HUMORAL IMMUNE RESPONDING OF THE ALBINO RAT DURING PHASES OF THE ESTROUS CYCLE

By

Kathleen J. Reid, H.B.Sc. ©

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Arts in Clinical Psychology

School of Graduate Studies

Lakehead University

Thunder Bay, Ontario

November, 1995

ISBN 0-612-09235-6

Canada

Abstract

The relationship between estrous phase and immune response in female rats was studied using two designs. In the between subjects design, groups of five rats were tested for antigen binding capacity and estrous phase on each of the ten days following secondary exposure to an HSA antigen. In the within subjects design, fourteen rats were tested for estrous phase at time of antigen exposure and for antigen binding capacity on each of the ten days following secondary exposure. Estrous phase was determined from microscopic examination of vaginal smears and phases were categorized according to high or low levels of estrogen and progesterone. Both designs showed a significant increase in immune response over the ten days. The within subjects design showed that higher estrogen levels at the time of antigen exposure were accompanied by elevated immune response. This finding shows that naturally occurring fluctuations in estrogen level at the time of a challenge to the immune system have an influence on the immune response.

ACKNOWLEDGEMENTS

I would like to express my appreciation to those faculty members at Laurentian University (Dr. Michael Persinger and Dr. Hermann Falter), whose support and guidance in the development of my research skills, were the original sources of inspiration behind this project.

I owe the successful completion of this undertaking to two faculty members of Lakehead University: Dr. Tony Thompson, who advocated for the continuance of this project following its prolonged political adversity, and Dr. John Jamieson, whose generous donations of time and effort as "surrogate" supervisor and whose statistical expertise, guidance and motivational talents contributed significantly to the completion of this project. These individuals represent a rare class of honorable academics who continue to operate with integrity and regard for fair treatment and ultimate success of the student.

Deepest thanks to Michael, who has been the source of great joy and inspiration in my life, and has sacrificed his own peace to move me to be my very best.

Finally, I dedicate this long-belabored, very costly document and everything it represents to my parents, whose unconditional emotional and financial support have paved the way for my successes. Their sacrifices will always be remembered.

Table of Contents

	Page
Abstract	ii
Acknowledgements	iii
List of Tables	v
List of Figures	vi
Chapter I **Introduction**	1
The Immune System: A Brief Overview	1
The influence of Gender on Immunity	3
The Influence of Estrogen & Progesterone on Immunity	6
The Influence of the Estrous Cycle on Immunity	12
The Present Study: Design and Main Hypotheses	16
Chapter II **Method**	18
Animals	18
Methodological Overview	18
Immunization and Blood Collection	18
Estrous Phase Determination	19
Radioiodination of Protein Antigen (HSA)	20
Biochemical Analysis	21
Calculation of Antigen Binding Capacity (ABC)	21
Procedure	22
Data Analysis	23
Chapter III **Results**	24
Cross-sectional (Between Subjects)	24
Longitudinal (Within Subjects)	28
Chapter IV **Discussion**	32
References	37

List of Tables

Table		Page
1	**Summary Table:** Means, Standard Deviations of Log ABC and Numbers of Subjects, Categorized According to Progesterone/ Estrogen Levels At Time of Testing, Over the 10 Day Period of Immune Measurement (Between Subjects)	25
2	**ANOVA Summary Table:** Log ABC by Group, Progesterone and Estrogen Levels @ Time of Testing (Between Subjects)	26
3	**Summary Table:** Means, Standard Deviations of Log ABC Numbers of Subjects, Categorized According to Progesterone/ Estrogen Levels @ Day 0, Over the Ten Day Period of Immune Response Measurement (Within Subjects)	29
4	**ANOVA Summary Table:** Log ABC (repeated) by Progesterone and Estrogen Levels @ Day 0 (Within Subjects)	30

List of Figures

Figure		Page
1	Overall Mean Log ABC as a Function of Days of Immune Response Measurement (Between and Within Subjects)	27
2	Mean Log ABC by Estrogen Levels @ Day 0 Over the Ten Day Period Immune Response Measurement (Within Subjects)	31

Introduction

Female mammals characteristically demonstrate immunological responses which are superior to and more variable than that of males (Terres, Morrison, & Habicht, 1968; Kenny & Gray, 1971; Krzych, Thurman, Goldstein, Bressler and Strausser, 1979; Ahmed, Penhale and Talal, 1985; Schuurs & Verheul, 1990; Falter, Persinger and Reid, 1991). Such sexual dimorphism in immunological responding may be either phylogenetically or ontogenetically determined. That is, the source of the dimorphism may be related to predetermined factors such as genetic programming of structural/functional contributants to immunity, or factors arising as the organism develops, such as the hormonal events triggered by sexual maturity. Clarification is required surrounding the origin of the dimorphism.

The Immune System: A Brief Overview

The immune system is a complex physiological system that has been broadly categorized into two types of immune responses, specific and nonspecific, involving different mechanisms of defense against invading, infectious organisms. Nonspecific immune responses, which are characterized as innate, are present prior to exposure to the invading organism and are indiscriminant. Specific immune responses, which are characterized as acquired, are induced or initiated by the presence of invading organisms and other molecules, known as antigens. Antigens are molecules or parts of molecules which induce a specific immune response. Specific immunity has been subdivided into two response systems according to the types of immune cells involved in a given immune response. These subdivisions are referred to as cellular immunity, involving immune cells called T-cells, and humoral immunity, involving immune cells called B-cells. When activated by the presence of an antigen, the humoral system produces protein molecules known as antibodies, or

immunoglobulins. Antibodies bind to antigens and produce a complex which triggers other cells of the immune system called phagocytes, macrophages and the complement system of proteins, to eliminate the antigen by destroying it (Grossman, 1984; 1985).

Numerous quantitative measures of humoral and cellular immune function have been developed. There are a number essential steps in quantification of an immune response, that are shared by all measurement systems. First, the immune system must be prompted to respond by administration of an antigen. The most common types of antigens include foreign proteins and cells, such as human serum albumin (HSA) and sheep red blood cells (SRBC) in rodents, and bacteria such as *E. Coli* or *Brucella.* Second, the immune response elicited by antigen administration must be measured. The nature of this measurement depends on the type of antigen used and the particular component(s) of the immune system that are stimulated by the antigen. Two commonly used assays, or measurement systems, of humoral immunity are radioimmunoassay (RIA) and hemolytic plaque assay (HPA). The RIA technique involves the principle that antigen (Ag) - antibody (Ab) binding are in dynamic equilibrium, as follows: Ab + Ag $\Leftrightarrow$ Ab·Ag. A mixture of radioactively- labeled antigen with antibody containing serum and known or unknown amounts of unlabeled antigen is incubated to allow antigen and antibody to reach binding equilibrium. The bound antigen is then separated from the unbound antigen. The amount of radioactivity of the bound antigen then provides a direct measure of antibodies in the serum. The HPA technique involves the use of red blood cells (RBC), or erythrocytes, which bind to antibody-producing cells, or lymphocytes, when incubated in a supportive medium with the lymphocytes. The addition of the cell-lysing protein, complement, then produces areas of cell lysis, or "plaques" in the medium surrounding the antibody-producing cells. Measurement of responses of T-cells and B-cells to substances called mitogens is another commonly used technique used to measure immune function. Mitogens, which are groups of

plant substances, stimulate immune cells by inducing cell division. The mitogen phytohemagglutinin (PHA) stimulates T cells, whereas the pokeweed mitogen stimulates B cells and T cells. This assay involves the addition of a known quantity of mitogen to a sample of immune cells, after which division of the immune cells is measured.

The Influence of Gender on Immunity

The 1960's and early 1970's heralded much of the seminal research that established the gender differences in immunity. Researchers explored the foundations of such differences using human and animal subjects. Terres, Morrison & Habicht (1968) observed and quantified differences in immune responses between male and female mice. They injected a foreign protein called bovine serum albumin (BSA), then measured the antibodies produced in response to the BSA antigen. The results indicated that females produced 10-85 times more antibody than the males, that female antibody responses were of longer duration and that females responded to smaller doses of the antigen than males. These findings suggested that differences existed in antibody levels between male and female mice. Terres *et al.* (1968) postulated that the differences they observed could have arisen from a greater efficiency of phagocytes in females. This explanation is consistent with the findings of Nicol (1932), (1935) and Nicol and Bilbey (1960), which showed that the action of phagocytes is greater in females and under the control of the sex hormone, or steroid, estrogen.

Rhodes, Scott, Markham and Monk-Jones (1969) examined the immunological responses of male and female rheumatoid arthritis patients, the patient's relatives, and a group of control subjects devoid of inflammatory disease. *Brucella* vaccine was administered to all three groups as a means of eliciting a measurable immune response. The results showed that in all groups the mean levels of antibody produced in response to the *Brucella* vaccine were higher in females.

Similarily, Michaels and Rogers (1971), examined the immune responses of two groups of infants, aged 6-9 and 11-14 months and assayed the blood samples for their reactivity to five *E. Coli* bacteria types. They found that the antibody levels, produced in response to the *E. Coli* bacteria, were higher for females in both age groups of infants. The greater antibody responses of infant females to bacteria, at least partially supports the phylogenetic origin of gender differences in immunity insofar as these subjects have not been exposed to the levels of circulating hormones associated with sexual maturation that have been hypothesized as a potential source of the sexual dimorphism in immunity.

Kenny and Gray (1971) examined the antibody responses of male and female mice to the bacteria *E. Coli*. In a series of experiments, the mice were infected with the *E. Coli* bacteria and antibody production by the spleen cells was measured using the hemolytic plaque assay. The results supported generally supported a better ability of female mice to respond immunologically, with the following observations: weanling female mice produced more antibodies than their male littermates, the antibody response of females, and not males, increased with age, female mice produced antibodies to a small amount of antigen for which the male mice were non-responsive, ovariectomized mice responded just as their sham-operated male counterparts to a small dose of antigen and estradiol administered to weanling male mice enhanced their antibody production. Additionally, administration of small doses of estrogen to weanling male mice enhanced antibody production. Kenny and Gray hypothesized that their results could be explained by the influence of female sex hormones on the immune system. They asserted that their observations of gender differences in immunity in sexually immature mice were consistent with the hypothesis, given the secretion of sex hormones in sexually immature mice. Similarily, Eidinger and Garrett (1972) examined the immune responses of male mice, female mice, and males castrated at 6 weeks of age. The responses to various

types of antigens were measured in order to quantify and compare different aspects of the immune response. Female mice exhibited greater antibody production than male mice. Castrated male mice demonstrated immune responding comparable to that of female mice, thus converting the males to females with respect to their immune responses. The investigators concluded that sex hormones influenced immune responding.

Krzych, Thurman, Goldstein, Bressler and Strausser (1979) evaluated the responses of immune cells from various immune system organs, including the thymus, lymph nodes, and spleen in newborn, weanling and young adult male and female mice. The results showed no gender differences in the immune response in the weanling mice. Gender differences in immunity observed in the young adult mice were assay-specific. The gender differences obtained were specific to the spleen. Krzych *et al.* (1979) concluded that the immune system organs exhibit a differential sensitivity to the influence of the sex hormones, and that this differential sensitivity underlies the sex-related differences in immunity. Additionally, the authors asserted that the direct relationship between strength of sex-related difference in immune response and age of the animal, strongly suggested a "parallel relationship between immune response and sexual maturity." (p. 2572). According to Krzych *et al.*, the superior immune responsiveness of females, dependent on or triggered by sexual maturation, could be attributed to the apparent immuno-enhancing properties of the female sex hormones, which become active at the time of sexual maturation.

Using male and female rats, Falter, Persinger and Reid (1991) examined antibody production in response to human serum albumin (HSA) antigen. The female rats showed higher and more variable antibody responses to HSA. Specifically, the females exhbited twice the antibody production of males, and a tenfold amplification of antibody production compared to males when the antigen activity of the HSA was enhanced by

the addition of rat spinal cord emulsion called Freund's adjuvant. Castration of post-weanling males failed to stimulate the level of antibody production exhibited by the female rats. The results with the castrated males ruled out testosterone as a factor mediating the immunological gender differences observed in rats. To test the potential influence of sex hormone fluctuation on antibody production in female rats, Falter *et al.* sacrificed the rats at the peaks and nadirs of their activity cycles, which cohere strongly to discrete stages of the estrous cycle. Antibody production was not altered according to peak or nadir of the activity cycle. The results supported a phylogenetic basis for immunological gender differences, given that measures and inferences of adult male and female gonadal hormonal fluctuations did not appear to mediate variable antibody production.

Collectively, the literature provides ample support for the immunological superiority of female rodents and humans relative to their male counterparts. Researchers have linked this superiority and greater variability to the influence of sex hormones, such as estrogen, that produce significant physiological changes at the time of sexual maturation (Krzych et al., 1979). However, the findings of other researchers suggests that fluctuating sex hormones in the adult female, associated with sexual maturation, are not the primary source of immunological gender differences, given the observations of sex differences in immunity prior to sexual maturation (Michaels & Rogers, 1971; Kenny and Gray, 1971).

The Influence of Estrogen & Progesterone on Immunity

Estrogen and progesterone have multifactorial and, in many respects, complementary roles in preserving the integrity and assuring the survival of the organism. Both hormones are involved in the maintenance of the reproductive cycle in mammals, exhibit cyclicity. Estrogen, an ovarian hormone, plays a role in cellular growth and proliferation, in addition to supporting the development of secondary sex characteristics of females. Progesterone, also an ovarian hormone, complements

estrogen by suppressing and limiting growth and proliferation of cells, except during pregnancy when progesterone acts to promote the growth and development of the fetus (Wade, 1971). Although in unique ways, both estrogen and progesterone contribute to survival and perpetuation of the species. During pregnancy in mammals, for instance, when progesterone levels are high relative to estrogen levels, some degree of maternal immunosuppression is beneficial and adaptive to the survival of the newly developing organism. Likewise just before ovulation, when estrogen levels are high relative to progesterone, it is of some advantage for the immune system to be alert to foreign material introduced by virtue of intercourse.

Whether estrogen produces immunofacilitation or immunosuppression appears to depend on the concentration of estrogen used in *in vitro* or administered *in vivo,* the timing of the injection of the hormone relative to activation of the immune response by antigen injection, the immune parameter measured and the developmental stage, and age of the organism. Kenny, Pangburn and Trail (1976), injected *E. Coli* bacteria into male weanling mice and measured antibody production by the spleen. To clarify the role of estrogen in modulating the antibody-producing process, 17β-estradiol, an estrogen analog, was administered at the time of injection of the *E. Coli* and following the development of an immune response to the *E. Coli.* The concentration of estradiol and the timing of administration were significant variables influencing the magnitude of antibody-production. Small concentrations of estradiol stimulated division of antibody-producing cells, whereas larger concentrations of estradiol suppressed division of antibody-producing cells. Administration of estradiol 1 day before and up to 3.5 days after injection of the *E. Coli* increased the numbers of antibody-producing cells, whereas administration of estradiol 2 days before the antigen had no effect. The differential effect of the timing of estradiol administration, was attributed to the relative serum levels of the

estradiol at the time of the antigen injection. Serum levels of estradiol slightly above the physiological range at the time of antigen delivery, as was obtained with injection of estradiol 2 days before antigen delivery, had no effect on antibody-producing cells. Administration of estradiol 1 day before and up to 3.5 days following injection of the antigen, however, increased numbers of antibody-producing cells.

To assess the effects of neonatal estrogen treatment on adult immuno-responsiveness, Kalland, Strand and Forsberg (1979) administered 17β-estradiol (10 μg) and diethylstilbestrol (DES) (0.01 μg, 0.1μg, 1 μg, 5μg), a synthetic estrogen, to 5 groups of female mice for 5 days post partum, with each group receiving a different dose. Treatment of neonatal mice with DES resulted in a protracted impairment of spleen cell responsiveness to mitogens, as far as 17 months later in DES treated females, compared to controls. Of all the DES doses, and including the estradiol, the 5 ug dose of DES was the only one which resulted in suppressed splenic mitogen responses post-infancy. DES (5μg) administered for five consecutive days from day 6 through 10 post-partum, did not influence the post-infancy splenic mitogen response. Luster, Boorman, Dean, Luebke, and Lawson (1980) administered 3 doses of diethylstilbestrol (DES), (0.2 mg/kg, 2.0 mg/kg, 8.0 mg/kg), to adult female mice and measured splenic antibody responses to SRBC antigen and found that only the 2.0 and 8.0 mg/kg doses of DES had reduced antibody levels relative to controls. Those mice that received lower doses of DES had antibody levels similar to controls. Luster, Hayes, Korach, Tucker, Dean, Greenlee, and Boorman (1984) provided further evidence of the potency and specificity of estrogen compounds in modulating immunity. They administered the compounds DES, 17β-estradiol, 5α-dienestrol, 5α-dihydro- testosterone (5α-DHT) and progesterone, classified by the investigators as estrogenic compounds, to adult female mice. Each group of mice was exposed to one of the five compounds for

five consecutive days. Macrophages and lymphocytes from the spleens of the mice, were quantified following exposure of the mice to the compounds described. The results showed a positive correlation between magnitude of influence on immune response and estrogenic activity. Specifically, both DES and 17β-estradiol activated lymphocyte and macrophage function of spleen cells, with DES the more potent activator. 5α-dienestrol also increased splenic macrophage and lymphocyte function, whereas 5α-DHT and progesterone had no effect on splenic macrophage /lymphocyte function. The results of all three studies support dose-dependent effect of exogenous, administered estrogens on cellular and humoral immune responses. Kalland, Strand and Forsberg (1979) demonstrate that the effect of exogenous estrogen is not only dose-dependent but dependent upon the maturity of the organism, and more specifically, the maturity of the organism's immune system.

Hu, Mitcho, and Rath (1987) investigated the effects of 17β-estradiol on the synthesis and secretion of interleukin 1 (IL-1), a chemical messenger in the immune system, in rats, and found an increased IL-1 synthesis by peritoneal macrophages in adult female rats relative to age-matched male and prepubescent female rat controls and increased IL-1 synthesis in ovariectomized female rats exposed to 17β-estradiol replacement therapy. Furthermore, treatment of male peritoneal macrophages with 17β-estradiol stimulated IL-1 synthesis at low doses and inhibited it at high doses, relative to controls. These results indicate that estradiol modulation of IL-1 synthesis by peritoneal macrophages may contribute to the greater immunological response observed in females.

Investigations exploring the immunomodulatory properties of progesterone are minimally represented in the literature relative to the documented immuno-modulatory properties of estrogen and related compounds. There is a significant amount of literature showing that progesterone suppresses immune function during pregnancy (Bratanov and Vulchanov, 1986), however the evidence demonstrating

the influence of progesterone in normal physiology is more mixed and less prevalent. An inference of this may be that the immunomodulatory role of progesterone is less obvious, masked by a possible co-immunomodulatory or synergistic function in which the more potent immunomodulator, estrogen, would dominate the immunomodulation.

Munroe (1971) injected monkeys with the *Rous chicken sarcoma* virus (RSV) and administered progesteroids, a group of compounds with progesterone activity, to groups of the monkeys as follows: progesterone (5 mg/kg), medroxyprogesterone (10 mg), nortestosterone (5-15 mg) and medrogestone (10 mg/lb). The latency for tumours to develop was measured for each group. Groups of pregnant monkeys and untreated control monkeys were also observed for latency of tumour induction. Thirty out of 34 progesteroid treated groups of monkeys, including 5 out of 5 pregnant monkeys, developed tumours, whereas 0 out of the 22 control monkeys developed tumours in response to inoculation with RSV. Munroe concluded that both exogenous, administered progesteroids and endogenous progesterone, as in the case of elevated levels of progesterone in pregnancy, have a potent immunosuppressive effects.

Wybran, Bogaert, and Govaerts (1977) examined the effects of the synthetic progesterone, lynestrenol, on *in vitro* human lymphocyte responses. Lymphocytes were obtained from the blood of normal adult male and female subjects. To quantify lymphocyte function, the lymphocytes were exposed to two types of mitogens, pokeweed mitogen and phytohemagglutinin (PHA), in order to measure B and T-cell responses. Lymphocytes were exposed to varying doses (0.1 μg/ml - 10^{-7} μg/ml) of synthetic progesterone for varying lengths of time, up to a maximum of 7 days. The results showed that lynestrenol did not modify the responses of the lymphocytes to either mitogen. However in the presence of PHA, the lynestrenol (10^{-3} to 10^{-1}) increased lymphocyte incorporation of a radioactively-labeled DNA

tracer, ^{3}H-thymidine. Although not directly involved in the modification of human lymphocyte responses to mitogens, lynestrenol seemed to activate lymphocytes in response to the T-cell specific mitogen, PHA, as indicated by the thymidine incorporation. Progesterone may then be involved in limited aspects of immune modulation, limited to amplification and not initiation of immune T-cell division.

In a subsequent examination of the effects of lynestrenol on immunity, Wybran and Thiry (1978) administered 1.0 mg/kg, 0.1 mg/kg and 0.01 mg/kg lynestrenol to groups of male and female newborn hamsters. The hamsters were inoculated with herpes simplex virus type 1 (HSV 1) one day after the beginning of lynestrenol treatment, and the development of subcutaneous tumours was monitored. The results indicated that the 1 mg/kg dose of lynestrenol delayed tumour growth in the cells affected by HSV 1. Another group of 4 week old hamsters were vaccinated 2 times at one week intervals with heat-killed HSV 1, treated with 1 mg/kg of lynestrenol from 2 days before the first vaccine to 2 days after the second vaccine, then challenged with viable HSV 1. The vaccination- facilitated tumour growth was inhibited in lynestrenol-treated hamsters. The results of delayed tumour growth appeared to suggest immunopotentiating properties of lynestrenol, while the data from the vaccinated hamsters appeared to suggest immunosuppressive activity. The authors reconcile this apparent contradiction by postulating that lynestrenol may indeed be immunosuppressive. Consistent with this, the immunopotentiating properties of lynestrenol can be accounted for by a mechanism involving the lynestrenol-facilitation of specific class of immune cells, called T-suppressor cells, which act to suppress immunity.

In summary, both natural and synthetic estrogens and progesterones have been shown to be immunoregulatory. The direction, extent, and nature of this regulation is likely dependent on a host of variables such as type of hormonal treatment, biochemical similarity of synthetic hormones to physiological hormones,

dose of hormonal treatment, timing of hormonal treatment relative to initiation of immune response, and parameter of immunity measured. Some evidence supports a synergistic role between progesterone and estrogen in modulating the immune system during normal, non-pregnant, physiological functioning. Estrogen clearly has very potent immunomodulatory properties, immunofacilitory for the most part, whereas progesterone may serve as a co-modulator under regular physiological conditions, possibly acting with subtle immunosuppressive properties, which become enhanced during specialized conditions such as pregnancy.

The Influence of the Estrous Cycle on Immunity

Collectively, the literature has demonstrated that estrogen and progesterone modulate immune function, estrogen to a greater extent, and that this capacity appears at the time of sexual maturation in the female, when the menstrual or estrous cycles become active. Serum levels of estrogen and progesterone fluctuate during the monthly reproductive (ovarian) cycle in female mammals, producing the menstrual cycle in humans and estrous cycle in rodents. Evidence that levels of estrogen and/or progesterone contribute to the superior and more variable female immune response, coupled with quantifications of estrogen and progesterone levels during the estrous cycle, such as those by Butcher, Collins and Fugo (1974), have prompted and assisted with investigations of the impact of estrous-related fluctuations in estrogen and progesterone on immune function.

One study that demonstrated the influence of the hormonal events associated with the estrous cycle on immune response was conducted by Black, Simon, McNutt and Casida (1953). The authors injected rabbit semen contaminated by *E. Coli* and *staphylococcus* bacterium into the uteri of two groups of rabbits: one group consisted of pseudo pregnant rabbits, in which there is no estrous as a result of an infertile copulation; the other group consisted of rabbits in the estrous, a fertile stage of the estrous cycle. The pseudo-pregnant rabbits showed pus formation in the

uterus in response to the infected sperm, whereas the rabbits in estrous did not. Rabbits treated with estrogen/progesterone or progesterone alone, developed an accumulation of pus in the uterus, whereas the rabbits treated with estrogen alone did not. These results suggest that under conditions where the influence of the ovarian cycle, and its associated hormonal correlates, is removed, as in the case of pseudo-pregnancy, the immune resistance is impaired. Accordingly, estrogen may promote immunofacilitation, thereby supporting the body's natural defense, while progesterone may inhibit this natural defense.

Nicol and Vernon-Roberts (1965) examined the influence of the estrous cycle and ovariectomy on immune function in female mice and rats. They measured activity in the reticuloendothelial system (RES), which is a population of immune cells located throughout the body in both immune-related organs and other tissues, that function to phagocytize invading organisms. Estrous stage was determined using a method based on examination of predominant types of epithelial cells obtained from a vaginal smear. The follicular phase (proestrous) was characterized by predominant round, nucleated epithelium, estrous was characterized by predominant cornified epithelium, the luteal phase (metestrous) was characterized by predominant cornified epithelium with the presence of leukocytes, or white blood cells, and diestrous was characterized by predominant round, nucleated epithelium and leukocytes. Nicol and Vernon-Roberts observed two peaks in the RES activity in both mice and rats during follicular and luteal phases of the estrous cycle, corresponding to proestrous and metestrous stages of the estrous cycle, respectively. Ovariectomy abolished the observed peaks of RES activity in mice. Without the benefit of quantification of hormone levels in mice and rats during stages of the estrous cycle, the authors assumed that the observed peaks in RES activity corresponded to peaks in serum estrogen in the rodents. This assumption was based upon their postulation that estrogen acted as the body's natural stimulant of

immunity. Later, Krzych, Strausser, Bressler and Goldstein (1978) measured the response of splenic lymphocytes to mitogens and splenic antibody production to SRBC antigen during the stages of the estrous cycle in female mice. The investigators knew from Butcher *et al.* (1974) that in the estrous cycle of the rodent, estrogen levels were highest during proestrous and diestrous phases whereas progesterone levels were highest during proestrous and metestrous phases. In their investigation, Krzych *et al.* (1978) showed that both mitogenic and antibody responses were elevated at proestrous relative to diestrous and at metestrous relative to estrous. Collectively, the findings of Krzych *et al.* (1978) and Nicol and Vernon-Roberts (1965) provide evidence of two peaks of immunoresponsiveness, occurring during proestrous and metestous phases of the estrous cycle. The peaks correspond to high estrogen/high progesterone (proestrous) and high progesterone/low estrogen (metestrous).

De and Wood (1990) analyzed the distribution of immune cells called macrophages through different stages of the estrous cycle in the mouse. The distribution of uterine macrophages changed from one stage to the next through the stages of the estrous cycle. In order to determine whether estrogen and progesterone were involved in producing these distributional differences, the distribution of uterine macrophages were analyzed in ovariectomized females. The investigators observed that the number of uterine macrophages was decreased relative to controls but could be restored with the administration of progesterone or estrogen. The results support the influence of the ovarian hormones estrogen and progesterone as having the capacity to affect number and distribution of immune system cells during the estrous cycle. Furthermore, Brown, Sundstrom, Komm, Teuscher, and Lyttle, (1990) investigated the hormonal regulation of complement (C3). Complement proteins comprise a complex system is activated by antibody-coated cells to lyse the antibody-coated cells. Uterine complement activity was

measured during stages of the estrous cycle. In a separate experiment, uterine C3 was measured following the administration of ovarian hormones according to the following protocol: estradiol alone, estradiol and progesterone, and progesterone alone and controls (untreated). The results showed that C3 activity was highest during the estrogen-dominated stages of the estrous cycle, namely, proestrous and estrous and lowest during diestrous. They also demonstrated that estradiol produced a progesterone-reversible increase C3 relative to controls, whereas the progesterone had no effect. The estradiol-induced increase in C3 could be reversed by the coadministration of progesterone. The findings of Brown et al. concur with those of De and Wood, who also observed immunomodulation by endogenous, estrous-associated ovarian hormones, corroborated by similar immune effects of externally administered ovarian hormones. Generally, the results support immuno-modulation by the ovarian hormones associated with the estrous cycle in rodents.

Mukherjee, Mastro, and Hymer (1990) demonstrated that in female rats the peptide hormone prolactin, which is normally secreted during pregnancy to promote the secretion of milk, induced the formation of receptors on spleen cells. The receptors were specific for IL-2, a specific chemical messenger in the immune system and the induction was dependent on estrous phase of the rat. Specifically, ovariectomized rats or rats in diestrous demonstrated a receptor-induction response to prolactin, while estrogen-treated ovariectomized rats or rats in estrous did not. These findings not only support Hu *et al.'s* (1987) observations that externally administered estrogen modulates the interleukins but that the resulting immunomodulation depends on the availability of circulating estrogen.

In addition to direct influence of naturally-occurring, estrous-associated estrogen and progesterone on immune function, there is evidence supporting the existence of a neuroanatomical pathway through which hormonally-determined immune regulation can occur. Pfaff and Keiner (1975), Rainbow, Parsons, MacLusky and McEwen (1982) and

Lipositis, Kallo, Coen, Paull and Flerko (1990) independently reported estrogen receptors in limbic and hypothalamic structures known to be involved in the central regulation of immunity (Jankovic and Isakovic, 1973; Schleifer and Shapiro, 1986; Besedovsky, Rey and Sorkin, 1985). Kelly, Moss and Dudley (1977) showed that infusion of 17β-estradiol into immuno- regulatory areas in the hypothalamus altered the electrical activity in an estrous-stage dependent fashion. The hypothalamic cells were electrically stimulated by 17β-estradiol during metestrous and electrically inhibited by 17 β-estradiol during diestrous, proestrous and estrous. Collectively, these data may indirectly support a mechanism whereby naturally-occurring circulating estrogen and progesterone associated with the estrous cycle may influence immunity. That is, estrogen and progesterone may influence immunity by their action in the central nervous system, as well as at the cellular level.

Although there is adequate support for the immunomodulatory properties of estrogen and progesterone, it remains unclear which phases of estrous are associated with enhanced or suppressed immune function, particularly for the modulation of antibody responses.

The Present Study: Design and Main Hypotheses

This study was designed to clarify what effects the estrous cycle exerts on antibody responding in intact female rats, and to determine if the estrous phases associated with higher levels of serum estrogen or progesterone had any differential impact on such responding. Relative levels of circulating ovarian steroids (estrogen, progesterone) were based on the findings of Butcher, Collins and Fugo (1974), who quantified levels of estrogen and progesterone during each phase of the estrous cycle in the rat.

Two designs were used. The immune response to be measured was initiated by two injections of a protein antigen called human serum albumin (HSA). The first injection of HSA acted to prime the rats, or set up an initial immune response. The

second (booster) injection, which typically results in an immune response that is more robust and measurable, was the response measured in this study. Because the immune response generally asymptotes around ten days following a booster injection of protein antigen, only the first ten days following the second injection were examined. The first design used a cross-sectional approach, in which different groups of 5 rats were tested on each of the 10 days following secondary exposure. This design yielded a direct measure of estrous phase at the time the immune response was being measured. The second design used a longitudinal approach in which the estrous stage at the time of secondary exposure was measured in 15 rats, and these same rats were repeatedly sampled for secondary immune response for the 10 days following exposure.

Based upon review of the literature, it was hypothesized that the estrous stages associated with high serum estrogen levels will increase antibody levels relative to those estrous stages associated with low serum estrogen levels, and that the estrous stages associated with high serum progesterone levels will decrease antibody levels relative to those estrous stages associated with low serum progesterone levels. In other words, it is hypothesized that estrogen will be immunofacilitating and progesterone immunosuppressing. The cross-sectional and longitudinal designs have been used to explore the dynamics of a possible interaction between estrous phase and antibody response. The cross-sectional and longitudinal designs were used to determine whether the estrous phase at the time of antigen exposure (cross-sectional), or at the time the antibody response is measured (longitudinal), has a greater influence estrous stage related immunomodulation.

Method

Animals

A total of 65 female Wistar rats (age 80 days) obtained from Charles River Suppliers, served as subjects. They were housed in standard wire cages (3/cage) in temperature and light (12:12 LD) controlled rooms, with food and water available ad libitum.

Methodological Overview

The method used in the study was adapted from previous investigations in which the current author was primary author and active researcher (Reid, Falter and Persinger, 1991; Falter, Persinger and Reid, 1991).

Immunization and Blood Collection

The animals were permitted ten days to habituate to housing conditions then inoculated subcutaneously in the right flank with an homogenized solution of 0.1 mL human serum albumin (HSA) (10 ug/mL) and 0.1 mL complete Freund's adjuvant (CFA). Eighteen days later, 100-200 uL of blood was removed from the caudal vein of each subject, which was followed by a second identical injection of HSA. The second HSA injection did not require CFA as an immune response facilitator because second exposures to antigens reliably produce a robust response. Blood collection involved immobilization of the subject using a plastic restraint cage, dilation of the vasculature by immersion of the tail in water at 50 °C for approximately 30 seconds, and withdrawal of blood using a 26.5 guage hypodermic tip. The blood was removed from the hypodermic tip using a pasteur pipette, placed in a Microtainer serum separator tube, to separate the antibody-containing serum from the cells, centrifuged at 3000 g for ten minutes, then stored at 4 °C for later analysis.

Estrous Phase Determination

Estrous phase was determined by microscopic examination of vaginal epithelium cells. The method for determining estrous phase by classification of predominant epithelial cell types was adapted from Long and Evans (1922) and Harris and Kesel (1990). Smears of vaginal epithelium were obtained by gently flushing the vagina with distilled water (35-40 °C) expelled from a smooth tipped dropper. Triplicate sampling was performed for each subject, smeared across a standard glass slide and then left to air dry. A 0.5 % Toluidine Blue (in 20 % ethanol) stain was used in a standard staining sequence of 20 % ethanol, stain, 40 % ethanol, 60 % ethanol, 80 % ethanol, 100 % ethanol (dehydration phase) and xylene (clearing agent). The slides were then left to air dry for subsequent examination. Phase of the estrous cycle was determined by microscopic examination of the slides. Rating criteria were according to the predominant epithelial cell type observed in the smears (as outlined by Nicol & Vernon-Roberts, 1965). Initial categorization of estrous stage included the four stages, proestrous, estrous, metestrous and diestrous stages, or phases. These four stages were incorporated in a 2x2 design with respect to high or low levels of estrogen or progesterone, since proestrous is associated with high estrogen and progesterone, estrous with low estrogen and low progesterone, diestrous, with high estrogen and low progesterone, and metestrous with low estrogen and high progesterone (Butcher et al. 1974). The data were collapsed as follows: diestrous/proestrous = estrogen high, estrous/metestrous = estrogen low, metestrous/proestrous = progesterone high, estrous/diestrous = progesterone low (Butcher et al., 1974).

Radioiodination of Protein Antigen (HSA)

Additional HSA antigen, to be used in the *in vitro* measurement of HSA antibodies, required labeling with a radioactive material in order to facilitate the identification of relative levels of HSA antibodies in the rat sera. A 6 mg/mL solution of freeze dried human serum albumin (HSA) dissolved in a sodium phosphate buffer, pH 7.2, 0.15 mol/L, was prepared immediately prior to the mixing of the following reagents in a single reaction vile, or small v-shaped tube: phosphate buffer, pH 6.5, 0.2 mol/L (50 μL), Enzymobead reagent (50 μL), Beta D-glucose solution (25 μL), NaI* specific activity 17.4 Ci/mg. Specific activity, measured in the unit Curie per unit mass, reflects the relative amount of radioactive disintegration per unit mass. A volume of 50 μL of the HSA solution was then added to the reaction vile, and the reaction was allowed to proceed for one hour at room temperature. The reaction was quenched, or brought to an end point, by applying the mixture to a G-25 superfine Sephadex gel filtration column.

The column was prepared in advance by adding 12 grams of G-25 superfine powder to phosphate buffer, pH 6.5, 0.2 mol/L (75 mL), which was gradually applied to the column and equilibrated by passing through a phosphate buffer. Prior to use of the column, nonspecific binding sites, which would inhibit the column from binding unlabelled HSA, were eliminated by passing through an HSA solution, and the flow rate was adjusted to 4-5 mL/tube/20 minutes. Samples (20 μL) of the fractions collected from the addition of the reaction mixture were then counted using a gamma scintillation counter to determine the location of the precipitated labeled protein (*I-HSA) (samples with highest cpm's).

Biochemical Analysis

The antigen binding capacity (ABC) of the rat sera was determined by modification of a method described by Farr (1958). Test serum (or appropriate dilutions of test serum) (20 μL), carrier serum from control animals (20 μL), 1.1 ug/mL *I-HSA (radioactively labeled) (20 μL), and phosphate buffer, pH 7.2, 0.15 mol/L (60 μL) were incubated for two hours at 37 °C. Phosphate buffered saline, pH 7.2 (380 μL) was added, followed by saturated ammonium sulphate. This was allowed to stand at room temperature for 15 minutes, then centrifuged at 3000 g for 30 minutes. The supernatant was discarded and the precipitate washed with saturated ammonium sulphate (50 %) (500 μL). This was followed by another centrifugation (room temperature, 3000 g, 10 minutes) and disposal of supernatant. The gamma emission of the resulting pellet was measured using a gamma scintillation counter.

Calculation of Antigen Binding Capacity (ABC)

In order to determine the antigen concentrations in unknown samples, namely the antisera from subjects, a standard curve was constructed as follows: for each series of dilutions, the percent of precipitate was plotted on a logarithmic scale against the inverse of the dilution. The dilution required to produce 33 % precipitate, reflecting a region of in the antigen-antibody reaction in which the antigen is in excess of the antibodies, was determined by graphical interpolation. The antigen binding capacity (ABC) expressed in μg/mL (serum), was calculated according to the formula:

$$ABC=(\mu g\ *I\text{-}HSA)\ (\%\ precipitate/100)\ (50)$$

Rat sera that gave less than 10 % precipitate were identified as non-responders and not included in the data. The *a priori* definition of a response (to the antigen) was an ABC of at least 0.11 μg/ml on the day of the baseline sample.

Procedure

Both cross-sectional (between subjects) and longitudinal (within subjects) groups received identical injection protocols: initial injection of antigen + adjuvant followed by a second injection of antigen alone, which produces the more robust secondary response that was measured in this study. The second injection of antigen, known as the booster injection, is typically administered as the primary response begins to attenuate, which is usually three weeks post primary injection (Falter at al., 1991), 18 days post-primary in the current investigation. The groups differed only with respect to the timing of vaginal epithelium and blood collection protocols.

Cross-sectional (between subjects) groups, which comprised 10 groups of 5 rats, were sampled (vaginal epithelium/blood collection) sequentially, with each of the 10 groups sampled on one day of the ten days used as the period over which the antibodies were measured. That is, with one group representing each day of the secondary immune response, day 1 being the first day following the booster injection, or beginning of the secondary response, through day 10, at which time plateau/attenuation of the secondary response occurs. Longitudinal (within subjects) groups, which comprised 5 groups of 3 rats, 15 rats in total, were sampled (as above) on a daily basis such that each subject in this condition had an estrous stage measure and antibody measure for each day of the ten days of the secondary response. The actual group size for the longitudinal study was n=14 due to the exclusion of n=1 (a non-responder according to the *a priori* criteria of ABC < 0.11 μg/ml).

Data Analysis

Since the raw ABC measures typically show heterogeneity of variance due to positive skewness (Terres et al., 1968; Rhodes et al., 1969; Falter et al., 1991) log transformations of the data were used to stabilize the variance. Cross-sectional (between subjects) data were used to examine the influence of hormonal level at the time of testing whereas longitudinal (within subjects) data were used to test the influence of hormonal level at the time of the booster injection. For the second design, three way anovas were performed on the dependent variable log ABC, with the independent variables being day (10 levels), estrogen (2 levels; high, low) and progesterone (2 levels; high, low). For the between subjects design, the small cell sizes did not allow for the examination of interaction terms. The within subjects design yielded a complete 3 way anova.

Estrous phase categorizations used in the analysis to test the first hypothesis involving the exploration of the immunomodulatory influence of estrous phase at time of testing, were directly determined by vaginal smear methodology on the day of blood collection. Estrous phase categorizations used in the analysis to test the second hypothesis involving the immunomodulatory influence of estrous phase at day 0, were determined directly by vaginal smear methodology for the longitudinal subjects on the day of the booster injection of HSA.

Results

Cross-Sectional (Between Subjects)

The means, standard deviations, and counts for the $\log_{10}$ transformed measures of antigen binding capacity (ABC) for cross-sectional analyses are shown in Table 1. There was significant increase in ABC as a function of time (Day 0 through Day 10), $F(9, 38) = 9.04$, shown in Figure 1. This is characteristic of the secondary immune response. The effect size for the change in ABC values over time (as indicated by Eta squared) was 0.66.

TABLE 1

Summary Table:

Means, Standard Deviations of Log ABC and Numbers of Subjects Categorized According to Estrogen/Progesterone Levels @ Time of Testing, Over the 10 Day Period of Immune Response Measurement

(Between Subjects)

	Estrogen ↙ ↘						*Progesterone* ↙ ↘					
	Low			High			Low			High		
Day	M	s.d.	n	M	s.d.	n	M	s.d.	n	M	s.d.	n
1	--	--	0	0.78	0.23	4	0.68	0.00	1	0.82	0.26	3
2	1.05	0.26	4	0.85	0.28	2	0.99	0.33	4	0.98	0.00	2
3	1.07	0.00	1	0.96	0.12	4	1.00	0.12	4	0.91	0.00	1
4	1.34	0.00	1	1.12	0.49	4	1.40	0.33	3	0.81	0.36	2
5	--	--	0	1.62	0.36	5	2.01	0.00	1	1.52	0.33	4
6	1.55	0.22	3	1.17	0.35	2	1.40	0.36	4	1.42	0.00	1
7	1.83	0.00	1	1.52	0.47	4	1.73	0.14	2	1.49	0.57	3
8	1.71	0.00	1	1.96	0.16	4	1.94	0.19	2	1.90	0.21	3
9	--	--	0	1.82	0.10	5	1.92	0.09	2	1.75	0.03	3
10	1.38	0.39	2	1.82	0.07	3	1.65	0.31	5	--	--	0

TABLE 2

ANOVA Summary Table:
Log ABC by Group, Progesterone and Estrogen Levels @ Time of Testing

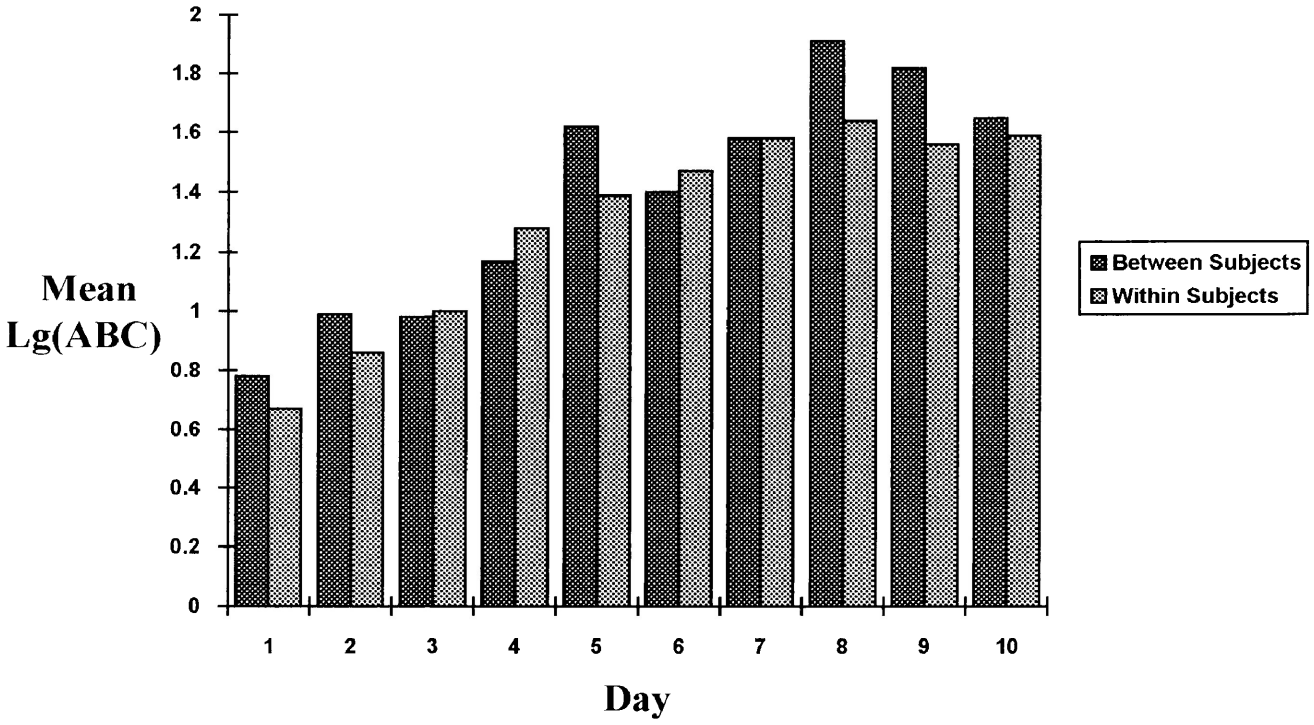
(Between Subjects)

	SS	df	MS	F	p
Main Effects	6.90	11	0.63	7.51	<.001
Group	6.80	9	0.76	9.04	<.001
Progesterone Level	0.29	1	0.29	3.46	=.071
Estrogen Level	0.04	1	0.04	0.49	
Explained	6.90	11	0.63	7.51	<.001
Residual	3.18	38	0.08		

FIGURE 1

Overall Mean Log ABC as a Function of Days of Immune Response Following Booster Injection

(Between and Within Subjects)



Longitudinal Results (Within Subjects)

The means, standard deviations and counts for the $\log_{10}$ transformed measures of antigen binding capacity (ABC) are shown in Table 4. Concurrent with the cross-sectional data, there was a significant increase in ABC as a function of time, $F(9, 90) = 38.15$, $p = 0.00$, shown in Figure 1. The effect size for the change in ABC values over time was 0.75.

Repeated measures analysis of variance (summarized in Table 5 and shown in Figure 2) demonstrated a significant effect of day 0 estrogen levels, $F(1, 10) = 7.65$, $p = 0.02$, and no significant differences for day 0 progesterone levels, $F(1, 10) = 0.04$, $p = 0.85$, on magnitude of subsequent humoral immune responding.

Subjects with higher estrogen levels at the time of the secondary booster injection (Day 0), exhibited a relatively greater antigen binding capacity, whereas subjects with lower estrogen levels at Day 0, exhibited a relatively lower antigen binding capacity. Eta values indicated that the strength of the effect of estrogen-dominant estrous phases at the time of the injection, on subsequent humoral immune response, was equal to 0.41. The interaction of estrogen and days approached significance ($p = .054$), but after applying the Greenhouse-Geisser correction, it was no longer near significance, and it will not be considered further.

TABLE 3

Summary Table:

Means, Standard Deviations of Log ABC and Numbers of Subjects Categorized According to Estrogen/Progesterone Levels @ Day 0, Over the Ten Day Period of Immune Response Measurement

(Within Subjects)

	Estrogen ↙ Low			↘ High			*Progesterone* ↙ Low			↘ High		
Day	M	s.d.	n	M	s.d.	n	M	s.d.	n	M	s.d.	n
1	0.50	0.12	9	0.97	0.32	5	0.89	0.41	4	0.58	0.22	10
2	0.70	0.20	9	1.14	0.40	5	1.10	0.42	4	0.77	0.30	10
3	0.84	0.23	9	1.28	0.48	5	1.25	0.55	4	0.89	0.28	10
4	1.05	0.27	9	1.68	0.33	5	1.60	0.45	4	1.15	0.35	10
5	1.15	0.45	9	1.83	0.28	5	1.69	0.47	4	1.27	0.50	10
6	1.25	0.40	9	1.88	0.41	5	1.79	0.60	4	1.35	0.43	10
7	1.32	0.34	9	2.06	0.41	5	1.92	0.67	4	1.45	0.39	10
8	1.37	0.37	9	2.13	0.44	5	1.92	0.76	4	1.53	0.41	10
9	1.32	0.44	9	2.00	0.38	5	1.83	0.66	4	1.46	0.47	10
10	1.30	0.43	9	2.11	0.42	5	1.95	0.71	4	1.44	0.47	10

TABLE 4

ANOVA Summary Table:
Log ABC (repeated) by Progesterone and Estrogen Levels @ Day 0

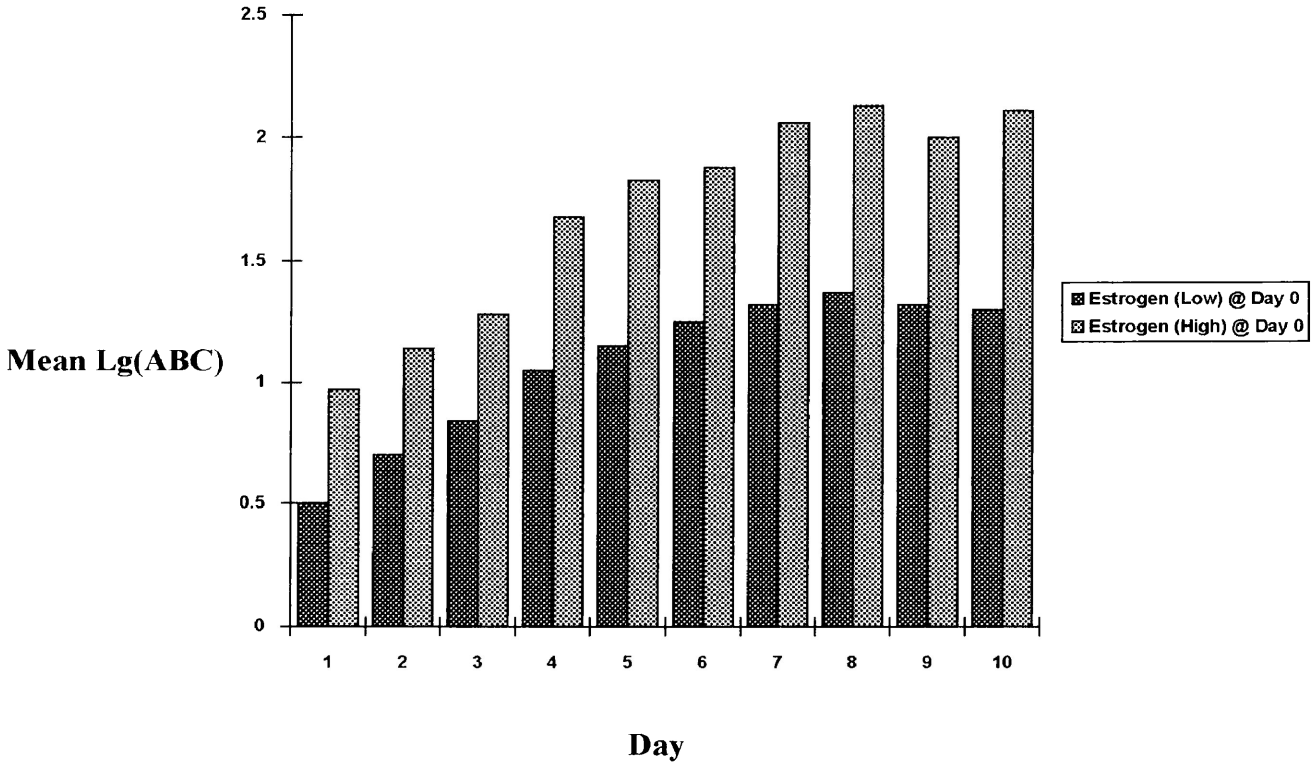
(Within Subjects)

	SS	**df**	**MS**	**F**	**p**
Between Subjects Effects					
Within Cells	11.90	10	1.19		
Progesterone Level	0.04	1	0.04	0.04	
Estrogen Level	9.10	1	9.10	7.65	<.05
Estrogen x Progesterone	0.95	1	0.95	0.80	
Within Subjects Effects					
Within Cells	2.40	90	0.03		
Days	9.17	9	1.02	38.15	<.001
Progesterone Level x Days	0.08	9	0.01	0.32	
Estrogen Level x Days	0.47	9	0.05	1.95	=.054
Estrogen x Progesterone x Days	0.08	9	0.01	0.33	

FIGURE 2

Mean Log ABC by Estrogen Levels @ Day 0
Over the Ten Day Period of Immune Response Measurement

(Within Subjects)



Discussion

This study explored the influence of estrous phase in female rats on antibody responses to a protein antigen. The intent was to examine the potential influence of the sex hormones estrogen and progesterone, associated with estrous, using a biologically intact system. Unlike previous investigations in this area, which have typically examined sex hormone influences in ovariectomized or neonatally castrated subjects, the design and methodology of the current investigation translates to the least amount of invasive procedures possible, such that the natural state of the animal may be preserved and observed accordingly. The current investigation was also designed to examine the role of estrous stage at the time of an immune system challenge as a critical factor in influencing the subsequent development of an immune response, as well as the role of estrous stage at any time during the development of an immune response as a critical factor in influencing that response. We proposed that the relative levels of estrogen and progesterone, as inferred from estrous phase, would influence the magnitude of humoral immune responding and that specific conditions (baseline and subsequent levels of estrogen, progesterone) would be associated with discrete and hormone-specific fluctuations in immunity.

Statistical analyses of the cross-sectionally sampled groups revealed an effect of increased magnitude of immune responding over time. The observed difference is an inherent characteristic of the development of a secondary immune response, which typically proceeds from an arbitrary zero at time of injection, follows a gradual upward slope as the appropriate antigen-specific immune cells are produced and recruited, and then rises abruptly to asymptote once the antibodies have reached maximum titre. Further analyses of the cross-sectionally sampled groups, categorized according to relevant estrous phase clustering,

(estrogen high/low and progesterone high/low), revealed no differences in antigen binding capacity. Analyses using these categories, which reflect estrogen and progesterone levels across time of second antigen delivery and time of testing, showed that the level of progesterone at time of testing approached significance ($p = .071$), with lower antigen binding capacity associated with higher progesterone levels. This tendency is consistent with other reports of an immunosuppressive influence from progesterone (Munroe, 1971; Nicol & Vernon-Roberts, 1965; Bratanov & Vulchanov, 1986).

Analyses of longitudinal measures indicated a relationship between estrogen status (as inferred by estrous phase at the time of secondary injection) and measures of antigen binding capacity. Females with estrogen-dominant hormonal status, at time of injection, demonstrated greater magnitude of humoral immune responding than those with diminished estrogen at time of injection (as indicated by relatively higher and lower magnitude of antigen binding capacity, respectively). Progesterone status at time of antigen injection did not influence the magnitude of subsequent humoral response.

It is unclear why the significant results obtained in the longitudinal design were not supported by the cross-sectional design. The two designs were focused on the effects of the estrous phase at two different times: time of testing versus time of antigen delivery. The inconsistency raises questions about the strength and generality of the estrous effects, and therefore creates caution in generalizing from the longitudinal design.

These observations are consistent with reports showing that challenges to the immune system (such as injection of antigen) influence subsequent immune response and that this response is dependent upon endocrine status at the time of the challenge (Dukor and Dietrich, 1968). The importance of timing of the delivery of behavioral/

pharmacological immune-altering treatment, relative to initiation of the immune response through antigen injection, was shown by Esterling and Rabin's (1987) in their investigation of the effects of rotational stress on splenic antibody production to SRBC antigen. The mice were either exposed to rotational stress and injected with the antigen simultaneously or stressed 24 hours following injection of the antigen. Timing of antigen injection relative to stress treatment, proved to be critical: antibody levels in mice exposed to stress 24 hours following antigen injection were 50 % lower than the mice stressed and injected with the antigen simultaneously. Mice and rats are valid models for studying endocrine and immune correlates of estrous (Nicol and Vernon-Roberts, 1965). In an investigation of endocrine changes which precede and follow immune responding, Pierpaoli and Maestroni (1977) administered a combination of drugs, phentolamine, haloperidol and 5HTP (L-5-hydroxytryptophan), as well as the hormones LH (leutenizing hormone), FSH (follicle stimulating hormone) and ACTH (adrenocorticotrophic hormone), to male and female mice. They measured antibody responses in the spleen to sheep red blood cells (SRBC) following the administration of the drug combination. They also measured LH and FSH levels before and after injection of SRBC antigen. The results showed that LH and FSH levels increased significantly following injection of SRBC. The combination of drugs phentolamine, haloperidol and 5HTP, which act centrally on the noradrenergic, dopaminergic and serotonergic neurotransmitter systems, respectively, completely prevented antibody production to SRBC. Pretreatment, but not subsequent injection, of LH, FSH and ACTH inhibited SRBC antibody production. The investigators hypothesized that the secretion of hormones involved in the initiation of the antibody response to SRBC could be effectively blocked by a combination of drugs acting in the central nervous system, likely at the level of the hypothalamus. This would support a central nervous system mediated mechanism whereby the sex hormones estrogen and progesterone may modulate immune function.

The literature examining the interactions of sex steroids and immunity implicate estrogen, as the source of, or at least major contributant to the observed female immunological superiority. Results of the current investigation support the findings that estrogen status at the time of an immune system challenge is a critical determinant of the immune response that ensues. Although quantitative differences in immunity at various stages of the estrous cycle in mice have been observed (Krzych *et al.* 1978), such differences were not observed in the current investigation. The absence of coherence between the findings of Krzych *et al.* 1978, and the findings of the current investigation may be attributable to a number of variables, including design and/or antigen and immune assay differences. Additionally, the validity of the findings in the current study may have been influenced by insufficient or uneven subject representation in each of the four estrous stages. Generally, a large proportion of studies exploring the influence of sex hormones on immune function have measured cellular immune function, compared with the measurement of humoral immune function, specifically in response to a protein antigen, as was used in this study. Cellular immune function may be more susceptible to the transient fluctuations of ovarian steroids that accompany estrous, whereas the influence on antibody production is less robust and influenced longitudinally, as was observed in this investigation. Inconsistencies within the measurement of either cellular or humoral immune responses may stem from variable sensitivity of the measurement assay as well as differences in sensitivity among subsets of immune system cells. These possible sources of inconsistency aside, in the absence of observations of fluctuations in immunity during discrete, hormonally-distinct stages of the estrous cycle, the current findings support a phylogenetic origin of the superior and more variable immunocapacity of females.

Further study is required to fully understand the influence of sex hormones on immunity, particularly given the range and complexity of interrelationships among the endocrine, immune, and central nervous systems. Subsequent research in this area should diverge with refinement of the roles of other physiological systems involved in the steroidal modulation of immunity, and converge with a focus at the cell and the gene level of interaction between steroids and the immune system. The implications of the effect of endogenous endocrine status as a determinant of subsequent immune response are extensive, and raise possibilities for future research not only in the broad area of stress-induced immunosuppression, but also in the growing field of women's health. An enhanced understanding of the influence of hormonal status, stress, the influence of other physiological systems and their interactions, will lead to improved preventative and prophylactic strategies in women's health care.

References

Ahmed, A., Penhale, W., Talal, N. (1985) sex hormones, immune responses, and autoimmune diseases, The American Journal of Pathology, 121, 531-551.

Besedovsky, H., delRey, A., Sorkin, E., (1985) immune-neuroendocrine interactions, The Journal of Immunology, 135, 750-753.

Black, W., McNutt, S., Casida, L., (1953) investigations on the physiological basis for the differential response of estrous and pseudopregnant rabbit uteri to induced infection, American Journal of Veterinary Research, 14, 318-323.

Bratanov, K., Vulchanov, V., (1986) reproductive immunology: a prospective marginal field of biomedical sciences, American Journal of Reproductive Immunology, 10, 68-73.

Brown, E.O., Sundstrom, S.A., Komm, B.S., Yi, Z., Teuscher, C., Lyttle, C.R., (1990) progesterone regulation of estradiol-induced rat uterine secretory protein, complement C3, Biology of Reproduction, 42, 713-719.

Butcher, R.L., Collins, W.E., Fugo, N.W., (1974) plasma concentration of LH, FSH, prolactin, progesterone, and estradiol-17β throughout the 4-Day estrous cycle of the rat, Endocrinology, 94, 1704-1708.

De, M., Wood, G. (1990) influence of oestrogen and progesterone on macrophage distribution in the mouse uterus, Journal of Endocrinology, 126, 417-424.

Dukor, P., and Dietrich, F.M., (1968) characteristic features of immunosuppression by steroids and cytotoxic drugs, International Archives of Allergy, 34, 32-48.

Eidinger, D., Garrett, T. (1972) studies of the regulatory effects of the sex hormones an antibody formation and stem cell differentiation, The Journal of Experimental Medicine, 136, 1098-1116.

Esterling, B., Rabin, B., (1987) stress-induced alteration of T-lymphocyte subsets and humoral immunity in mice, Behavioural Neuroscience, 101, 115-119.

Falter, H., Persinger, M.A., Reid, K.J (1991) sex differences in primary humoral responses of albino rats to human serum albumin, Immunology Letters, 28, 143-146.

Farr, R.S. (1958) a quantitative immunochemical measure of the interaction between I*BSA and antibody, Journal of Infectious Diseases, 103, 239-262.

Grossman, C. (1984) regulation of the immune system by sex steroids, Endocrinology Reviews, 5, 435-455.

Grossman, C. (1985) interactions between the gonadal steroids and the immune system, Science, 227, 257-261.

Harris, M., Kesel, M., (1990) an improved method for accurately timed mating in rats, Laboratory Animal Science, 40, 424-425.

Hu, S.K., Mitcho, Y.L., and Rath, N.C., (1987) effect of estradiol on interleukin-1 synthesis by macrophages, International Journal of Immunopharmacology, 10(3), 247-252.

Jankovic, B., Isakovic, K., (1973) neuro-endocrine correlates of immune response, International Archives of Allergy, 45, 360-372.

Kalland, T., Strand, O., Forsberg, J., (1979) long-term effects of neonatal estrogen treatment on mitogen responsiveness of mouse spleen lymphocytes, Journal of Clinical Immunology, 63, 413-421.

Kelly, M., Moss, R., Dudley, C. (1977) the effects of microelectrophoretically applied estrogen, cortisol and acetylcholine on medial preoptic-septal unit activity throughout the estrous cycle of the female rat, Experimental Brain Research, 30, 53-64.

Kenny, J.F., Pangburn, P.C., and Trail, G., (1976) effect of estradiol on immune competence: in vivo and in vitro studies, Infection and Immunity, 13(2), 448-456.

Kenny, J., Gray, J. (1971) sex differences in immunological response: studies of antibody production by individual spleen cells after stimulus with *E. Coli* antigen, Pediatric Research, 5, 246.

Krzych, U., Strausser, H., Bressler, J., Goldstein, A. (1978) quantitative differences in the immune responses during the various stages of the estrous cycle in female BALB/c mice, Journal of Immunology, 121, 1603.

Krzych, U., Thurman, G., Goldstein, A., Bressler, J., Strausser, H. (1979) sex-related immunocompetence of BALB/c mice: study of immunologic responsiveness of neonatal, weanling, and young adult mice, The Journal of Immunology, 123, 2568-2574.

Lipositis, Z., Kallo, I., Coen, C., Paull, W., Flerko, B. (1990) ultrastructural analysis of estrogen receptor immunoreactive neurons in the medial preoptic area of the female rat brain, Histochemistry, 93, 233-239.

Long, J., Evans, H. (1922) the oestrous cycle in the rat and its associated phenomena, Memoirs of the University of California, 6, 1-148.

Luster, M.I., Boorman, G.A., Dean, J.H., Luebke, R.W., and Lawson, L.D., (1980), the effect of adult exposure to diethylstilbestrol in the mouse: alterations in immunological functions, Journal of the Reticuloendothelial Society, 28(6), 561-568.

Luster, M.I., Hayes, H.T., Korach, K., Tucker, A.N., Dean, J.H., Greenlee, W.F., and Boorman, G.A., (1984) estrogen immunosuppression is regulated through estrogenic responses in the thymus, The Journal of Immunology, 133(1), 110-116.

Michaels, R., Rogers, K. (1971) a sex difference in immunologic responsiveness, Pediatrics, 47, 120-123.

Mukherjee, P., Mastro, A.M., and Hymer, W.C. (1990) prolactin induction of interleukin-2 receptors on rat splenic lymphocytes, Endocrinology, 126(1), 88-94.

Munroe, J.S., (1971) progesteroids as immunosuppressive agents, Journal of the Reticuloendothelial Society, 9, 361-375.

Nicol, T. (1932) response of the reticuloendothelial system to stimulation with estrogens Journal of Anatomy, 66, 181.

Nicol, T. (1935) the female reproductive system in the guinea-pig: intravitam staining: fat production: influence of hormones, Transatlantic Royal Society, Edinburgh, 58, 449.

Nicol, T., Bilbey, D. (1960) the effect of various steroids on the phagocytic activity of the reticuloendothelial system, in "Reticuloendothelial Structure and Function" (J. Heller, ed.), p.301, Ronald Press New York.

Nicol, T., Vernon-Roberts, B. (1965) the influence of the estrous cycle, pregnancy, and ovariectomy on RES activity, Journal of the Reticuloendothelial Society, 2, 15-29.

Pierpaoli, W., Maestroni, G., (1977) pharmacological control of the immune response by blockade of the early hormonal changes following antigen injection, Cellular Immunology, 31, 355-363.

Ptaff, D., Keiner, M., (1975) atlas of estradiol-concentrating cells in the central nervous system of the female rat, Journal of Comparative Neurology, 151, 121-158.

Rainbow, T., Parsons, B., MacLusky, N., McEwen, B., (1982) estradiol receptor levels in rat hypothalamic and limbic nuclei, The Journal of Neuroscience, 2, 1439-1445.

Reid, K., Falter, H., Persinger, M. (1991) humoral (immunological) responses in female albino rats during rotating magnetic field exposures, International Journal of Biometeorology, 34, 239-241.

Rhodes, K., Scott, A., Markham, R., Monk-Jones, M. (1969) immunological sex differences: a study of patients with rheumatoid arthritis, their relatives, and controls, Annals of the Rheumatic Diseases, 28, 104-119.

Schleifer, S., Shapiro, R., (1986) central nervous system modulation of immunity, Integrative Psychiatry, 4, 32-40.

Schuurs, A., Verheul, H. (1990) effects of gender and sex steroids on the immune response, Journal of Steroid Biochemistry, 35, 157-172.

Terres, G., Morrison, S., Habicht, G. (1968) a quantitative difference in the immune response between male and female mice, Proceedings in Social and Experimental Biology and Medicine, 127, 664.

Wade, G. (1971) gonadal hormones and behavioural regulation of body weight, Physiology and Behaviour, 8, 523-534.

Wybran, J., Van Bogaert, E., and Govaerts, A., (1977) lynestrol, an amplifier of lymphocyte stimulation, Biomedicine, 27, 16-19.

Wybran, J., Thiry, L., (1978) delay of tumour growth in hamsters treated with lynestrenol and effect of *Staphylococcus aureus* cowan a, Journal of the National Cancer Institute, 61, 173-176.