFFECTS OF TESTOSTERONE AND CASTRATION ON ORGAN WEIGHTS ANTIBODY FORMATION^a
IN SUBLETHALLY IRRADIATED ADULT (Balb/c X DBA/2)F₁ MALES

Treatment	Sem. Ves. wt(mg) ± S.D.	Kidney wt(mg) ± S.D.	Thymus wt(mg) ± S.D.	Spleen wt(mg) ± S.D.	PFC/Spleen ± S.D.	PFC/mg Spleen ± S.D.
Intact	66.2±7.4	415.6±40.0	27.1±14.1	97.7±6.9	33,375±4664	341.5±40.5
Castrated Sham	4.2±0.5	285.6±11.7	46.7±20.4	138.0±5.9	70,222±5004	510.0±53.7
Castrated Test.	81.5±15.5	578.6±80.6	26.3±20.8	81.5±13.8	15,633±8325	173.0±95.8
Statistical Evaluation						
Intact vs. Castrated Sham	p<.001	p<.005	p<.2	p<.001	p<.001	p<.025
Intact vs. Castrated Test.	p<.2	p<.025	p<.5	p<.5	p<.01	p<.005
Castrated Sham vs. Castrated Test.	p<.001	p<.005	p<.4	p<.005	p<.001	p<.01

^aMice were castrated at one month of age and implanted one week later. Irradiation was given at a dose of 400 rads two weeks subsequent to implantation. Data were collected 10 weeks after irradiation and represent 3-4 mice per point.

TABLE IX

EFFECTS OF TESTOSTERONE ON LYMPHOID ORGAN WEIGHTS AND ANTIBODY FORMATION
IN SUBLETHALLY IRRADIATED (Balb/c X DBA/2)_{F1} FEMALES^a

Treatment	Time after Irradiation	Thymus wt(mg) ± S.D.	Spleen wt(mg) ± S.D.	PFC/Spleen ± S.D.	PFC/mg Spleen ± S.D.
Sham-implanted	—	—	123.7 ± 17.1	30,600 ± 11,357	245.6 ± 81.8
	8 weeks		p < .01	p < .01	p < .01
Test.-implanted	—	—	86.4 ± 13.7	6152 ± 5,209	73.6 ± 58.9
Sham-implanted	23.7 ± 7.9	147.7 ± 25.7	53,867 ± 12,867	374.4 ± 120.0	
	12 weeks	p > .5	p < .2	p < .001	p < .01
Test.-implanted	26.2 ± 5.7	122.3 ± 13.4	17,375 ± 2,907	141.0 ± 12.0	

^a Implantation was performed on one month old females which were irradiated two weeks later with a dose of 400 rads. Data represent 4-5 mice per point.

correlated with observed changes in lymphoid organ weights and immune capacity. Initial studies using castrated male mice compared to intact controls demonstrated that castration can modify blood cell counts. Table X shows the lymphocyte and polymorphonuclear leucocyte (PMN) counts of castrated three month old DBA/2 and NZB males castrated at two months of age compared to their intact litter mates. Lymphocyte counts of both intact and castrated DBA/2 males were comparable. However, castration produced a significant decrease in the numbers of PMN's. Interestingly, in the NZB strain a small, but significant, increase in the lymphocyte count followed castration, whereas no difference was observed in the numbers of PMN's between the two groups. An observable decrease in the red cell population following castration was also noted in the NZB strain, but not detectable in the DBA/2, with average hematocrit values of 44 per cent for the castrated and 47 per cent for the normal mice. No differences in numbers of total white cell counts, monocytes, or eosinophils were observed in either strain.

Figure 2 compares the lymphocyte and PMN numbers of the hormone-treated and irradiated Balb/c males and females whose lymphoid organ weights and PFC responses were previously described in Tables V-VII. All mice received 400 rads of whole-body x-irradiation. At seven weeks following irradiation, the male intact control group shows an approximate PMN:lymphocyte ratio of 3.5:4.5. The castrated, sham-implanted males exhibited a significant elevation in lymphocyte count, but no evident difference in the absolute number of PMN's compared to the irradiated controls. This suggests that physiological levels of testosterone in the male delays the post-irradiation recovery of the lympho-

TABLE X

EFFECTS OF CASTRATION ON MALE NZB AND DBA/2
WHITE CELL POPULATIONS^a

Treatment	No. of Mice	No. WBC $\pm$ S.D.	No. PMN $\pm$ S.D.	No. Lymphocyte $\pm$ S.D.
NZB-	Intact	4	4900 $\pm$ 1061	1547 $\pm$ 612
			$p < .5$	3111 $\pm$ 635
	Castrated	8	5775 $\pm$ 2303	1965 $\pm$ 456
			$p < .4$	3977 $\pm$ 547
<hr/>				
DBA/2-	Intact	6	17,133 $\pm$ 2989	5047 $\pm$ 1446
			$p < .5$	11,513 $\pm$ 2614
	Castrated	9	17,333 $\pm$ 2733	2701 $\pm$ 730
			$p < .1$	13,893 $\pm$ 2140

^aMice were castrated at two months of age. Data were collected two weeks after castration in the NZB strain and four weeks after castration in the DBA/2 males.

Figure 2. Effects of testosterone on hematological recovery of Balb/c males and females seven weeks following sublethal irradiation (400). Data represent 4-8 mice per point.

cyte population. Testosterone treatment, with pharmacological doses, following castration resulted in a reversal of the PMN:lymphocyte ratio (3:1) seven weeks after radiation exposure. The PMN's were significantly higher than for both the castrated, sham-implanted males and control groups, and the lymphocyte count was significantly decreased. Sham-implanted, age-matched, females seven weeks after irradiation produced a PMN:lymphocyte ratio of approximately 1:2, values closely resembling those of the castrated males. Here again, testosterone treatment of intact females resulted in a reversal of the granulocyte:lymphocyte ratio (2:1). This was almost entirely due to a lymphocytopenia since a PMN elevation similar to that seen in the testosterone-treated male castrate was not observed here. No significant differences in hematocrit values averaging about 46 per cent, total white counts, monocytes, or eosinophils were observed between any of the male or female groups.

Figure 3 shows PMN and lymphocyte numbers obtained eight and 12 weeks following 500 rads of irradiation and testosterone treatment for the (Balb/c X DBA/2) F_1 hybrid females whose organ weights and plaque values were previously described in Table IX. Testosterone treatment resulted in a delay in lymphocyte recovery at eight weeks after irradiation with a concurrent increase in PMN's. By 12 weeks, this effect was no longer evident and no statistical differences were observed between the sham- and testosterone-treated groups suggesting that the effect on the peripheral blood picture of a single implant of testosterone had worn off. It should be recalled (Table IX) that at 12 weeks post-irradiation, immunosuppression was still evident in the testosterone-treated group.

Figure 3. White cell counts in testosterone- and sham-treated (Balb/c X DBA/2)F₁ females following sublethal irradiation (500R). Data represent 8-10 mice per point.

Otherwise untreated male and female (Balb/c X DBA/2) F_1 hybrids also showed innate sex differences in hematological recovery following 400 rads of x-irradiation (Figures 4 and 5). Normal PMN and lymphocyte numbers of untreated mice of both sexes were approximately 3000 and 9000 cells per cubic millimeter, respectively. At four weeks after irradiation, lymphocyte counts for both males and females were approximately 3000 cells per cubic millimeter. By nine weeks, lymphocyte numbers were further approaching normal and no significant differences between the males and females was observed. Although unirradiated normal males and females do show slight, but insignificant, differences in numbers of PMN's, there were significant differences in these cell numbers during recovery. Males consistently showed significantly higher numbers of PMN's at four, nine and 18 weeks post-irradiation.

ANTINUCLEAR ANTIBODY FORMATION

Previous work in this laboratory has not shown any sex differences in the development or levels of antinuclear antibodies (ANA) in the NZB strain. Furthermore, results of ANA assays performed over a period of time revealed no differences between castrated and control groups of male NZB mice. Orchidectomy had been performed on these mice at three months of age. Autoantibody levels rose evenly in both groups as the animals aged. Orchidectomy was not observed to precipitate ANA formation in non-autoimmune strain mice either. For example, no ANA was detected in (Balb/c X DBA/2) F_1 mice castrated at two months of age and assayed periodically over the next nine months.

Figure 4. Sex differences in polymorphonuclear neutrophil counts of five month old (Balb/c X DBA/2) males and females following sublethal irradiation (400R).

Figure 5. Sex differences in lymphocyte counts of five month old (Balb/c X DBA/2) males and females following sublethal irradiation (400R).

NZB females receiving injections of testosterone dissolved in corn oil or corn oil alone were also assayed periodically for ANA. Exogenous testosterone had no effect on ANA titers, which increased with age in a similar manner in both groups.

Comparable results were observed in irradiated, testosterone- and sham-treated NZB males. Here, testosterone treatment afforded no apparent protection against the development of antinuclear antibodies during recovery from x-irradiation.

CHAPTER V

DISCUSSION

ORGAN WEIGHT CHANGES

The seminal vesicles are, of course, dependent on androgen for their growth and maintenance throughout life. In all of these experiments, the size of the seminal vesicles was reduced to only a few milligrams after removal of androgen by orchidectomy. Testosterone supplementation acted to increase their size over that in intact controls. In the female mice, the uteri in many cases also increased in weight with testosterone supplement. Giannopoulos (65) has shown that there are testosterone-specific receptors in rat uteri which may mediate this effect.

The kidneys are also a target organ for testosterone as reflected in the fluctuations in weight observed here upon hormone treatment. Testosterone supplements were capable of stimulating large increases in size and weight. And, since castration resulted in significant decreases in kidney weights, androgen would appear to play a role, not only in stimulation of growth, but also in the continued maintenance of normal kidney size and weight. In this regard, normal NZB female kidneys were significantly smaller ($p < .025$) than kidneys of NZB males. But, receptors for testosterone also appear to be present in female kidneys (66), since kidney weights increased in females treated with

testosterone. This may, in part, be due to the androgen-induced production of elevated levels of erythropoietin by the kidneys (51,52).

It has been known for several decades that steroid treatment will produce thymic involution (13,14,15). Our experimental data are in agreement with these early investigations. In vitro studies had suggested lymphocytolysis as a mechanism of initial thymic degeneration (24,25,26). However, our hematological data showed no increase in peripheral smudge or degenerative cells in testosterone-treated mice. A viable explanation for this finding could be that our earliest time point for the collection of hematological data was not soon enough after hormone administration. Degenerative changes in thymic structure have been detected as early as six hours following a single injection of ACTH (22,23). Sustained thymic atrophy could be related to the observed decreases in peripheral lymphocyte numbers with testosterone treatment. The Balb/c males and females described in Tables V and VII exhibited significant differences in thymic weight recovery five weeks following irradiation. The lymphocyte recovery at seven weeks after irradiation paralleled the pattern of thymic weight recovery. In testosterone-treated male and female mice, both the thymic weights and lymphocyte counts were decreased, while in castrated, sham-treated males, both parameters increased. This parallel between thymic density and peripheral lymphocyte numbers during recovery from irradiation is to be anticipated, since T cells undergo their initial maturation in the thymus and then seed the periphery. Furthermore, Fujii (37) has shown that irradiation and a single 10 milligram injection of testosterone severely depletes T-independent regions of spleen, lymph nodes and

Peyers' patches. A parallel between B-cell regeneration and peripheral blood lymphocyte restoration would require examination of recovery patterns of cellularity of T-cell independent areas of the spleen and lymph nodes following irradiation. Quantitation of peripheral lymphocytes for relative numbers of T- and B-cells by assessment of the presence of theta antigen and immunoglobulin, respectively, would also clarify the question of relative rates of restoration of the different lymphocyte subpopulations and their sensitivity to testosterone. These are subjects for future investigation.

Thymus weight also appeared to vary with time after irradiation. The relative size of thymuses at eight and nine weeks after irradiation of the females and males, respectively, were smaller compared to corresponding groups at the five week interval (Tables V-VII). The larger weights observed at five weeks may be the result of an overshoot phenomenon associated with the initial regenerative burst of the marrow following irradiation. By eight or nine weeks, the rate of proliferation of the marrow, and hence the number of thymus-bound lymphocytes may have returned more to normal and thymus weights are decreased as mature thymocytes leave the organ.

Spleen weights were observed to be far more variable. Castration resulted in a consistent augmentation of spleen weight recovery following irradiation. But testosterone supplementation was, in many cases, unable to produce a corresponding decrease. A likely explanation may involve the complicating factor of erythropoiesis. The spleen is a site of active red cell formation in the mouse as well as being rich in immunocytes. As testosterone has also been shown to have potent

erythrogenic properties, any loss of splenic weight due to decreased lymphocyte numbers may not always be visible because of activated red blood cell formation.

ANTIBODY FORMATION AND RELATION TO STEM CELL DIFFERENTIATION

Most importantly, this study has demonstrated that testosterone can markedly suppress antibody formation under certain conditions. When testosterone was administered by silastic implants over a period of seven weeks to castrated DBA/2 and NZB males that had not been irradiated, it had a minimal effect on the numbers of plaque-forming cells in the spleen (Tables I and II). However, NZB females receiving daily injections of 250 micrograms of testosterone in corn oil for 10 weeks, showed a marked suppression of PFC response (Table III). Those differences may be due, in part, to length of time of testosterone treatment. The failure of testosterone and castration to produce marked changes in the male NZB and DBA/2 may have been related to mouse strain differences in responsiveness to these manipulations. Cohn and Hamilton did demonstrate strain differences in sensitivity to androgen (67).

In contrast, all the mice studied here, male or female, that received sublethal irradiation subsequent to hormone treatment were immunosuppressed. These results suggested that the immunosuppressive effects of testosterone may be mediated primarily by the bone marrow stem cell and its differentiation. These events are magnified during recovery from irradiation and have thus provided a very sensitive experimental system for discerning the influence of testosterone on the immune system. Hematological studies during radiation recovery showed that testosterone

can modify the peripheral lymphocyte population and result in decreased numbers of lymphocytes. Radiation treatment is primarily cytotoxic to stem cells of the bone marrow, but is also believed to cause selective depletion of B-cell dependent areas of spleen and lymph nodes. Mature thymocytes and neutrophils are relatively radioresistant (68). The effect of irradiation is, therefore, to produce a rapidly regenerating hemopoietic system. The hematology picture following irradiation would suggest that testosterone may be acting at the level of stem cell or committed progenitor cell. Byron (54) had shown that testosterone can initiate bone marrow stem cells into cycle. It has also been observed bound to a nuclear receptor in the nucleus of stem cells (58). It may be selectively stimulating the generation of erythrocytes and granulocytes, particularly neutrophils, at the expense of the lymphocytic series. This would indicate an hormonal modulation of stem cell differentiation and produce competition between the myelopoietic and lymphoid pathways. This mechanism could explain the increase in PMN's and the concurrent decrease in lymphocyte numbers following testosterone and radiation treatment. The selective loss of B-cells upon irradiation, combined with a diminished capacity for their regeneration produced by testosterone treatment could conceivably account for the suppressed antibody response. Without irradiation to produce an initial depletion of cells and stimulate rapid regeneration, testosterone may only act on the relatively slow, natural process of continual regeneration occurring in the adult animal. This may account for the lack of an observable effect of seven weeks of hormone treatment on the PFC response of the unirradiated DBA/2 and NZB mice. However, the NZB females that received

testosterone over a period of 10 weeks did show differences in antibody formation. Time could be an important factor in this case.

Even after the peripheral blood picture (Figure 3) and spleen and thymus weights (Table IX) had returned to normal, female (Balb/c X DBA/2) mice that had been irradiated and testosterone-treated 12 weeks earlier remained significantly immunosuppressed. This has suggested a possible selective sensitivity of some immunoreactive cell type to the influence of testosterone, particularly during its differentiation and maturation.

ANTINUCLEAR ANTIBODY FORMATION

No protective effects of testosterone on ANA titers in the autoimmune-prone NZB strain were observed, either in intact mice or in sublethally irradiated animals. It is interesting to note that plasma cells are thought to be radioresistant (68). It seems possible that although radiation and testosterone treatment have marked immunosuppressive effects on antibody formation to newly introduced antigens, previously active plasma cells would be unaffected by the treatment. However, an early post-irradiation drop in ANA titers followed by rapid recovery has been noted. This has suggested that the ANA-forming cell has a rapid turn-over rate, which may not be testosterone sensitive.

CHAPTER VI

CONCLUSIONS

Immunological tissues are receptive to androgenic influence, since testosterone produces marked changes in the lymphoid cellularity of the thymus and the peripheral blood, and, to a lesser extent, the spleen. Removal of hormone by castration produced an environment capable of enhancing lymphoid organ growth and cell production.

At the doses and under the amplifying conditions of sublethal irradiation, used in these experiments, testosterone could produce severe depression of antibody formation to sheep erythrocytes in the spleen. It also appears to selectively stimulate myeloid regeneration in irradiated mice at the expense of lymphocytopoiesis. It is not yet clear whether there is an inhibitory effect of testosterone on the differentiation and maturation of one or more lymphocyte subpopulations, or whether the effect is a passive diversion of a limited number of hemopoietic stem cells along myeloid pathways of differentiation.

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