CYTOKINE EXPRESSION DURING DIFFERENT PHASES OF THE MENSTRUAL CYCLE

CENTRE FOR NEWFOUNDLAND STUDIES

TOTAL OF 10 PAGES ONLY
MAY BE XEROXED

(Without Author's Permission)

CHRISTINE APRIL KING

INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps. Each original is also photographed in one exposure and is included in reduced form at the back of the book.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

UMI

A Bell & Howell Information Company
300 North Zeeb Road, Ann Arbor MI 48106-1346 USA
313/761-4700 800/521-0600

CYTOKINE EXPRESSION DURING DIFFERENT PHASES OF THE MENSTRUAL CYCLE

by

Christine April King

A thesis submitted to the

School of Graduate Studies

in partial fulfilment of the

requirements for the degree of

Master of Science

Faculty of Medicine
Memorial University of Newfoundland

March 1998

St. John's Newfoundland

National Library of Canada
Acquisitions and Bibliographic Services
395 Wellington Street
Ottawa ON K1A 0N4
Canada

Bibliothèque nationale du Canada
Acquisitions et services bibliographiques
395, rue Wellington
Ottawa ON K1A 0N4
Canada

Your file Votre référence

Our file Notre référence

The author has granted a non-exclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of this thesis in microform, paper or electronic formats.

L'auteur a accordé une licence non exclusive permettant à la Bibliothèque nationale du Canada de reproduire, prêter, distribuer ou vendre des copies de cette thèse sous la forme de microfiche/film, de reproduction sur papier ou sur format électronique.

The author retains ownership of the copyright in this thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without the author's permission.

L'auteur conserve la propriété du droit d'auteur qui protège cette thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

0-612-34195-X

Canada

ABSTRACT

Previous studies have shown that cultured and circulating cells from women may differ in immune responses, the differences relating to the phase of the menstrual cycle in which they were harvested. In this investigation, specific enzyme-linked immunosorbent assays for IL-2, IL-4, IFNγ, IL-1ra and IL-1β were used to determine if there are differences in levels of cytokine expression at different phases of the menstrual cycle.

A sample set of mononuclear cells and serum were isolated from nine healthy, cycling females (19-29 years) at known points in their cycle and from four male controls. The gonadal hormones 17β-estradiol, progesterone and luteinizing hormone, as well as serum cytokine levels and cytokine production by unstimulated and stimulated cells in culture were monitored. Results indicated that the levels of all the cytokines measured varied in relation to the menstrual cycle. IL-2, IL-4, and IFNγ secretion by PHA stimulated PBMC was low at time of ovulation and high in the luteal phase of the menstrual cycle. There appears to be a T_H1 to T_H2 shift from the follicular to the luteal phase. Secretion of IL-1β and IL-1ra in LPS stimulated cultures changed during the menstrual cycle. The IL-1 ratio (IL-1β/IL-1ra) was low at ovulation in unstimulated cultures and low at the late follicular and the midluteal phases of the cycle in LPS stimulated cultures. The IL-1 ratio in serum was lowest at ovulation with higher levels in the follicular and luteal phases. Variability in the levels of the cytokines detected in successive samples, both in serum and in culture supernatants, was greater for women than

men for IL-4, IFNγ in culture supernatants, and for IL-1ra and IL-1β in serum. In addition, females had significantly higher mean values for IL-1ra in nonstimulated cultures and lower mean IL-1 ratios in serum as compared to males. Finally, multiple regression analysis of the cytokine data with the hormones estrogen, progesterone, and luteinizing hormone as well as with age of the volunteer, day in the cycle the sample was obtained, length of cycle, and day of ovulation demonstrated that IL-4 and possibly IFNγ production by stimulated PBMC and IL-1ra production by unstimulated PBMC are positively correlated with age. In contrast, IL-2, production by stimulated PBMC and serum levels of IL-1ra and IL-1β are negatively correlated with age. IL-1ra secreted by both stimulated and nonstimulated cells is positively correlated with day in the cycle the sample is taken whereas IL-1β correlates with day of ovulation and length of cycle. All of the correlations that were found to be significant in this study suggest that the menstrual cycle has a marked effect on the levels of cytokine production as detected *in vivo* in serum samples and *in vitro* in the supernatants of cells in culture.

In summary, these data demonstrate that a) there is greater variation over time in amounts of cytokine produced by women than by men b) there are changes in cytokine production in women related to the menstrual cycle c) the lowest cytokine production occurs at time of ovulation (the exception being IL-1 in stimulated cultures) and d) age influences the amounts of cytokines produced by women.

The data presented here support the hypothesis that (i) there are cyclical changes in immune responses in women (ii) the immune response is influenced by changes in

hormone status and (iii) regulation of cytokine production is fundamentally different in women than men.

ACKNOWLEDGEMENTS

I wish to say a heartfelt thank you to my supervisor, Dr. Bodil Larsen, who has been there with support, encouragement and sound advice throughout these past years.

A special thank you to the other members of my supervisory committee, Dr. Verna Skanes and Dr. Ehud Ur, who provided me with useful advice about my research and this manuscript.

To Dr. Veerabhadra Gadag, who helped me decipher the enormous amount of data I had collected and to mould it into something understandable, thank you for all the help you gave me on the statistical aspects of my analysis.

To all my volunteers, both the ladies who so graciously gave an enormous amount of their time and energy, as well as blood, for many months during this study and to the gentleman who reliably turned up three times per week and donated blood, I thank you for your cooperation; without you I would not have been able to do this project.

To Carolyn Murphy, thank you for helping in obtaining the blood samples needed from all the volunteers in the study.

To everyone in Immunology, thank you for helping me whenever you could and for never tiring of my seemingly endless questions.

To my parents, Dianne and Wayne King, for providing me with all the support I needed to continue on this path that I have chosen, a loving thank you.

And finally, to Stephen Hennessey, a sincere and heartfelt thank you for being

there through these last three years, for putting up with my worries about my research and for listening to me talk about immunology every day of my life.

Financial support was provided by Dr. Bodil Larsen and the Faculty of Medicine.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

Ab	Antibody
ACD	Acid Citrate Dextrose
Ag	Antigen
CPM	Counts per minute
DHEA	Dehydroepiandrosterone
DHT	5α-di-hydrotestosterone
E2	17β Estradiol
EBV	Epstein Barr Virus
EF	Early Follicular phase
EL	Early Luteal phase
ELISA	Enzyme Linked Immunosorbent Assay
FcR	Fc receptor
FCS	Foetal Calf Serum
FSH	Follicle Stimulating Hormone
GM-CSF	Granulocyte Monocyte Colony Stimulating Factor
GnRH	Gonadotropin Releasing Hormone
HLA	Human Leukocyte Antigen
IFNγ	Interferon gamma
Ig	Immunoglobulin
IgA	Immunoglobulin A
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IL-1	Interleukin 1
IL-1β	Interleukin 1 beta
IL-1ra	Interleukin 1 receptor antagonist
IL-2	Interleukin 2
IL-4	Interleukin 4
IL-6	Interleukin 6
i.m	Intramuscular
i.n.	Intranasal
i.p.	Intraperitoneal
K_d	Disassociation Constant
L	Litre
LF	Late Follicular Phase
LH	Luteinizing Hormone
LL	Late Luteal Phase
LPS	Lipopolysaccharide
M	Menses
M	Molar

MCP-1	Monocyte Chemotactic Protein-1
MF	Midfollicular Phase
MHC	Major Histocompatibility Complex
ML	Midluteal Phase
ml	Millilitres
MLR	Mixed Lymphocyte Reaction
mM	Millimolar
mRNA	Messenger Ribonucleic Acid
mg	Milligrams
ng	Nanograms
NK	Natural Killer Cells
nm	Nanometers
O	Ovulation
OD	Optical Density
OS	Donor's own serum
P	Progesterone
PAC	Peritoneal Adherent Cells
PBL	Peripheral Blood Lymphocyte
PBMC	Peripheral Blood Mononuclear Cells
PFC	Plaque Forming Cells
PG	Prostaglandins
pg	Picograms
PHA	Phytohaemagglutinin
PMA	Phorbal 12-myristate 13-acetate
PWM	Pokeweed mitogen
RIA	Radioimmunoassay
SC	Secretory Component
s.c.	Subcutaneous
SD	Standard Deviation
SRBC	Sheep Red Blood Cells
Te	Testosterone
T_H1	T Helper one
T_H2	T Helper two
TNFα	Tumor Necrosis Factor alpha
TNFR	Tumor Necrosis Factor Receptors
TNFR1	TNF Receptor Type I
TNFRII	TNF Receptor Type II
U	Units
µg	Micrograms
µl	Microlitres
VCAM-1	Vascular Cell Adhesion Molecule 1
vs	Versus

CHAPTER I

INTRODUCTION

It is generally recognized that women have more vigourous immune responses than men. It would appear that gender influences both humoral and cell mediated immune responses since a number of autoimmune diseases occur with higher frequency in women than in men.

It is suggested that a woman's immune responses are influenced by hormones, and, since hormone levels change throughout the menstrual cycle one would expect her immune responses to also change.

I have followed nine women through a full menstrual cycle. Hormone levels and cytokines were measured in blood samples taken in different phases of the cycle. The aim of the study was to see if changes in cytokine production correlated with the phases of the menstrual cycle.

1.1 The Menstrual Cycle

Females undergo complex cycling of reproductive hormones; this cycling is referred to as the menstrual cycle (or perhaps more correctly, the ovarian cycle).

The menstrual cycle alternates between two major phases, the follicular phase, which typically persist for 12 to 16 days and is characterized by the presence of maturing follicles, and the luteal phase, which most commonly persists for 10 to 16 days and is characterised by the presence of the corpus luteum in the ovary (Marshall, 1995).

The cycling that is exhibited in females is due to the ovarian and pituitary hormones that are produced. The levels of these hormones that are seen in a typical female cycle are illustrated in Figure 1.1 (modified from Asso, 1983) and the plasma concentration of various ovarian steroids as well as their production and secretion rates are shown in Table 1.1 (modified from Hsueh *et al.*,1995).

1.1.1 The Feedback Relationship between Ovarian and Pituitary Hormones

The feedback relationship that exists between the ovarian hormone estrogen and the pituitary hormones, follicle stimulating hormone (FSH) and luteinizing hormone (LH), determines the events of the menstrual cycle (Asso, 1983). The main estrogenic product of the ovary is 17β-estradiol (E2) and it also produces progesterone (P). FSH and LH are controlled by the hypothalamus through gonadotropin releasing hormone (GnRH).

The control mechanisms are a combination of negative and positive feedback systems (Clarke, 1995). Estrogen exerts negative feedback control on FSH and LH through GnRH. If estrogen is increased, the tendency is to suppress GnRH, release in the hypothalamus, thus in turn suppressing FSH and LH production and release. In addition, the ovarian hormones also act at the pituitary level to decrease the sensitivity of FSH and LH producing cells to GnRH (Asso, 1983). However, there seems to be a unique switch in the effect of estrogen at the time of the preovulatory GnRH/LH surge. At this time the situation is reversed and estrogen exerts a positive feedback effect on the system (Clarke, 1995).

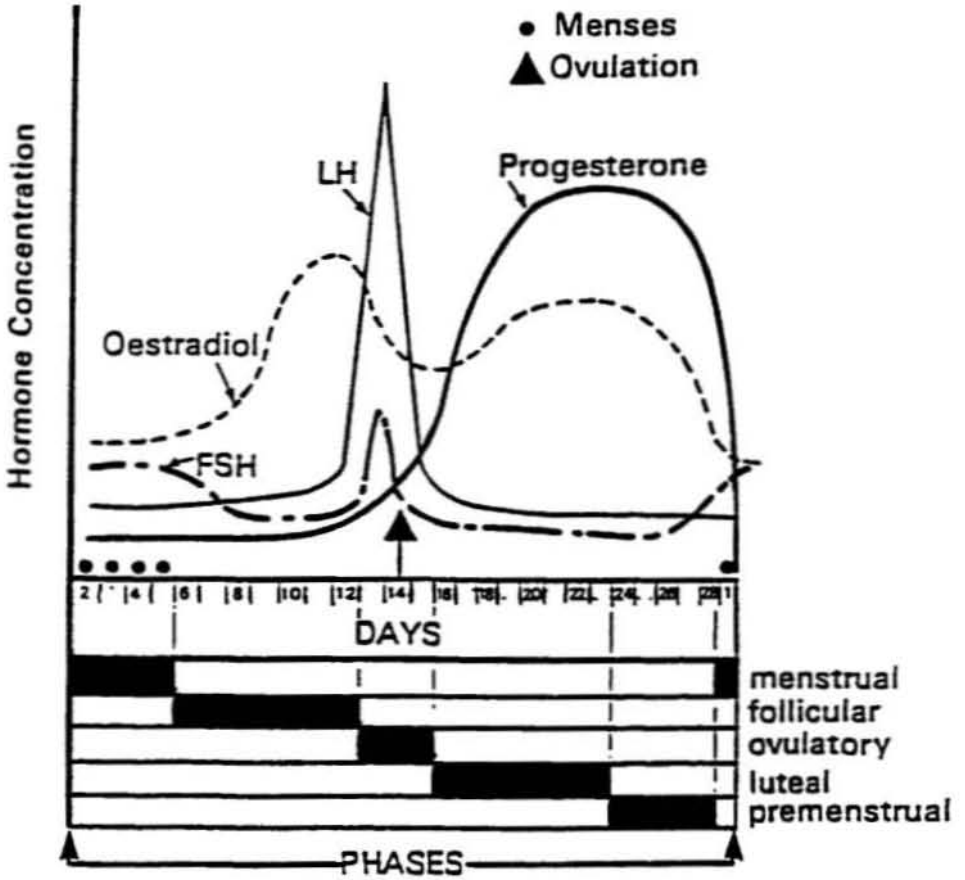

Figure 1.1. Relative levels of estradiol, progesterone, FSH and LH throughout the menstrual cycle.

Table 1.1. Plasma concentrations, production rates, and ovarian secretion rates of ovarian steroid hormones.

Steroid	Phase of Menstrual Cycle	Plasma conc. (ng/100ml)	Production rate (mg/day)	Secretion rate (mg/day)
Estradiol	Early follicular	6	0.08	0.07
	Late follicular	33-70	0.5-1.0	0.4-0.8
	Midluteal	20	0.27	0.25
Estrone	Early follicular	5	0.11	0.08
	Late follicular	15-30	0.3-0.7	0.25-0.50
	Midluteal	11	0.24	0.16
Progesterone	Follicular	50-100	2.1	1.5
	Luteal	1000-1500	25.0	24.0
17α-hydroxyproges terone	Early follicular	30	0.6	0.2
	Late follicular	200	4.0	3-4
	Midluteal	200	4.0	3-4
Androsten-edione		130-160	3.2	0.8-1.6
Testosterone		38	0.3	-

1.1.2 Beginning of the Cycle

The beginning of the cycle is referred to as Day 1 and is the first day of menstruation. The levels of hormones at the end of one cycle are preparing the body for the next cycle. This can be seen in the hormone FSH. As estrogen levels decline toward the late luteal phase, FSH is beginning to rise commencing the growth of follicles that will be involved in the next cycle (Asso, 1983).

1.1.3 Follicular Phase

The follicular phase begins with Day 1 of the cycle and ends with ovulation. Throughout this phase the follicles that can eventually give rise to a mature ovum influence the changes seen in this phase and the hormonal changes that occur in this phase provide the correct environment for maturation of the follicle.

FSH stimulates the growth of these follicles which in turn become the source of estrogen in the cycle (Asso, 1983). Thus the early follicular phase is characterized by relatively high plasma levels of FSH and low levels of LH, estrogen, progesterone, and inhibin. FSH and LH exert a combined effect on the follicles to stimulate secretion of estradiol which by the middle to the late follicular phase is secreted by the dominant follicle (Marshall, 1995).

In the midfollicular phase estradiol concentrations in plasma are rising and suppressing secretion of FSH by action on the pituitary. As well, inhibin levels are rising possibly contributing to the suppression of FSH (Marshall,1995).

Estradiol is increased in the late follicular phase and exerts a positive feedback effect on LH by enhancing LH responsiveness to GnRH and by increasing GnRH secretion (Marshall, 1995).

Progesterone, at this time in the cycle, is also beginning to rise and exerts a positive feedback effect on LH. It is the "ovarian estradiol-progesterone signal system" that induces the "GnRH-LH-FSH" ovulatory surge (Marshall, 1995).

Several follicles develop in the ovary at the same time but only one will go on to become the ovum that is released at ovulation. The other follicles will undergo atresia. The developing or atretic stage of a follicle is characterized by the follicular fluid content of estrogens and androgens (Hsueh *et al.*, 1995). As they undergo atresia the follicles will begin to switch from secreting principally estrogen to the secretion of androgens. One of the androgens that is secreted in this stage is testosterone and it is seen to peak just prior to ovulation (Asso, 1983).

The menstrual cycle of a woman is generally stated to be 28 days in length. However, the length varies greatly between women. On average the cycle is 29.5 days with a range of 26-34 days in normal females (Asso, 1983).

1.1.4 Ovulation

As estrogen declines in the late follicular phase a surge in LH is seen and maintained for approximately 24 hours. This LH surge causes the largest follicle of the ovum to be released from the ovary thereby allowing for possible fertilization and

implantation (Asso, 1983).

The rupture of the follicle at ovulation signals the end of the follicular phase and the beginning of the luteal phase (Marshall, 1995).

1.1.5 The Luteal Phase

The ruptured follicle that is left behind in the ovary following release of the oocyte undergoes a process called luteinization resulting in the formation of the corpus luteum (Marshall, 1995).

This corpus luteum is responsible for the production and secretion of progesterone and estrogen that are seen in the luteal phase of the menstrual cycle.

This increased secretion of progesterone and estrogen lasts for a number of days after ovulation during which time FSH secretion is inhibited and remains low (Marshall, 1995).

This increase in progesterone stimulates the growth of the endometrium that will allow for development and growth of the fertilized ovum (Asso, 1983). If fertilization does not take place, plasma levels of estradiol and progesterone decline leading to shedding of the endometrium and is seen as menstrual flow (Asso, 1983).

At this time FSH levels begin to rise initiating the development of a new set of follicles, beginning the cycle all over again (Asso, 1983).

1.2 Sex hormones and Immune Response

It has long been known that the sex hormones influence or are interrelated with the immune response. Research in the field of immune-endocrine interactions has been ongoing for some time. Four main areas of research today have provided significant evidence for the role of sex hormones in the immune response, including: 1) the differences in immune responses between males and females; 2) the knowledge that gonadectomy and hormone replacement alter the immune response; 3) the observation that the immune response is altered during pregnancy; and 4) the presence of receptors for sex hormones on cells of various arms of the immune system (Grossman, 1984).

1.2.1 Sex Related Differences in Immune Response

Sex related differences in immune response have been observed in several strains of mice and in humans (Huber *et al.*, 1981; Shuurs and Verheul, 1990). It has been observed that males are less likely to develop autoimmune diseases than females (Lillehoj *et al.*, 1981; Ahmed and Penhale, 1982), that autoimmunity in males can be induced by injection of estradiol (Gershwin *et al.*, 1980) and that females have higher titres of autoantibodies in serum (Inman, 1978; Ahmed *et al.*, 1985). The immune response of females is greater than that of males (Grossman, 1985). Females have higher serum immunoglobulin levels than males, mount higher antibody (Ab) responses to various antigens (Schuurs and Verheul, 1990) and reject allogeneic skin grafts faster than males (Butterworth *et al.*, 1967; Graff *et al.*, 1969). In addition, it has been noted that

prevalence of predisposition to allergy changes at about age 15 from males to females (Schuurs and Verheul, 1990).

The evidence that there is a difference between the immune system of males and females suggests that the influence of gonadal steroids is very important. Much of the work done has been on determining the influence of these hormones on various arms of the immune response.

It is important to note here that the outcome of *in vivo* studies done in various laboratories is influenced by the dose and type of administration of the hormone as well as by age of the animals used (Ahmed *et al.*, 1985).

1.2.2 Humoral Immunity

1.2.2.1 Direct Action of Gonadal Hormones

Estrogen has been shown to enhance the immune response to various antigens in several species (Stern and Davidson, 1955; Kenny and Gray, 1971; Myers and Petersen, 1985; Erbach and Bahr., 1988). Nikolaevich *et al.*, 1991 demonstrated that injection of estradiol into ovariectomized mice stimulated the immune response as indicated by increased Ab response to sheep erythrocytes, while injection of progesterone did not have such an effect. *In vitro* studies indicate that estrogen has a direct effect on the immune system by influencing lymphocyte function (Erbach and Bahr, 1991). Kenny *et al.*, 1976, incubated, *in vitro*, *E. coli* primed murine spleen cells with various concentrations of estrogen. They found that physiological levels of E2 caused an increase in the number

of Ab secreting cells in male mice compared to untreated cells as demonstrated in a haemolytic plaque assay (PFC), by examining the ratio of total plaques from E2 treated cells with control cells. The authors suggest that the phenomenon observed is independent of phagocytic function and that E2 directly affects lymphoid cells in the spleen.

Myers and Petersen., 1985, found that intraperitoneal (i.p.) injection of E2 in male rats resulted in a dose-related increase in anti- sheep red blood cell (SRBC) antibody titres during the primary immune response (characterized by the production of anti-SRBC IgM). Unlike Kenny, these investigators found no increase in the number of Ab producing cells between control and estradiol treated animals as measured by a plaque forming assay.

Similarly, in humans, incubation of peripheral blood cells (PBL) with E2 caused an increase in the number of cells secreting IgM (Paavonen *et al.*, 1981) and IgG (Weetman *et al.*, 1981).

Ainbender *et al.*, 1968, examined differences in specific Ab to poliovirus in the serum of males and female and found a difference between men and women in the proportion of IgA and IgG polio antibody. Specifically, it was found that in women the IgA polio antibody titre was equal to or greater than the IgG titre and that in men it was less. There was no difference in the serum concentrations of IgA or IgG between the two groups.

Konstadoulakis *et al.*, 1995, compared *in vitro* and *in vivo* immune parameters between female and male rats using a complicated experimental design involving hormone injections and gonadectomy. The results of the study considering only the *in vivo* IgG

levels in serum were as follows: IgG levels in serum of male pups treated with testosterone propionate (TP) were increased as compared to controls and IgG levels in serum of female pups treated with TP decreased; gonadectomy in both sexes produced effects similar to those as animals treated with TP. Thus in male rats the increase of IgG production *in vivo* after castration indicates that testosterone suppresses T and B cell functions.

Another study examined the *in vitro* effect of physiological concentrations of E2 and testosterone (Te) on Ag non-specific differentiation of human peripheral blood mononuclear cells (PBMC) stimulated with pokeweed mitogen (PWM). Assessment of B cell differentiation was indicated by the number of Ab secreting cells assayed by reverse hemolytic PFC assay using SRBC as indicator cells. It was found that *in vitro* differentiation of B cells, from both men and women, stimulated with pokeweed mitogen and cultured with physiological levels of E2 was significantly augmented as compared to controls and that physiological concentrations of Te inhibited PWM-induced B cell differentiation (Sthoeger *et al.*, 1988).

The variable effects of estrogen on the humoral immune response is illustrated in the observation that production of Ab to *Candida albicans* in mice is enhanced by E2 at low levels but depressed at high levels of the hormone (Mathur *et al.*, 1979).

Similarly, it has been found that women have better Ab response to the Hepatitis B vaccine than men do as indicated by the greater geometric mean titers for anti-HBs (Struve *et al.*, 1992).

Evidence for the direct effect of sex hormones on the immune response is also supported by the fact that receptors for estrogen have been found in mouse spleen (Detlefsen *et al.*, 1977), human spleen (Danel *et al.*, 1983; Stimson, 1988) and on human PBL's (Danel *et al.*, 1983; Weusten *et al.*, 1986).

1.2.2.2 Indirect Action of Gonadal Hormones

Several papers, illustrating both *in vivo* and *in vitro* experiments, have indicated that the influence of hormones on humoral immunity is indirect (Paavovnen *et al.*, 1981; Barnes *et al.*, 1974; Stimson and Hunter, 1976; Holdstock *et al.*, 1982).

Erbach and Bahr, 1991, investigated the requirement of the thymus in the enhancement of humoral immune responses by estrogen. Adult female rats were ovariectomized and thymectomized or sham thymectomized and s.c. given E2 or left untreated. Results showed that E2 enhancement of *in vivo* humoral immunity requires the thymus. Both male and female rats need an intact thymus to achieve E2 enhancement of Ab titres.

1.2.3 Cellular Immunity

Estrogens and progestins inhibit proliferative responses of T cells to certain mitogens and antigens [phytohaemagluttin (PHA), Con A or purified protein derivatives (Mendelsohn *et al.*, 1977; Wyle and Kent., 1977)]. Athreya *et al.*, 1993, evaluated the effect of the presence or absence (controls) of various sex steroids on the proliferative

responses to PHA, IL-2 or anti-CD3 of PBMC's taken from normal human adult males and found that E2 and Te had no consistent effect on the proliferative response to any of the stimuli tested. E2 + PHA caused a decrease in the percentage of $CD4^+$ T cells and E2 + IL-2 an increase in the number of $CD8^+$ T cells. Te + IL-2 increased the percentage of $CD4^+$ cells indicating a potential role of hormones in T cell activation. In contrast to this, Gilbody *et al.*, 1992, studied dose related effects of estradiol on rat thymic and splenic T lymphocyte responsiveness to mitogens and found that estradiol stimulated responsiveness of lymphocytes.

Konstadoulakis *et al.*, 1995, did *in vitro* studies on rats and the cellular response during self MLR (p.10). They found that *in vivo* administration of Te had a negative effect on self MLR and E2 injection had a positive effect as shown by an increase in MLR after castration and a decrease in MLR after oophorectomization.

Baral *et al.*, 1996, found that pretreatment of P815 target cells (a murine mastocytoma cell line) with estradiol or tamoxifen (estrogen antagonist and potent immunomodulatory agent) makes them significantly more susceptible to lymphokine activated (LAK) cell mediated cytotoxicity.

1.2.4 The Cycle and Immune Response

1.2.4.1 Humoral and Mucosal Immunity

Studies done in mice and rats indicate that the levels of Ab's fluctuate during the estrous cycle. Total IgA levels in rat uterine secretions obtained by uterine flushing are

higher during the estrus and proestrus when compared to the diestrus; total IgG levels in uterine secretions are higher in the proestrus phase of the cycle as compared to the other phases (Wira and Sandoe, 1977, 1980). In addition, migration of lymphocytes to the genital tract, specifically the uterus, vagina and cervix, in female mice is influenced by the estrous cycle; it has been found that there is an increased number of IgA producing plasma cells at these sites during proestrus and estrus (McDermott *et al.*, 1980; Rachman *et al.*, 1983).

Several groups have shown that progesterone and estrogen directly influence the fluctuating levels of secretory component (SC) and antibodies, as well as the number of B cells in the genital tract of female rats and mice (Wang *et al.*, 1996; Wira and Sandoe, 1977; Sullivan *et al.*, 1983). Similarly, cyclical changes in SC, measured in uterine secretions, and total Ab levels have been demonstrated in humans (Sullivan *et al.*, 1984; Usala *et al.*, 1989). Specifically, Usala *et al.*, 1989, investigated immunoglobulin levels in cervicovaginal secretions during the menstrual cycle using the microradial immunodiffusion method. Results indicated that IgG levels in vaginal fluid samples were high in the follicular phase with a steady decline to low levels throughout the luteal phase. IgA was present at levels 10-fold less than IgG and near the assays limit of detection though it was seen that the lowest vaginal concentration was around the midluteal phase.

Several different systemic routes of administration (s.c., i.m. and i.p.) of antigen have been shown to induce specific Ab's in the female genital tract of several species including humans (Gallichan and Rosenthal, 1996; Miller *et al.*, 1992; Ogra and Ogra,

1973; Parr and Parr, 1990).

Intravaginal and intrauterine immunization are mucosal routes of immunization and have been able to induce both specific IgG and IgA in the genital tract, vaginal secretions, and in serum (Milleigan and Bernstein, 1995; Ogra and Ogra, 1973). Other mucosal sites, for example, intranasal (i.n.) generates both IgG and IgA in the vaginal secretions of the genital tract (Gallichan and Rosenthal, 1996; Muster *et al.*, 1995).

Few of these studies take into account the stage of the cycle. Gallichan and Rosenthal, 1996, examined the influence of the estrous cycle in mice on the levels of Herpes Simplex Virus Type 2 glycoprotein (HSV-gb) specific IgA and IgG Ab's in the female genital tract, specifically vaginal fluid. The mice were immunized i.n. with AdgB8, a recombinant adenovirus type 5 vector. Results indicated that there was a cyclical fluctuation in the Ab production, IgG was highest in diestrus and lowest at estrus, IgA was highest in estrus and lowest in diestrus. Susceptibility of unimmunized mice to intravaginal HSV-2 infection was studied during various estrous cycle stages and it was found that mice infected during estrus were protected from infection since no viral replication or genital pathology was observed. In addition, results showed that i.n. immunization during a progesterone-induced diestrus-like state protects against subsequent intravaginal challenge as significantly less virus replication and genital pathology was observed. The discovery that levels of IgG and IgA antibodies in the genital tract of i.n. immunized mice vary inversely with each other and are dependent on the stage of the estrous cycle reflects the changes that occur in the reproductive tract during the course of

the cycle. Estrus is the time of mating and it follows that high levels of IgA should be present to take care of the pathogens associated with mating (Gallichan and Rosenthal, 1996). These increased levels of IgA seen at estrus are supported by the finding that there is an increase in the number of IgA producing plasma cells in the uterus, vagina and cervix during proestrus and estrus (McDermott *et al.*, 1980; Rachman *et al.*, 1983) and an increase in SC in the uterus during estrus (Sullivan *et al.*, 1983).

Similarly, Parr *et al.*, 1994 investigated the effect of the estrous cycle and sex hormones on vaginal infection of adult mice by HSV-2. Results indicated that inoculation during estrus resulted in no infection whereas inoculation in the progesterone dominated phase resulted in mice becoming infected with the virus (Parr *et al.*, 1994).

1.2.4.2 Prostaglandins

Prostaglandins (PG) are also involved in the immune response. They are mediators at several levels (Kunkel, 1988). Leslie *et al.*, 1987 noted that female mice synthesized a greater amount of PG than male mice and with greater variation between females in the group, therefore leading to the hypothesis that PG synthesis was modified by hormones during the estrous cycle. A later study found that hormonal status of human females is associated with changes in PG metabolism. Leslie and Dubey, 1994, found that both PGE_2 and PGI_2 were increased in the medium of cultured monocytes isolated during the luteal phase compared to medium from follicular phase cultures and to monocytes cultured from males donors.

1.2.4.3 Cellular Immunity

Herrera *et al.*, 1992, examined the response of human lymphocytes to mitogenic stimulation over the course of the menstrual cycle and in response to *in vitro* addition of physiological concentrations of E2 and P. The study showed that there was large intra-individual variation over the course of the menstrual cycle and that *in vitro* administration of estradiol and progesterone at physiological concentrations inhibited proliferation of male and female PHA stimulated human lymphocytes.

Similarly, Bjune, 1976, investigated *in vitro* lymphocyte responses to PHA during the menstrual cycle and pregnancy. In this study, 2 healthy women were bled twice weekly for 2 months. Response of PBMC to PHA were recorded as incorporation of ^{3}H-thymidine in the presence of 10% autologous plasma and 10% pooled human serum. One pregnant woman and two males served as controls. The results indicated that in the two cycling women, PHA responses varied greatly throughout the cycle. They found that at the time of ovulation the responses were low and that during menses they were high. They also found that addition of autologous serum resulted in strong suppression in the pregnant woman and in cycling women in the luteal phase of the cycle, whereas it had an augmenting effect in the follicular phase. The PHA responses to autologous plasma were constant in the males and the pregnant woman.

In contrast, another study investigated nine women over the course of one menstrual cycle measuring reactivity of blood lymphocytes to PHA during the menstrual (low levels of E2 and P), midfollicular (peak levels of estradiol), and midluteal phases

(peak levels of progesterone). Data indicated that there were no differences in reactivity to PHA over the three menstrual cycle phases and no relationship with any of the hormones measured (Caggiula *et al.*, 1990).

In addition to studies done on the PHA response during the menstrual cycle, a recent study has been done on cytotoxic lymphocyte (CTL) activity during the menstrual cycle. In this study CTL activity was monitored in the reproductive tract in relation to the menstrual cycle. Human female reproductive tract cells were isolated from hysterectomy patients from the fallopian tube, uterine endometrium, endocervix, ectocervix and vaginal mucosa. The cells were cultured overnight in the presence of IL-2 to yield effector cells and then these effector cells were incubated with or without OKT3 (anti-CD3 mAb) in a chromium release assay. This assay measured total $CD3^+$ T cell mediated lytic activity by use of FcRII/III bearing, ^{51} Cr-labelled P815 target cells. Results indicated that $CD3^+$ $CD8^+$ cytolytic T cells are found throughout the reproductive tract and, when the proliferative phase (follicular) and the secretory phase (luteal) were compared, that the capacity for $CD3^+$ T cell cytolytic activity in the uterine endometrium is present during the proliferative phase but absent during the secretory phase. This indicated that $CD3^+$ $CD8^+$ cytolytic T cells are hormonally regulated. It was also found that in postmenopausal women, the entire reproductive tract retains the capacity for strong $CD3^+$ T cell cytolytic activity. The authors suggest that the data support the idea that high levels of E2 and P present in the luteal phase of the menstrual cycle downregulate CTL activity in the uterus thus allowing for implantation of the semiallogeneic embryo. They

also conclude that CTL activity is regulated differently in different regions of the reproductive tract (White *et al.*, 1997)

Fluctuations in the WBC counts have been observed during the menstrual cycle of humans. Mathur *et al.*, 1979 investigated cyclic variations in white cell subpopulations in serial blood samples from males and females. Monocytes and granulocytes were increased in the luteal phase as compared to the follicular phase; WBC, total T cells and lymphocyte counts were decreased at the pre-ovulatory E2 peak in the follicular phase. The conclusion of Mathur *et al.*, 1979 was that lymphocyte counts were negatively correlated with E2 and monocyte and granulocyte counts were positively correlated with progesterone. Results also showed that there were no cyclical variations in WBC populations in men and in women with nonovulatory cycles.

There has also been some interest in whether the immune response differences that are seen between males and females exist at the level of extrathymic T cells, defined as T cells expressing T cell receptor, (CD3) of intermediate intensity (i.e.intermediate Tcells) and a high level of IL-2 receptor β-chain, in various organs of mice. It was found that the $CD4^+/CD8^+$ T cells ratio is higher in females as compared to males and that intermediate T cells of possibly extrathymic origin were more predominant in the liver and other organs of female mice than in males (Kimura *et al.*, 1994).

Sex hormones play a role in regulating antigen presentation by uterine cells (obtained by uteri enzymatic digestion) in female rats as demonstrated by Wira and Rossoll. Antigen presentation was measured by ovalbumin-sensitized T cells cultured in

the presence of irradiated epithelial or stromal cells and ovalbumin with proliferation measured by ^{3}H thymidine uptake. Results indicated that antigen presentation by uterine epithelial cells is high at diestrus, low at estrus. Antigen presentation by uterine stromal cells was high at estrus, low at diestrus. In addition, if estradiol is given to ovariectomized rats this causes an increase in antigen presentation by the epithelial cells and a decrease in antigen presentation by stromal cells as compared with controls (Wira and Rossoll, 1995).

1.2.5 Conclusions

It is apparent from the volume of work that has been done on the influence of gonadal hormones on the immune system that there is much interest in the area but the picture is still unclear. Contributing to this phenomenon is the knowledge that many investigations yield different results depending on the route of administration and dose of the hormones used for studies. As well, different results are obtained from different sites including uterine, vaginal, mucosal, and systemic areas of the system. Although the results differ in these circumstances it has been shown conclusively that gonadal steroids do indeed influence the immune response in humans and animals.

1.3 Cytokines

Cytokines are peptides or glycoproteins that act as soluble mediators of immune response. They regulate local and systemic immune and inflammatory responses as well as many other biological processes (Oppenheim *et al.*,1994).

They are highly potent molecules that act at concentrations of 10^{-10} to 10^{-15}M influencing the immune system as autocrine, paracrine and endocrine hormones (Oppenheim *et al.*, 1994).

All the information in the following sections on cytokines has been obtained from two primary sources i.e. Abbas *et al.*, 1994, and Oppenheim *et al.*, 1994. Due to this fact the publications will not be sited throughout the text.

1.3.1 Interleukin 1

Interleukin 1 is a cytokine that is principally produced by activated mononuclear phagocyctes but can be produced by other cell types. These include: B lymphocytes, natural killer cells (NK), T cell clones grown in culture, keratinocytes, dendritic cells, astrocytes, fibroblasts, neutrophils, endothelial cells and smooth muscle cells. Significant amounts of IL-1 have been found in skin, amniotic fluid, sweat and urine.

Lipopolysaccharide (LPS), a bacterial cell wall product, tumour necrosis factor and interleukin 6, other cytokines, and $CD4^+$ T cells interacting with antigen presenting cells, can all initiate production of IL-1 by mononuclear phagocytes.

The principle function of IL-1 is as a mediator of the host inflammatory response.

The actions of IL-1 depend on the concentration of IL-1 present in the system.

At low concentrations of IL-1, IL-1 acts on mononuclear phagocytes in an autocrine fashion to further stimulate synthesis of IL-1 and to induce synthesis of IL-6. It also acts on mononuclear phagocyctes to synthesize chemokines, molecules that stimulate and direct leucocyte movement.

When IL-1 is present at higher concentrations it can exert endocrine effects by causing fever, synthesis of acute phase proteins and cachexia.

IL-1 exerts its actions through two distinct forms, those of IL-1α and IL-1β. These two types of IL-1 are peptides that are encoded by separate genes and share 26% amino acid sequence homology. Although these are distinct forms of IL-1 they are virtually identical in potency and activity.

In addition to having two distinct forms of IL-1 present in the system there is also a naturally occurring inhibitor of IL-1. This inhibitor is called IL-1 receptor antagonist (IL-1ra) and acts to inhibit IL-1 by binding to IL-1 receptors preventing the functional IL-1 from binding and exerting it's influence. IL-1ra is a competitive inhibitor that acts to endogenously regulate IL-1 in the system.

IL-1α and IL-1β bind to high affinity receptors (K_d~10^{-10} M) that are found on all IL-1 target cells. Two distinct types of receptors, IL-1RI and IL-1RII, have been found to bind to IL-1α and IL-1β equally. These receptors are 28% homologous in sequence similarity and have three extracelluar domains.

IL-1RI is the type I receptor and it has a 217 a.a. cytoplasmic tail that can transmit

signals to the cytoplasm of the cell when IL-1 is bound.

IL-1RII is the second distinct type of receptor found that will bind IL-1 and, unlike IL-1RI, it does not have a cytoplasmic tail. Thus it cannot transduce signals to the interior of the target cell.

It is also important to note that one of the major functions of IL-1RII is thought to be as an endogenous inhibitor of IL-1β at inflammatory sites due to the release of the extracelluar domain of the receptor into solution at times of inflammation. This soluble IL-1RII can bind IL-1β more strongly than IL-1α.

1.3.2 Tumour Necrosis Factor α

Tumour necrosis factor α is a cytokine that is primarily produced by activated mononuclear phagocytes but can also be produced by antigen stimulated T lymphocytes, activated NK cells and activated mast cells. TNFα is the mediator of response to gram negative bacteria with the response due directly to the presence of LPS in the bacterial cell wall. The effects of TNFα on the systems of the body depend largely on the concentration of TNFα present. TNFα can act in an autocrine, paracrine and endocrine manner and has effects in much the same way IL-1 does.

At low concentrations TNFα acts locally as a paracrine and autocrine regulator of leukocytes and endothelial cells. TNFα, at these low concentrations, causes activation of neutrophils; increase in expression of adhesion molecules contributing to accumulation of leukocytes at the area of inflammation; production of other cytokines like IL-1, IL-6 and

TNFα itself as well as chemokines; and augmentation of expression of Class I MHC molecules thereby assisting in cytotoxic lymphocyte (CTL) mediated killing of infected cells.

At high concentrations, TNFα acts in an endocrine manner and enters the blood stream. When TNFα is acting in this manner it produces different effects on biological systems. These effects include the induction of fever by acting on the hypothalamus, induction of mononuclear phagocytes to secrete IL-1 and IL-6 into circulation, increased secretion of serum proteins like serum amyloid A protein thus contributing to the acute phase response, activation of the coagulation system, and suppression of bone marrow stem cell division.

As with IL-1, two types of high affinity receptors have been identified to bind TNFα. The type II receptor has a higher affinity for TNFα than Type I. Each receptor is composed of a large extracelluar domain, a hydrophobic transmembrane region and an intracellular transducing piece. It is thought that the two types of receptors induce different effector functions. Binding of TNFα to Type I receptor promotes cytotoxic activity and Type II promotes T cell proliferation.

1.3.3 Interleukin 6

Interleukin 6 is a cytokine that has a wide variety of biological effects. It is mainly produced by mononuclear phagocytes, vascular endothelial cells and fibroblasts. The secretion of IL-6 is often in response to IL-1 and TNFα secretion.

IL-6 synergizes IL-1 and TNFα to induce the acute phase response. IL-6 participates in this response by inducing hepatocyctes to produce serum proteins like fibrinogen. It is also known to serve as a growth factor for activated B cells. It enhances B cell replication, differentiation, and immunoglobulin production.

IL-6 has the ability to synergize with IL-1 and TNFα augmenting the mitogenic effects of these cytokines on T helper cells. This effect is in part due to the ability of IL-6 to increase expression of IL-2 receptors.

The high affinity receptors for IL-6, (K_d~ 10^{-10} - 10^{-12} M), are expressed on a variety of cell types. These include macrophages, myelomonocytic cell lines, hepatocytes, resting T cells, activated or EBV infected B cells, and plasma cells. The receptor is composed of an α and a β chain. The IL-6Rα chain does not have a cytoplasmic domain. This chain binds IL-6 with low affinity producing the IL-6Rα-IL-6 complex which then binds with high affinity to IL-6Rβ chain. This chain does contain a cytoplasmic tail and thus the signal can be transduced.

1.3.4 Interleukin 2

IL-2 is the principle cytokine responsible for the growth of T cells, specifically the induction of T cells to move from the G_1 phase to the S phase of the cell cycle. This cytokine is produced by T cells, specifically $CD4^+$ T cells but also by $CD8^+$ T cells when antigen and costimulatory factors are present.

IL-2 has the ability to act in an autocrine and paracrine manner. It functions in an

autocrine manner by acting as a growth factor for the T cells that produce it but can also act on nearby T cells to induce IL-2 thus acting in a paracrine manner. It does not circulate in the blood during an immune response thus does not act as an endocrine growth factor.

Because of its effects on T cells, a major component of the immune system, it is thought to be a critical immunoregulatory cytokine. It influences human lymphocytes to promote not only proliferation but also production of other lymphokines like IFNγ, TNFβ, IL-6, IL-4, IL-3, IL-5, and GM-CSF. IL-2 will also stimulate NK cells to produce other cytokines that in turn activate macrophages and enhance the cytolytic activity of NK cells. The effect of IL-2 on NK cells produce a superior killing cell called the lymphokine activated killer cell (LAK). IL-2 influences B cells as well by enhancing proliferation and antibody production, preferentially promoting production of IgG2 antibodies.

The IL-2 receptor is composed of three polypeptides, α, β and γ. Each polypeptide on its own can bind IL-2 but with low affinity. Together they bind with a high affinity ($K_d \sim 10^{-11}$ M).

The α chain has only a small cytoplasmic domain and thus cannot transduce signals to the interior of the cell. The β and γ chains have larger cytoplasmic domains and can transduce signals to the interior of the cell.

1.3.5 Interleukin 4

Interleukin 4 is a cytokine that is principally produced by $CD4^+$ T lymphocytes,

specifically those of the T_H2 subset. It is also produced by mast cells. IL-4 was initially described as a B cell growth factor as it helps in B cell proliferation and as a B cell stimulatory factor because of its ability to induce Class II MHC expression on resting B cells. IL-4 is a major regulator of heavy chain class switching as it is required for the production of IgE antibodies. In addition, IL-4 influences expression of low affinity Fcε receptors on B cells.

IL-4 acts on macrophages to inhibit activation and block the effects of another cytokine, IFNγ, produced by the T_H1 subset. IL-4 acts as a growth and differentiation factor for T_H2 cells and suppresses the induction and function of T_H1 cells.

IL-4 also influences expression of some of the adhesion molecules like VCAM-1 on endothelial cells and causes endothelial cells to secrete MCP-1, a chemotactic protein. These functions allow for inflammatory reactions that are rich in monocytes and eosinophils. Mast cells are influenced by IL-4 as it promotes growth of this type of cell.

IL-4, through all these functions, seems to be a principle mediator of allergic reactions and, as it is characteristically produced by the T_H2 subset, is responsible for the prevention of many cell-mediated immune responses by preventing the T_H1 subset functions.

1.3.6 Interferon γ

IFNγ is produced by both naive (T_H0) and T_H1 $CD4^+$ helper T cells, by $CD8^+$ T cells and NK cells when activated. It upregulates expression of Class I and Class II MHC

thus aiding in cytotoxic killing and promoting antigen presentation to $CD4^+$ T lymphocytes. It acts as a potent activator of mononuclear phagoctyes inducing the microbicidal and cytotoxic activity of macrophages and production of other cytokines such as IL-1, IL-6, IL-8, and TNFα. NK cells, neutrophils, and vascular endothelial cells are also activated by this cytokine.

IFNγ acts on T and B lymphocytes to promote differentiation, specifically IFNγ promotes differentiation of naive T cells to the T_H1 subset and inhibits proliferation of the T_H2 subset. $CD8^+$ cells are aided in maturation by IFNγ and B cells exposed to IFNγ produce IgG2a and IgG3 subclasses of antibodies preventing production of IgG1 and IgE.

The effects of IFNγ described above all contribute to cell mediated immune responses and the suppression of humoral mediated immune response.

1.4 Sex Hormones and Cytokines

There are several studies that indicate the influence of sex hormones on cytokine production. This influence has not been explained although a great deal of evidence exists for the relationship. Much of the work has been done on the inflammatory cytokine IL-1 and some information is available on several other cytokines. The focus of this section will be on the cytokines measured in this study.

1.4.1 Sex Hormones and IL-1

One of the earliest studies done to show the influence of gonadal steroid on

cytokine production was by Cannon and Dinarello in 1985. They examined IL-1 activity as measured by the effect on proliferation of D10 cells, a murine T cell line, stimulated with PHA. When IL-1 activity was assayed using plasma from healthy human females, there was significant IL-1 activity in the plasma isolated during the luteal phase as compared to the follicular phase of the menstrual cycle. Subsequently, an experiment done with plasma from five healthy women who were bled once during the follicular and luteal phase of their cycle (as determined by progesterone concentrations) showed that the luteal phase plasma had greater IL-1 activity than the follicular phase plasma. They postulated that this increase in IL-1 activity during the luteal phase of the menstrual cycle was consistent with the 0.2 to 0.6 °C rise in body temperature that is seen in the luteal phase and the inflammatory focus present after follicular rupture (Cannon and Dinarello, 1985).

Since then, there have been several studies done on the influence of gonadal steroids on the production of cytokines both by humans and animals in both *in vitro* and *in vivo* systems.

Hu *et al.*, 1988 examined the effect of estradiol on IL-1 synthesis/secretion by rat peritoneal macrophages as measured by a thymocyte proliferation assay. Using peritoneal adherent cells (PAC) isolated from five female and male rats they analyzed the amount of IL-1 spontaneously secreted in culture and found that cells from adult females secreted more IL-1 than cells from males or prepubescent females. In addition, ovariectomy led to reduced synthesis of IL-1 by PAC and *in vivo* estradiol administered subcutaneously

effectively increased IL-1 synthesis. To further show the influence of estradiol on IL-1 secretion, it was demonstrated that IL-1 secretion could be increased in PAC isolated from male rats when the PAC were incubated *in vitro* with physiological concentrations of estradiol. When PAC were incubated with E2 and LPS the enhanced IL-1 secretion was greater than with E2 alone.

The effect of steroids on monocytes has also been demonstrated. Human monocyte IL-1 activity as a function of increasing E2 and P levels have been examined. Human peripheral monocytes isolated from males were incubated with increasing concentrations of both steroids in separate cultures. IL-1 was measured by the D10 thymocyte stimulation bioassay and dose response curves as a function of E2 and P were established. It was found that low concentrations of E2 (10^{-9} - 10^{-10}M) and P (10^{-8} - 10^{-9}M) resulted in maximal IL-1 production. At higher concentrations of E2 (10^{-7}M) and P ($\geq 10^{-7}$M, this being the physiological range of P seen in the luteal phase and early pregnancy) there was a significant reduction in IL-1 activity (Polan *et al.*,1988a).

Similarly, Flynn, 1984, demonstrated that monocytes isolated from placental tissue, both cultured and not cultured (lyzed by sonication to determine cellular IL-1 content), produce IL-1. In addition, Flynn also found that with estrogen and progesterone stimulation of the monocytic tumor line P338D, production of IL-1 was stimulated at physiological concentrations but suppressed at high levels of these steroids.

The ability of peripheral monocytes isolated from varying endocrine milieux to secrete IL-1 has been investigated. Polan *et al.*, 1990, examined the levels of IL-1

secreted by cultured peripheral monocytes isolated from eight women before ovulation (follicular phase) and during the late luteal phase (Day 12 of luteal phase) in human menopausal gonadotropin and human chorionic gonadotropin stimulated cycles a well as during pregnancy, n=6. IL-1 activity was measured by the D10 thymocyte proliferation assay. Significantly more IL-1 was secreted by monocytes isolated during the luteal phase of the cycle than by cells isolated during the follicular phase or the third trimester of pregnancy. This observation is consistent with previous work (Polan *et al.*, 1988a; Polan *et al.*, 1988b). These experiments were designed to determine the effect of physiological levels of E2 and P on IL-1 secretion by isolating the monocytes at various time points representing the concentration of circulating steroids. The results of the *in vitro* culture studies suggest that IL-1 production by circulating monocytes may be controlled in part by circulating steroid levels. Specifically, that progesterone is the critical factor in the control of IL-1 production.

Trying to tie the two studies together it is important to realize that monocytes isolated during the second and third trimester of pregnancy in the study done by Polan and associates in 1990 were isolated at a time point where the concentration of P in serum, is approximately 1.5×10^{-7} and 2.9×10^{-7} M, respectively. This approaches the concentration where *in vitro* bioactivity is decreased (Polan *et al.*, 1988a) and where IL-1β mRNA content is decreased (Polan *et al.*, 1988b).

The relationship between E2 and P concentration and the level of IL-1β mRNA in cultured human peripheral monocytes and pelvic macrophages has also been addressed.

Human peripheral monocytes and peritoneal macrophages isolated during the luteal phase of the cycle and stimulated with LPS and increasing amounts of P or E2 show an inverse relationship between IL-1β mRNA levels and steroid concentration. IL-1β mRNA levels decrease as P or E2 increase to 10^{-5}M indicating that regulation by gonadal steroids occur at the level of transcription (Polan *et al.*, 1989).

In females undergoing *in vitro* fertilization, Polan *et al.*, 1994, showed that luteinized granulosa and cumulus cells isolated from the follicular fluid of five women in the luteal phase stained for the presence of IL-1 mRNA but that cells isolated during the follicular phase did not and that IL-1 mRNA protein appeared in the follicle coincident with imminent ovulation. In addition, this study found that IL-1 mRNA levels in peripheral macrophages isolated during the follicular phase were equivalent to those seen in male controls, but that a three-fold increase in IL-1β mRNA was seen in monocytes isolated during the luteal phase.

The effects of menopause and ovarian steroid treatment on IL-1 release by monocytes has also been studied. Monocytes were obtained from venous blood, cultured, and IL-1 activity measured by the D10 bioassay. IL-1 activity from cells isolated from postmenopausal, n=14, (low estrogen) women was higher than in either premenopausal women, n=7, or estrogen/progesterone treated postmenopausal women, n=8, and treatment with estrogen/progesterone for 1 month caused a substantial decrease in IL-1 activity in the postmenopausal women (Pacifici *et al.*, 1989) further establishing a relationship between gonadal steroids and IL-1 production.

Pacifici *et al.*, 1991, evaluated the effects of oophorectomy and subsequent estrogen replacement on spontaneous secretion of IL-1 and TNFα from peripheral blood mononuclear cells. They demonstrated that women who underwent oophorectomy, n=15, had increased production of IL-1 and TNFα and that those who started estrogen replacement therapy showed a decrease in both cytokines. It was also demonstrated that in women who had hysterectomy without oophorectomy, n=9, no change in cytokine production was seen. This study indicates that changes in estrogen status *in vivo* are associated with changes in secretion of mononuclear cell cytokines *in vitro*.

Another study, (Lynch *et al.*, 1994) looked at gender and menstrual cycle dependent variation on IL-1 production. Secretion of IL-1α, IL-1β and IL-1ra by cultured PBMC and urine cytokine concentrations were examined to determine if *in vitro* secretion corresponds to *in vivo* status. Mononuclear cells were isolated from five women, once in the follicular phase and once in the luteal phase of the menstrual cycle, and from six men. In unstimulated cell cultures it was found that there were significantly higher levels of each of the three isoforms in females as compared to males and that in females cytokine production was higher in the cells isolated during the follicular phase of the cycle. The agonist/antagonist ratio (IL-1α + IL-1β/IL-1ra) was higher in the luteal samples consistent with Polan *et al.*, 1990 and Cannon and Dinarello, 1985. In the LPS stimulated cultures there was no significant gender or menstrual cycle related difference seen among any of the isoforms. The agonist/antagonist ratio (IL-1α + IL-1β/IL-1ra) was three-fold higher in the follicular phase as compared to the other groups indicating that although women

have elevated IL-1 activity in the luteal phase they have a more vigourous response to challenge in the follicular phase (Lynch *et al.*, 1994).

1.4.2 Sex Hormones and TNFα

The effects of E2 on TNFα release, measured by bioassay, from human PBMC *in vitro* has been demonstrated by Ralston *et al.*, 1990. Ralston examined TNFα release from eight healthy premenopausal and postmenopausal women and from men. It was found that in nonstimulated cultures of cells isolated from postmenopausal females with E2 added at concentrations between 10^{-12} - 10^{-6} M, TNFα release was significantly inhibited but the male hormone DHT had no effect. In cells isolated from males and premenopausal women E2 and DHT had no effect on TNFα release. In cultures stimulated with LPS in the presence of either E2 or DHT no effect was seen in either of the groups. Ralston also established that cytotoxcicity mediated by the PBMC supernatants was due to the TNFα as it was abolished by a specific monoclonal Ab to TNFα.

The effect of female sex hormones on peritoneal macrophage TNFα release was shown in a study on five male rats. In this study resident peritoneal macrophages obtained by peritoneal lavage or oil-elicited macrophages were used to determine TNFα release after treatment with E2, P or LPS. The results indicated that resident peritoneal macrophages treated with concentrations of E2 or P greater than 10^{-1} ng/ml had significantly reduced TNFα release compared to untreated controls. Using oil-elicited macrophages it was found that TNFα release was dose dependent. Maximal TNFα release

was induced by E2 concentrations of 10^{-2} ng/ml and this release was greater than that of the controls. Concentrations less than 10^{-4} or greater than 10^{-1} reduced TNFα release by the macrophages. Progesterone results were similar to the E2 results. They also showed that Te depressed TNFα release from male rat peritoneal cells (Chao et at al., 1995).

In addition to studies showing the effects of hormones on TNFα production other studies have shown alterations in TNFα mRNA levels. Loy *et al.*, 1992, tested whether TNFα mRNA levels in human peripheral monocytes were regulated by gonadal steroids. In this study, monocytes isolated from peripheral blood of 2 healthy females during their respective luteal phases were cultured with LPS and P or E2 in dose response experiments. The results showed that an inverse relationship existed between TNFα mRNA production and concentrations of E2 or P, that is, the higher the dose of hormone in the culture medium the lower the TNFα mRNA levels.

1.4.3 Sex Hormones and Other Cytokines

Tamoxifen and tormifene are anti-estrogenic chemicals that bind to estrogen receptors. Jarvinen *et al*, 1996 studied whether these compounds had any effect on phorbal 12-myristate 13-acetate (PMA) induced cytokine production of B, T or myeloid cell lines. When tamoxifen or tormifene were added to B cell line cultures it was found that both stimulated overall production of TNF, IFN-γ, IL-4, IL-10, and IL-1β by the B cell line. Results using a T cell line indicated that only tamoxifen stimulated cytokine production, specifically IL-1β, IL-6 and IFN-γ, whereas tormifene inhibited cytokine

production. Cultures with a myeloid cell line showed no effect of the compounds on cytokine production.

A study of fourteen normal women showed that there is no significant variation in serum levels of IL-10, measured by ELISA, during the menstrual cycle in humans. It is important to note that this study made use of multiple samples during the menstrual cycle, specifically, 61 successive samples were taken in total from the 14 women (Blakemore *et al.*, 1996).

Matsuzaki *et al.*, 1995 investigated the changes in soluble IL-6R and IL-6 levels in serum of eighty pregnant and forty-five nonpregnant women by ELISA (n=18 in the follicular and luteal phase, n=9 at ovulation). The group found that sIL-6R in nonpregnant women remained unchanged during the menstrual cycle; that the level in pregnant women, although higher than in nonpregnant women did not show changes with respect to stage of pregnancy and that serum IL-6 concentration did not differ between the groups or during the cycle.

Another study quantitated the amounts of IL-6 present in the cervical mucus of women with regular menstrual cycles both before and after ovulation. Cervical mucus was obtained from 15 ovulating women during the 5 days before ovulation (follicular) and during the 5 days after ovulation (luteal). Analysis of IL-6 showed a wide range of individual variation in the levels of IL-6 and trend analysis demonstrated an increase in IL-6 levels in samples obtained from the follicular phase with a decrease at ovulation (Kutteh *et al.*, 1996).

Although much work has been done on the influence of female sex hormone less has been done on the influence of male hormones on cytokine production. *In vivo* i.p. injection of mice with DHEA or DHEAS (sulfonated form of DHEA) resulted in greater levels of IL-2 produced by T cells in subsequent *in vitro* cultures stimulated with anti-CD3 than in untreated controls. This study also showed that direct *in vitro* culture with DHEA at low doses enhanced the capacity to secrete IL-2 following stimulation (Daynes *et al.*, 1990).

1.4.4 Pilot Study

Our unpublished pilot study, similar to this project, suggested that there were cyclical changes in cytokine production in females in relation to the menstrual cycle. In the pilot project we measured the cytokines IL-2, IL-4 and IFNγ, in cultures of mononuclear cells stimulated with anti-CD3. We isolated PBMC from 5 women four times during their cycle, twice in the follicular phase and twice in the luteal phase. Our results indicated that production of IL-2 fluctuated during the menstrual cycle with a peak in the follicular phase and possibly a second peak in the luteal phase. The pilot study failed to take into account length of cycle, health of the volunteers, and whether or not the volunteers ovulated.

1.4.5 Conclusions

A summary of the relevant human cytokine information in relation to gonadal hormones is presented in Table 1.2. This table includes the cytokine examined, the number of participants in the study, the source of the material tested, the results that are applicable to this project and the reference. The table is organized by cytokine.

It would thus appear that i) estrogen and progesterone do influence IL-1 and TNFα production in *in vitro* cultures, the effect depending on the concentration of the hormones. ii) IL-1 production by unstimulated cells is higher when cells are isolated from women in the luteal phase than in the follicular phase, the reverse is true when the cells are stimulated with LPS (what happens in between the two points is unknown). iii) Plasma IL-1 is higher in women in the luteal phase than in the follicular phase, but again, what happens between the two time points is unknown. iv) There is no significant change in serum IL-10 concentration during the menstrual cycle.

It is apparent that many of the studies on hormonal influence of cytokines employed only few subjects and only one time point. In about half of the studies 2 points were obtained in the cycle, one in the follicular phase and one in the luteal phase. We wanted to investigate the influence of hormones on cytokine expression at different phases of the menstrual cycle using a larger sample size and many more sampling days. Differences are observed between the follicular and luteal phases in several studies but whether changes occurred that were not being picked up as a result of taking only two time points is unknown. In addition, in several studies the women who provided the

follicular phase samples were not the same as those who provided the luteal phase samples. It was our intent to control for the extra variable by using the same volunteers and sampling them throughout their menstrual cycle so we could see the differences that occur in individual women.

Table 1.2. Summary of human cytokine data in relation to the female gonadal hormones. The cytokine examined, the number of participants in the study, the source of the material tested, the results and the references are given.

Cytokine	n*	Source	Results	Reference
IL-1				
	5 ♀[a]	plasma	IL-1 activity ↑[c] in L[d]	Cannon and Dinarello, 1985
	♂[b]	peripheral monocytes	E2 10^{-9}-10^{-10} M ↑ IL-1 P 10^{-8}-10^{-9} M ↑ IL-1 E2 or P 10^{-7} M ↓[c] IL-1	Polan *et al.*, 1988b
	♀	placental monocytes	produce IL-1	Flynn, 1984
		P338D cell line	↑ IL-1 at physiol. conc. of E2 and P ↓ IL-1 at > conc.	
	8 ♀ F[e] 8 ♀ L 6 ♀ pregnant	peripheral monocytes	↑ IL-1 in L	Polan *et al.*, 1990
	♀ L	peripheral monocytes	↑ in IL-1β mRNA as E2 or P ↓	Polan *et al.*, 1989
		peritoneal macrophages		

	5 ♀	granulosa and cumulus cells from F fluid	IL-1 mRNA in L phase not in F phase	Polan *et al.*, 1994
		peripheral macrophages	IL-1 mRNA in F = men 3-fold > in L	
IL-1	14 pm[a] ♀ 7 prm[b] ♀ 8 E2/P pm ♀	peripheral monocytes	IL-1 activity in pm > prm or E2/P pm	Pacifici *et al.*, 1989
	15 ♀ oophorectomy	PBMC	↑ IL-1 and TNFα E2 treatment ↓ IL-1 and TNFα	Pacific *et al.*, 1991
	5 ♀ 6 ♂	PBMC 1 sample in F and L	unstimulated IL-1α ↑ F vs L IL-1β ↑ F vs L IL-1ra ↑ F vs L IL-1α + IL-1β /IL-1ra ↑ L	Lynch *et al.*, 1994
			stimulated all isoforms of IL-1 →[c] IL-1α + IL-1β /IL-1ra ↑ F	
TNFα				
	8 prm ♀ 8 pm ♀ 8 ♂	PBMC	unstimulated pm + E2 ↓ TNFα ♂ or prm + E2 TNFα →	Ralston *et al.*, 1990
			stimulated no effect	
	2 ♀	PBMc in L	↓ TNFα as ↑ in E2 or P	Loy *et al.*, 1992

IL-10	14 ♀ multiple samples	serum	IL-10 →	Blakemore *et al.*, 1996
IL-6	15 ♀	cervical mucus	IL-6 ↑ F IL-6 ↓ at Ovul	Kutteh *et al.*, 1996
	80 pm ♀ 45 prm ♀ (18 F and L, 9 at O)	serum	sIL-6R and IL-6 → in prm or in stages of pregnancy	Matsuzaki *et al.*, 1995

[a] number of participants in the study if known
[b] female
[c] male
[d] increases or greater
[e] Luteal phase
[f] Follicular phase
[g] decreases or lower
[h] postmenopausal
[i] premenopausal
[j] no difference

1.5 Aims and Objectives

Based on the literature available and our pilot study data (section 1.4.4) our hypothesis was that cytokine expression/secretion is regulated in females by ovarian hormones and therefore affected by hormonal changes of the menstrual cycle. Specifically, that the capacity of cells to produce cytokines, as demonstrated either by circulating cytokine levels or secretion by cells in culture, is affected by the changing levels of steroid hormones *in vivo*.

We chose to examine production of IL-2, IL-4, IL-1ra, IL-1β, IL-6, TNFα and IFNγ with a view to determining whether one or more of these displayed identical patterns of change in all the women studied. The one or ones with the most predictable pattern could then be used for future studies.

The objectives of this project were :

1) to establish culture and stimulation conditions allowing detection in supernatants of PBMC of various cytokines by commercial ELISA's

2) to create a sample set of serum and cells from normal healthy females at known points in their menstrual cycle (ovarian cycle)

3) to create a similar sample set of controls using normal healthy males

4) to measure levels of estrogen, progesterone, and luteinizing hormone in the females and testosterone in the males

5) to compare variability of cytokine production in circulation and in supernatants

of stimulated and unstimulated cells in culture, in males and females

6) to monitor serum cytokine levels and cytokine production by unstimulated and stimulated cells in culture over the course of the menstrual cycle

7) to determine if there is cyclical variation, in females, of cytokine levels in serum or cytokines produced by cells in culture

8) to determine if variation in cytokine expression is linked to cyclical changes in the hormones estrogen, progesterone and luteinizing hormone

9) to select and use appropriate statistical methods for analysis of multiple data points from a small number of individuals.

The parameters that were measured in serum were: IL-1β, IL-1ra, IL-6, TNFα.

The parameters that were measured in culture were: IL-1β, IL-1ra, IFNγ, IL-2, and IL-4.

CHAPTER II

MATERIALS AND METHODS

2.1 Volunteers

2.1.1 Cycling Women Selection Process:

The cycling volunteers were recruited from the university population through the use of posters and presentations to various classes. They were screened using an Human Investigation Committee approved questionnaire designed by us (Appendix A). Volunteer criteria were as follows:

Healthy (subjective, based on the report of the individual)
Caucasoid (subjective, based on appearance)
Women between the ages of 19-29 years
Not known to be pregnant
Not taking any medication
Not taking oral contraceptives
Stable menstrual cycles, appr. 28 days
No history of major medical problems
Normal height and weight (Body Mass Index, BMI)

Based on the above criteria 19 cycling volunteers were chosen to enter the study and proceed to Phase I out of 24 who volunteered to be screened.

2.1.2 Control Selection Process

Four males between the ages of 20-25 and 2 post-menopausal women, 57 and 59

years old, respectively, were chosen through the use of a similar questionnaire. It was intended to recruit 4 postmenopausal women but that could not be accomplished. In the end we had only 2 and one women was not proved conclusively to be post-menopausal, thus we did not include this data in the results though the raw data can be found in Appendix B. The criteria for the controls were as follows:

Healthy (subjective, based on the self report)
Caucasoid (subjective, based on appearance)
No history of major medical problems
Normal height and weight (BMI)
Not taking any medication

2.2 Phase I

Phase I of the study was designed to determine which volunteers were acceptable to proceed to the quantification stage (Phase II) of the study. Volunteers were required to monitor the symptoms they experienced during the course of three menstrual cycles using a Menstrual Cycle Diary (2.2.1 and Appendix A). In addition, the volunteers monitored their ovulation time with the aid of a commercial OvuQuick self-test kit (Pharmasience Inc., Montreal) that detects the presence or absence of luteinizing hormone in urine. This is useful because it is known that when LH peaks then ovulation proceeds 24 to 36 hours later.

2.2.1 Menstrual Cycle Diary

All the cycling volunteers, 21 in total, were required to complete the Diary in the evening for each day during the cycle. This was done for 3 full cycles. The diary demonstrated the symptoms that each volunteer had, health status, and medication consumed. The diary is a single page, self report questionnaire that measures the magnitude of symptom severity on a scale from 1-3. There are 21 symptoms assessed in the diary. The instructions for completing the Diary as well as the Diary itself can be seen in Appendix A. Briefly, if the volunteer did not experience any symptoms the space corresponding to that symptoms was left blank, if the symptom was mild (noticeable but not troublesome), the number 1 was recorded, if the symptom was moderate (interferes with normal activity) a 2 was recorded, and if the symptom was severe (temporarily incapacitating) a 3 was recorded.

2.2.2 OvuQuick Kit

Materials:

Urine Dropper- To add the urine to the test pad
Urine Cup- For collection of urine.
Test Pads- Pads that test the urine.
Bottle A-D-Chemicals that allow detection of LH.
Buffer, Enzyme conjugate, Substrate, Stop solution.

Methods:

Urine was collected between 10:00 and 20:00 and was not the first urine of the

day. Date, cycle day and time were recorded when the sample was obtained. The enzyme conjugate was then prepared and left to stand for 10 minutes. The test pad was removed from the pouch and 6 drops of urine placed in the centre. Three drops of buffer were added to the test pad and allowed to soak in. Six drops of the enzyme conjugate followed, timed for 1 minute exactly. Six drops of substrate were added and the reaction allowed to proceed for exactly 3 minutes with the reaction terminated by adding stop solution(6 drops). A color change to blue at the site of urine application was considered a positive reaction, i.e. luteinizing hormone was present in the urine.

2.3 Phase II

Nine cycling females were chosen to enter into Phase II of the study based on the data gathered from Phase I. During this phase the subjects were also asked to continue to monitor their ovulation times and continue to complete the Menstrual Cycle Diary. In addition, both the female volunteers and the male controls were asked to complete a questionnaire every time they were bled (Appendix A). This ensured information on the health, sexual activity and general well-being of the individual.

2.3.1 Collection of Blood Samples

The 9 cycling female volunteers were bled three times a week for five weeks. Blood was obtained by venepuncture in vacutainer tubes between the hours of 8:30-20:00. A total of 5 x 10 ml Vacutainer (Becton-Dickenson,NJ) tubes of blood was collected from

the subject; 3 tubes contained ACD (acetate-citrate-dextrose) as an anticoagulant and 2 Vacutainer tubes contained no additive (serum separating tubes).

The control volunteers were bled 3 times a week for 3-4 weeks, in the same manner as the cycling females.

Before they could consent to the above all subjects were required to have their hemoglobin levels measured (Hematology Laboratory, Health Sciences Centre, St. John's, NF). A minimum value of 90g/l was required for entry into the study. As well hemaglobin levels were measured at the end of the five weeks. This data can be seen in Appendix A, Table A.1. This table also includes information on cell populations at the beginning and the end of the study.

2.3.1.1 Preparation of Cells

Peripheral blood mononuclear cells (PBMC) were isolated from fresh blood using density gradient centrifugation in Lymphoprep ™ (Cederlane,Ontario). The ACD Vacutainer tubes were spun at 2000 rpm (1000 x g) for 10 minutes and the buffy coat removed. The resulting 9 ml from the 3 tubes were combined and diluted 1:2 in sterile PBS (Phosphate Buffeted Saline) then layered onto 4.5 ml of Lymphoprep™ in each of three 15-ml centrifuge tubes at room temperature. The tubes were then centrifuged for 20 minutes at 2000 rpm. The PBMC layer was removed with the aid of a Pasteur pipette and washed twice in Hanks Balanced Salt Solution (IBN Biomedicals, California),and once in culture medium, counted and resuspended at a concentration of 5 x 10^6/ml or 2.5 x 10^6/ml in culture medium.

2.3.1.2 Culture Medium

The culture medium used for the cytokine stimulations was prepared sterile and consisted of:

500 ml RPMI 1640 (Gibco/BRL,Burlington)
5 ml FCS (Foetal Calf serum) (Gibco/BRL)
5 ml L-Glutamine 200mM (Gibco/BRL)
5 ml Sodium Pyruvate 100mM (ICN Biomedicals, CA)
5 ml Penicillin Streptomycin 5000I.U./ml (ICN Biomedicals)

2.3.2 Stimulation Protocol

The stimulation protocols varied depending on which type of cytokine we wished to measure. The systems were worked out prior to the beginning of Phase I and II (see section 3.5)

2.3.2.1 Induction of IL-2 and IL-4 Secretion

IL-2 and IL-4 secretion was induced with 50 μg/ml PHA (Phytohemagglutinin, Boehringer Mannheim, Montreal) for 24 hours. Aliquots of 1ml of freshly isolated PBMC at a concentration of 5 x 10^6 cells/ml were deposited in 2 sterile Falcon tubes (Becton/ Dickinson, Montreal). The cells in one tube were stimulated with 10μl PHA (5mg/ml) dissolved in sterile water, while the other cells served as a control, having no known stimulant, to see if the cells were naturally secreting IL-2 and IL-4. The cells were incubated at 37°C in a 5% humidified CO_2-incubator for 24 hours.

2.3.2.2 Induction of IL-1β and IL-1ra Secretion

2.5 x 10^6 cells/ml were stimulated with Lipopolysaccharide (Sigma, NJ, stock solution at 1mg/ml in RPMI) in round-bottom sterile polystyrene plates (Life Technologies, Burlington) at 200µl of cell suspension per well with 8µg/ml LPS per well for 24 hours at 37°C in a humidified 5% CO_2-incubator. There were 16 wells in total, 8 stimulated and 8 unstimulated.

2.3.2.3 Induction of INF-γ Secretion

5 x 10^6 cells/ml were incubated in 0.5 ml aliquots, in 2 sterile Falcon tubes, for 48 hours at 37°C in a 5% humidified CO_2-incubator. To one tube, PHA had been added (50µg/ml), the other tube served as an unstimulated control.

2.3.2.4 Harvest of Culture Supernatants

At the end of the stimulation time the tubes were centrifuged at 1500 rpm for 10 minutes. The supernatants were removed with a sterile pipette, under sterile conditions and frozen at -70°C in 250µl (IL-2) and 500µl (IL-4, INF-γ, IL-1ra, IL-1β) aliquots to be tested later in ELISAs.

2.3.3 Serum samples

The serum-separating tubes were left at room temperature to clot for at least 1 hour before spinning the tubes at 2000 rpm for 20 minutes. The serum was then removed from

the tubes and divided into several aliquots which were frozen at -70°C. There were approximately 12 aliquots per person and these were later used to determine the levels of LH, estrogen, progesterone, testosterone, IL-1β, IL-1ra, IL-6 and TNFα (also total hemolytic complement and C3, see discussion).

2.4 ELISA Assays

The cytokines in this study were all measured by commercial ELISA assay. Table 2.1 summarizes the manufacturer of each ELISA kit, the sample assayed, the sample volume used, and the sensitivity of the assay. All samples were run in parallel and all the samples collected for one volunteer during the menstrual cycle were run on the same plate. Specific details about the procedure can be found in the following sections.

2.4.1 General Principle of the IL-2 Immunoassay

The plates were purchased from R&D Systems Minneapolis, MN. This assay employs the quantitative "sandwich" enzyme immunoassay technique. A monoclonal antibody specific for IL-2 has been coated onto the microtiter plate provided. Standards and samples are pipetted into the wells and any IL-2 present is bound to the immobilized antibody. After washing away any unbound proteins, an enzyme-linked polyclonal antibody specific for IL-2 is added to the wells to "sandwich" the IL-2 immobilized during the first incubation. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and color develops in

Table 2.1 Summary of the cytokines examined for each of the volunteers with reference to the commercial source of the ELISA kit, sample and sample size, and sensitivity of the assay as determined by the manufacturer.

Cytokine	Commercial Source	Sample	Sample Volume	Sensitivity
IL-2	R&D Systems	Supernatant	100µl	6.0 pg/ml
IL-4	R&D Systems	Supernatant	200µl	3.0 pg/ml
IL-4	Biosource	Supernatant	50µl	2.0 pg/ml
IL-1ra	R&D Systems	Supernatant Serum	200µl 200µl	22.0 pg/ml 22.0 pg/ml
IL-1β	Biosource	Serum	100µl	0.083 pg/ml
IL-1β	Biosource	Supernatant	50µl	1.0 pg/ml
IFNγ	R&D Systems	Supernatant	200µl	3.0 pg/ml
IL-6	Biosource	Serum	100µl	0.104 fg/ml
TNFα	Biosource	Serum	100µl	0.112 fg/ml

proportion to the amount of IL-2 bound in the initial step. The color development is stopped and the intensity of the color is measured.

2.4.1.1 Procedure:

The materials were provided by R&D Systems and accompanied the kit. The IL-2 standard curve was generated by preparing doubling dilutions from 2000pg/ml to 31.3pg/ml in the 'calibrator diluent RD5' provided. The first dilution was prepared by reconstituting the 10ng of recombinant human IL-2 with 5 ml of 'calibrator diluent RD5' to give a concentration of 2000pg/ml. This stock solution was left to stand for 15 minutes before the dilutions were made.

After the samples needed to generate the standard curve were made, 100µl of Assay Diluent RD1A was added to the wells prior to adding the samples (the supernatants resulting from the PHA stimulation) and standards. 100µl of standard or sample were added to the wells in duplicate, mixed, covered and incubated at room temperature for 2 hours.

Each well was then washed with the wash buffer provided by alternately filling and aspirating the wells 3 times. After the final wash the plate was inverted and blotted on paper towel to remove any remaining liquid.

200µl of IL-2 'conjugate', Horse Radish Peroxidase(HRP) labelled anti-IL-2, were then added to all the wells with a multi-pipette. The plate was covered and incubated at room temperature for 2 hours.

After washing the plate 3 times, 200µl of 'substrate' (chromogen and hydrogen peroxide) were added per well and incubated for 20 minutes.The reaction was stopped by adding 50µl of the 'stop solution' provided. The optical densities (OD) were read at 450nm on a Bio-Rad Microplate Reader.

Standard curves were generated with Log OD's plotted against Log standard IL-2 concentrations. IL-2 concentrations in the culture samples were determined with the Microplate Manager software (Bio-Rad, Mississauga, ON). The minimum detectable dose of the assay was 6.0pg/ml.

2.4.2 General Principle of the IL-4 Immunoassay(I)

The plates were purchased from R&D Systems, Minneapolis, MN. This assay employs the quantitative sandwich enzyme immunoassay technique and is identical to that of IL-2 with the exception that the cytokine assayed for is IL-4.

2.4.2.1 Procedure:

The materials were provided by R&D Systems and were included in the kit. The IL-4 standard curve was generated by preparing doubling dilutions from 2000pg/ml to 31.3pg/ml in the 'calibrator diluent RD5' (culture media diluent) provided. The first dilution was prepared by reconstituting the 10ng of recombinant human IL-4 with 5 ml of 'calibrator diluent RD5' to give a concentration of 2000pg/ml. This stock solution was left to stand for 15 minutes before the dilutions were made.

After the dilutions were made, 200µl of standard or sample (the supernatants resulting from the PHA stimulation) were added to the wells in duplicate, covered and incubated at room temperature for 2 hours.

Each well was then washed with the wash buffer provided by alternately filling and aspirating the wells 3 times. After the final wash the plate was inverted and blotted on paper towel to remove any remaining liquid.

200µl of IL-4 'conjugate' (HRP labelled anti-IL-4) were then added to all the wells with a multi-pipette. The plate was covered and incubated at room temperature for 2 hours.

After washing the plate 3 times, 200µl of 'substrate' were added per well and incubated for 20 minutes. The reaction was stopped by adding 50µl of the 'stop solution' provided. The optical densities (OD) were read at 450nm on a Bio-Rad Microplate Reader.

Standard curves were generated with Log OD's plotted against Log standard IL-4 concentrations. IL-4 concentrations in the culture samples were determined with the Microplate Manager software (Bio-Rad, Mississauga, ON). The minimum detectable dose of the assay was 3.0 pg/ml.

2.4.3 General Principle of the IL-4 Immunoassay (II)

The plates were purchased from Biosource International, Camarillo, CA. This IL-4 assay is slightly different from the R&D Systems one. The use of a different assay was

due to monetary constraints as the Biosource plates are less expensive. As in the previous assay the wells of the plate are coated with anti-IL-4. The difference lies in the fact that once the samples are added to the wells a biotinylated second antibody is added and incubated. During the first incubation the IL-4 antigen binds simultaneously to the immobilized antibody on one site and to the solution-phase biotinylated antibody on a second site.

After removal of the excess second antibody, Streptavidin-Peroxidase (enzyme) is added. This binds to the biotinylated antibody to complete the four-member sandwich. The remainder of the assay is the same as for the R&D plates.

2.4.3.1 Procedure:

The materials were provided by Biosource International and were included in the kit. The IL-4 standard curve was generated by preparing doubling dilutions of IL-4 from 500pg/ml to 3.9pg/ml in the 'standard diluent buffer' provided. The first dilution was prepared by reconstituting the recombinant human IL-4 with 'standard diluent buffer' to give a concentration of 2.5ng/ml. This stock solution was left to stand for 15 minutes before the dilutions were made. Then 100µl of this stock solution was diluted in 400µl to give a concentration of 500pg/ml IL-4.

100µl of the 'standard diluent' were added to each well and an additional 50µl of the 'standard diluent' were added to the zero wells (no IL-4) prior to adding the standards and samples. 50µl of the standards and samples were added in duplicate followed by 50µl

of 'biotinylated anti-IL-4' (biotin conjugate) solution into each well except the blank wells (no anti-IL-4). The contents of each well was mixed by tapping the plate gently. The plate was covered and incubated for 2 hours at 37°C.

The plate was washed with the buffer provided by alternately filling and aspirating the wells. After the final wash the plate was inverted and blotted on paper towel to remove any excess liquid. 100µl of 'streptavidin-HRP working conjugate' were added to all the wells except the blank , the plate was covered and incubated at 37°C for 2 hours.

After the plate was washed 4 times, 100µl of 'stabilized chromogen' were added to each well and the plate incubated in the dark for 25 minutes. The reaction was terminated with 100µl of the 'stop solution' provided. The optical densities (OD) were read at 450nm on a Bio-Rad Microplate Reader.

Standard curves were generated with OD's plotted against standard IL-4 concentrations. IL-4 concentrations in the culture samples were determined with the Microplate Manager software (Bio-Rad, Mississauga, ON). The minimum detectable dose of the assay was 2.0 pg/ml.

2.4.4 General Principle of the IL-1ra Immunoassay

The plates were purchased from R&D Systems, Minneapolis,MN. This assay employs the quantitative sandwich enzyme immuncassay technique and is identical to that of IL-2 and IL-4 with the exception that the cytokine assayed for is IL-1ra.

2.4.4.1 Procedure:

The materials were provided by R&D Systems and were included in the kit. The IL-1ra standard curve was generated by preparing doubling dilutions of IL-1ra from 3000 pg/ml to 46.9 pg/ml in the 'calibrator diluent RD6G' (serum sample diluent) provided. The first dilution was prepared by reconstituting 6ng of recombinant human IL-1ra with 2 ml of 'calibrator diluent RD6G' to give a concentration of 3000 pg/ml. This stock solution was left to stand for 15 minutes before the dilutions were made.

After the dilutions for the standard curve were made, 50µl of 'assay diluent RD1E' were added to each of the wells prior to adding the serum samples and standards. When assaying the culture supernatants this step was not necessary. 200µl of standard or sample (serum or culture supernatant) were added to the wells in duplicate, mixed, covered and incubated at room temperature for 2 hours.

Each well was then washed with the wash buffer provided by alternately filling and aspirating the wells 4 times. After the final wash the plate was inverted and blotted on paper towel to remove any remaining liquid.

200µl of IL-1ra 'conjugate' (HRP labelled anti-IL-1ra) were then added to all the wells with a multi-pipette. The plate was covered and incubated at room temperature for 2 hours.

After washing the plate 4 times, 200µl/well of 'substrate' were added to the plate and incubated for 20 minutes. The reaction was stopped by adding 50µl/well of the 'stop solution' provided. The optical densities (OD) were read at 450nm on a Bio-Rad

Microplate Reader.

Standard curves were generated with Log OD's plotted against Log standard IL-1ra concentrations. IL-1ra concentrations in the serum samples were determined with the Microplate Manager software (Bio-Rad, Mississauga, ON). The minimum detectable dose of the assay was 22.0 pg/ml.

This assay was used for both the serum and culture supernatants. In the serum analysis, the procedure was as stated. In the analysis of the culture supernatants, a dilution of the sample was needed. For the unstimulated supernatants a dilution of 1:10 was used and for the stimulated supernatants a dilution of 1:20 was used. In addition, the standard curve ranged from 2000 pg/ml to 15.6 pg/ml when assaying the culture supernatants. Samples that were above or below the sensitivity of the plate were noted in the tables of data in Appendix B and were omitted from the subsequent calculations. When considering the raw data, that is, the values obtained from the ELISA, for IL-1ra, the dilution allowed for the highest value measured to be 40,000 pg/ml, although, extrapolation of the standard curve gave values in the 60,000 pg/ml range. Values that are seen in the tables as greater than signs (>) indicate that these samples exceeded the 3.0 optical density of the plate reader.

2.4.5 General Principle of the IL-1β UltraSensitive Immunoassay

The plates were purchased from Biosource International, Camarillo, CA. The principle of this assay is the same as that of the Biosource IL-4 ELISA with the exception

that the cytokine assayed for was IL-1β. This assay was used to measure IL-1β in serum samples.

2.4.5.1 Procedure:

The materials were provided by Biosource International and were included in the kit. The IL-1β standard curve was generated by preparing doubling dilutions of IL-1β from 10 pg/ml to 0.08 pg/ml in the 'standard diluent buffer' provided. The first dilution was prepared by reconstituting 5.9 ng of recombinant human IL-1β with 2.36 ml of 'standard diluent buffer' to give a concentration of 2500 pg/ml. This stock solution was left to stand for 15 minutes before the dilutions were made. Then 25µl of this stock solution was diluted in 6.23ml to give a concentration of 10 pg/ml IL-1β.

150µl of the 'standard diluent' were added to the blank wells and 100µl was added to the zero wells (0 pg/ml). 100µl of the standards and samples (serum) were added in duplicate followed by 50µl of 'biotinylated anti-IL-1β' (biotin conjugate) solution into each well except the blank wells. The contents of the wells were mixed by tapping the plate gently. The plate was covered and incubated for 2 hours at room temperature.

The plate was washed 4 times with the buffer provided, inverted and blotted on paper towel after the final wash. 100µl of 'streptavidin-HRP working conjugate' were added to all the wells except the blank wells , the plate was covered and incubated at room temperature for 30 minutes.

After the plate was washed 4 times, 100µl/well of 'stabilized chromogen' were

added and incubated in the dark for 25 minutes. The reaction was terminated with 100µl/well of the 'stop solution' provided. The optical densities (OD) were read at 450nm on a Bio-Rad Microplate Reader. The blank wells contained only chromogen and stop solution and were used to blank the plate reader.

Standard curves were generated with OD's plotted against standard IL-1β concentrations. IL-1β concentrations in the serum samples were determined with the Microplate Manager software (Bio-Rad, Mississauga, ON). The minimum detectable dose of the assay was 0.083 pg/ml. Samples that were above or below the sensitivity of the plate were noted in the tables of data in Appendix B and were omitted from the subsequent calculations.

2.4.6 General principles of the Il-1β Immunoassay

The plates were purchased from Biosource International, Camarillo, CA. The principle of this assay is the same as that of the Biosource IL-4 ELISA with the exception that the cytokine assayed for was IL-1β. This assay was used to measure IL-1β in culture supernatants.

2.4.6.1 Procedure

The materials were provided by Biosource International and were included in the kit. The IL-1β standard curve was generated by preparing doubling dilutions of IL-1β from 2000 pg/ml to 3.9 pg/ml in the 'standard diluent buffer' provided. The first dilution

was prepared by reconstituting the recombinant human IL-1β with 'standard diluent buffer' to give a concentration of 2500 pg/ml. This stock solution was left to stand for 15 minutes before the dilutions were made.

100µl of the 'standard diluent' were added to the blank wells and 50µl were added to the zero wells (0 pg/ml). 50µl of the standards and samples (culture supernatants, nonstimulated supernatants were assayed neat and stimulated supernatants were diluted 1:10) were added in duplicate followed by 50µl of 'biotinylated anti-IL-1β' (biotin conjugate) solution into each well except the blank wells. The contents of the wells were mixed by tapping the plate gently. The plate was covered and incubated for 1 hour at room temperature.

The plate was washed with the buffer provided 4 times, inverted and blotted on paper towel after the final wash. 100µl of 'streptavidin-HRP working conjugate' were added to all the wells except the blank wells, the plate was covered and incubated at room temperature for 30 minutes.

After the plate was washed 4 times, 100µl/well of 'stabilized chromogen' were added and incubated in the dark for 25 minutes. The reaction was terminated with 100µl/well of the 'stop solution' provided. The optical densities (OD) were read at 450nm on a Bio-Rad Microplate Reader. The blank wells contained only chromogen and stop solution and were used to blank the plate reader.

Standard curves were generated with OD's plotted against standard IL-1β concentrations. IL-1β concentrations in the culture samples were determined with the

Microplate Manager software (Bio-Rad, Mississauga, ON). The minimum detectable dose of the assay was 1.0 pg/ml. Note: the samples that were above or below the sensitivity of the plate were noted in the tables of data in Appendix B and were omitted from the subsequent calculations.

2.4.7 General Principle of the IFN-γ Immunoassay

The plates were purchased from R&D Systems, Minneapolis, MN. This assay employs the quantitative sandwich enzyme immunoassay technique and is identical to that of IL-2 with the exception that the cytokine assayed for is IFN-γ.

2.4.7.1 Procedure

The materials were provided by R&D Systems and accompanied the kit. The IFN-γ standard curve was generated by preparing doubling dilutions from 1000 pg/ml to 15.6 pg/ml in the 'calibrator diluent RD6R' provided. The first dilution was prepared by reconstituting 5.0 ng of recombinant human IFN-γ with 5 ml of 'calibrator diluent RD6R' to give a concentration of 1000 pg/ml. This stock solution was left to stand for 15 minutes before the dilutions were made.

After the samples needed to generate the standard curve were made, 50µl of 'Assay Diluent RD1Z' were added to the wells prior to adding the samples (the supernatants resulting from the PHA stimulation) and standards. 200µl of standard or sample were added to the wells in duplicate, mixed, covered and incubated at room temperature for 2.5

hours.

Each well was then washed with the wash buffer provided by alternately filling and aspirating the wells 4 times. After the final wash the plate was inverted and blotted on paper towel to remove any remaining liquid.

200µl of IFN-γ 'conjugate', Horse Radish Peroxidase (HRP) labelled anti-IFN-γ, were then added to all the wells with a multi-pipette. The plate was covered and incubated at room temperature for 2 hours.

After washing the plate 4 times, 200µl of 'substrate' (chromogen and hydrogen peroxide) were added per well and incubated for 20 minutes. The reaction was stopped by adding 50µl of the 'stop solution' provided. The optical densities (OD) were read at 450nm on a Bio-Rad Microplate Reader.

Standard curves were generated with Log OD's plotted against Log standard IFN-γ concentrations. IFN-γ concentrations in the culture samples were determined with the Microplate Manager software (Bio-Rad, Mississauga, ON). The minimum detectable dose of the assay was 3.0 pg/ml.

2.4.8 General Principle of the IL-6 Ultrasensitive Immunoassay

The plates were purchased from Biosource International, Camarillo, CA. The principle of this assay is the same as that of the Biosource IL-4 ELISA with the exception that the cytokine assayed for was IL-6.

2.4.8.1 Procedure

The materials were provided by Biosource International and were included in the kit. The IL-6 standard curve was generated by preparing doubling dilutions of IL-6 from 10 pg/ml to 0.156 pg/ml in the 'standard diluent buffer' provided. The first dilution was prepared by reconstituting the recombinant human IL-6 with 'standard diluent buffer' to give a concentration of 310 pg/ml. This stock solution was left to stand for 15 minutes before the dilutions were made.

150µl of the 'standard diluent' were added to the blank wells and 100µl was added to the zero wells (0 pg/ml). 100µl of the standards and serum samples were added in duplicate. The plate was covered and incubated for 3 hours in a 37°C chamber.

The plate was washed with the buffer provided 4 times, inverted and blotted on paper towel after the final wash. 100µl of 'biotinylated anti-IL-6' (biotin conjugate) solution into each well except the blank wells. The plate was covered and incubated for 45 minutes at room temperature.

The plate was washed with the buffer provided 6 times, inverted and blotted on paper towel after the final wash. 100µl of 'streptavidin-HRP working conjugate' were added to all the wells except the blank wells , the plate was covered and incubated at room temperature for 45 minutes.

After the plate was washed 4 times, 100µl/well of 'stabilized chromogen' were added and incubated in the dark for 25 minutes. The reaction was terminated with 100µl/well of the 'stop solution' provided. The optical densities (OD) were read at 450nm

on a Bio-Rad Microplate Reader. The blank wells contained only chromogen and stop solution and were used to blank the plate reader.

Standard curves were generated with OD's plotted against standard IL-6 concentrations. IL-6 concentrations in the serum samples were determined with the Microplate Manager software (Bio-Rad, Mississauga, ON). The minimum detectable dose of the assay was 0.104 pg/ml.

2.4.9 General Principle of the TNF-α Ultrasensitive Immunoassay

The plates were purchased from Biosource International, Camarillo, CA. The principle of this assay is the same as that of the Biosource IL-4 ELISA with the exception that the cytokine assayed for was TNF-α.

2.4.9.1 Procedure

The materials were provided by Biosource International and were included in the kit. The TNF-α standard curve was generated by preparing doubling dilutions of TNF-α from 32 pg/ml to 0.5 pg/ml in the 'standard diluent buffer' provided. The first dilution was prepared by reconstituting the recombinant human TNF-α with 'standard diluent buffer' to give a concentration of 2000 pg/ml. This stock solution was left to stand for 15 minutes before the dilutions were made.

100µl of the 'standard diluent' were added to the blank wells and to the zero wells(0 pg/ml). 100µl of the standards and serum samples were added in duplicate. The

plate was covered and incubated for 3 hours in a 37°C chamber.

The plate was washed with the buffer provided 4 times, inverted and blotted on paper towel after the final wash. 100µl of 'biotinylated anti-TNF-α' (biotin conjugate) solution into each well except the blank wells. The plate was covered and incubated for 1 hour at 37°C.

The plate was washed with the buffer provided 6 times, inverted and blotted on paper towel after the final wash. 100µl of 'streptavidin-HRP working conjugate' were added to all the wells except the blank wells , the plate was covered and incubated at room temperature for 45 minutes.

After the plate was washed 4 times, 100µl/well of 'stabilized chromogen' were added and incubated in the dark for 10 minutes. The reaction was terminated with 100µl/well of the 'stop solution' provided. The optical densities (OD) were read at 450nm on a Bio-Rad Microplate Reader. The blank wells contained only chromogen and stop solution and were used to blank the plate reader.

Standard curves were generated with OD's plotted against standard TNF-α concentrations. TNF-α concentrations in the serum samples were determined with the Microplate Manager software (Bio-Rad, Mississauga, ON). The minimum detectable dose of the assay was <0.112 pg/ml.

2.5 Hormone Radioimmunoassays (RIA)

The hormones in this study were measured by commercial RIA assay. Table 2.2

Table 2.2. Summary of the hormones measured for each of the volunteers with reference to source of the RIA, sample and sample size, and sensitivity of the assay.

Hormone	Commercial Source	Sample	Sample Size	Sensitivity
17β-Estradiol	ICN Biomedicals	Serum	50µl	5 pg/ml
Progesterone	ICN Biomedicals	Serum	100µl	0.1 ng/ml
Luteinizing Hormone	Immunocorp	Serum	100µl	0.46 U/L
Testosterone	ICN Biomedicals	Serum	50µl	0.05 ng/ml

summarizes the source of the RIA, the sample assayed, the sample volume used, and the sensitivity of the assay. All samples were run in parallel and all the samples collected for one volunteer during the menstrual cycle were run on the same kit. Specific details about the procedures can be found in the following sections.

2.5.1 General Principle of the 17β-Estradiol RIA

The RIA kit was purchased from ICN Biomedicals, California. The Estradiol(E2) RIA kit detects the unconjugated form of the steroid. Radioimmunoassays depend on the ability of an antibody to bind its antigen. To quantitate the antigen, the radioactive and nonradioactive form of the antigen compete for binding sites on a specific antibody. As more nonradioactive antigen is added, less radioactive antigen remains bound until equilibrium between free and antibody bound antigen occurs.

In the assay a limited amount of specific antibody is reacted with ^{125}I labelled estradiol. Upon addition of an increasing amount of estradiol, a correspondingly decreased amount of labelled estradiol is bound to the antibody due to the competitive binding principle. After separating the antibody bound ^{125}I estradiol from the unbound, the amount of radioactivity in the antibody-hormone complex is measured and used to construct the standard curve from which the unknown sample concentrations are measured.

2.5.1.1 RIA Protocol:

The materials were provided by ICN Biomedicals and accompanied the kit. The

appropriate number of disposable glass 2ml tubes were labelled and placed in a test tube rack. All the reagents and samples were allowed to come to room temperature before use. The standard curve was constructed using the commercial standards provided and consisted of concentrations 0, 10, 30, 100, 300, 1000, and 3000 pg/ml. To the Non Specific Binding (NSB) tubes 500µl of 'diluent buffer' were added and 50µl of the 0 pg/ml standard. The remaining tubes received 50µl each of standards or samples (serum) in duplicate.

To each tube 500µl of Estradiol ^{125}I were added followed by 500µl of 'anti-estradiol' to all the tubes except the NSB ones. The tubes were vortexed and incubated at 37°C for 90 minutes.

After the incubation 500µl of 'precipitant solution' were added to all the tubes. The tubes were spun at 1000 x g for 15 minutes and supernatants aspirated. The tubes were counted in a gamma counter calibrated for ^{125}I for 2 minutes and the CPM (counts per minute) recorded.

Standard curves for 'percent bound (%B)' versus 'log concentration' were generated with the InPlot (GraphPad, San Diego) software. Estradiol concentrations in the serum samples were determined by the software used. The minimum detectable dose of the assay was 5 pg/ml, as determined by the manufacturer. The formula for %B is:

$$\%B = \frac{CPM(sample) - CPM(NSB)}{CPM(0\ pg/ml) - CPM(NSB)} \times 100$$

2.5.2 General Principle of the Progesterone RIA

The assay was purchased from ICN Biomedicals, California. This assay employs the same quantitative technique as the 17β-estradiol RIA and is identical to that of E2 with the exception that the hormone assayed for is progesterone.

2.5.2.1 RIA Protocol:

The materials were provided by ICN Biomedicals and accompanied the kit. The appropriate number of disposable glass 2ml tubes were labelled and placed in a test tube rack. All the reagents and samples were allowed to come to room temperature before use. The standard curve was constructed using the commercial standards provided and consisted of concentrations 0, 0.2, 0.5, 2.0, 5.0, 10.0, 25.0, and 50.0 ng/ml. To the Non Specific Binding (NSB) tubes 500µl of 'diluent buffer' were added and 100µl of the 0 ng/ml standard. The remaining tubes received 100µl each of standards or samples (serum)in duplicate.

To each tube 500µl of 'anti-Progesterone' were added to all the tubes except the NSB ones, followed by 200µl of progesterone ^{125}I. The tubes were vortexed and incubated at 37°C for 60 minutes.

After the incubation 500µl of 'precipitant solution' were added to all the tubes. The tubes were spun at 1000 x g for 15 minutes and supernatants aspirated. The tubes were counted in a gamma counter calibrated for ^{125}I for 2 minutes and the CPM recorded.

Standard curves for 'percent bound' (%B)' versus 'log concentration' were generated

with the InPlot (GraphPad, San Diego) software. Progesterone serum values were determined by the software used. Calculation of %B is the same as for Estradiol. The minimum detectable dose of the assay was 0.1 ng/ml, half the lowest standard.

2.5.3 General Principle of the Testosterone RIA

The assay was purchased from ICN Biomedicals, CA. This assay employs the same technique as the estradiol and progesterone ones. The protocol is very similar.

2.5.3.1 RIA Protocol:

The materials were provided by ICN Biomedicals and accompanied the kit. The appropriate number of disposable glass 2ml tubes were labelled and placed in a test tube rack. All the reagents and samples were allowed to come to room temperature before use. The standard curve was constructed using the commercial standards provided and consisted of concentrations 0, 0.1, 0.25, 0.5, 1.0, 2.5, 5.0, and 10.0 ng/ml. To the Non Specific Binding (NSB) tubes 500µl of 'diluent buffer' were added and 50µl of the 0 ng/ml standard. The remaining tubes received 50µl each of standards or samples (serum) in duplicate.

To each tube 100µl of 'SBGI solution' (sex binding globulin inhibitor) were added, followed by 500µl of Testosterone ^{125}I. Following the addition of Testosterone, 500µl of 'anti-Testosterone' were added to each of the tubes except the NSB ones. The tubes were vortexed and incubated at 37^{0}C for 120 minutes.

After the incubation 100µl of 'second antibody' were added, the tubes were then vortexed and incubated at 37°C for 60 minutes. The tubes were then spun at 2300 rpm for 15 minutes and supernatants aspirated. The tubes were counted in a gamma counter calibrated for ^{125}I for 2 minutes and the CPM recorded.

Standard curves for 'percent bound' (%B) versus 'log concentration' were generated with the InPlot (GraphPad, San Diego) software. Testosterone serum values were determined by the software used. Calculation of %B is the same as E2. The minimum detectable dose of the assay was 0.05 ng/ml, half the lowest standard.

2.5.4 General Principle of the Luteinizing Hormone RIA

The assay was purchased from Immunocorp, Montreal. The Immunocorp coated tube assay is a two site immunoradiometric assay that utilizes a solid phase coupled antibody and a second antibody labelled with ^{125}I.

2.5.4.1 RIA Protocol:

The materials were provided by Immunocorp and accompanied the kit. The coated tubes provided were labelled and placed in a test tube rack. All the reagents and samples were allowed to come to room temperature before use. The standard curve was constructed using the commercial standards provided and consisted of concentrations 0, 2, 5, 20, 50, and 100 U/L. In addition, there were 2 non-coated tubes in the assay that were used to determine total radioactivity.

To each tube 100µl of standard, control, or sample (serum) were added in duplicate , followed by 100µl of a mixture consisting of equal volumes of 'Anti-hLH ^{125}I and mouse IgG'. The tubes were vortexed and incubated at 37°C for 60 minutes.

After the incubation the tubes were washed twice by alternately filling with 4ml wash buffer and aspirating. After the final wash the tubes were inverted for 5 minutes, then counted in a gamma counter calibrated for ^{125}I for 2 minutes and the CPM recorded.

Standard curves 'CPM' versus 'concentration (U/L)' were generated with the InPlot (GraphPad, San Diego) software. LH serum values were determined by the software used. The sensitivity of the assay was 0.46 U/L.

2.6 C'H50 and C3

One assay tested the percent of C'H50 in the serum of the volunteers. The analysis was performed by student, Trina Fournier, as part of a another project. The method of Mayer was used (Kabat and Mayer, 1971). The coefficient of variation for the intra-assay control was 2.1%, the mean for inter-assay variation was 7.0%.

The concentration of serum C3 was measured by nephelometry in a Beckman automated Specific Protein Analyzer (Beckman GH-3427 Array 360, Model 4480) using Beckman C3 kits by Meredith Steeves. The intra-assay coefficient of variation values were less than 5% and inter-assay values less than 8%. Normal values are given as 0.88-2.01 mg/ml.

Both of these assays were done with the sample set I had created. The results for

these factors are given in Tables B.1-B.15 in Appendix B. There will be a brief discussion in the last chapter of this document.

2.7 Coefficient of Variation (CV)

The CV was determined by including the same sample on all the ELISA plates and hormone assays carried out. This sample had been prepared identical to the volunteer samples, aliquoted, and stored at -70°C. The concentration of the sample read from the standard curve was used to calculate the CV. As a control, this sample could be used to examine inter-assay variation day to day and technique on a particular day.

2.8 Statistical Methods

2.8.1 Coefficient of variation

The coefficients of variation for the ELISA and Hormone assays were determined by the formula:

$$\frac{\text{Standard Deviation}}{\text{Mean}} \times 100$$

The standard deviation and mean were calculated using the statistical software InStat (GraphPad, San Diego).

2.8.2 F Test

The F test was used to assess variation over time comparing the males to females, testing equality of variances between the two groups. This test is done manually and the resulting F value is then referenced in statistical tables to determine if it is significant at α=0.05. If the F value is large then one group has significantly larger variation than the other. If the F value is not found to be significant then we have no evidence against the hypothesis that they are the same. All data gathered on the volunteers was used to calculate this value. The following formula was used:

$$\frac{\sum_{i=1}^{9} (n_i-1)(s_i^2)/ \sum_{i=1}^{9} (n_i-1)}{\sum_{i=1}^{4} (n_i-1)(s_i^2)/ \sum_{i=1}^{4} (n_i-1)} \sim F$$

The numerator of the above equation represents the variation of the cycling females.
The denominator of the above equation represents the male variation.

Σ the sum of
n_i the number of data values for each volunteer
s_i^2 the variance for each volunteer squared

2.8.3 t Test

The t test (two-tailed) was used to compare the means of the cycling females to the male controls for each cytokine measured. This statistical test is used when the two samples are independent and when they have been drawn from a normally distributed population. Due to the large number of samples we obtained from the volunteers we were able to assume normal distribution within the sample set. All the data obtained from the

males and the raw data for one full cycle in the females was used to compare the means. The statistical significance level was set at 5% (α=0.05) which is the probability of not rejecting the hypothesis that the two means are the same when in fact they are different. The t test was calculated with the statistical software SPSS, Version 6.1.3 and the computer software automatically corrected calculations when the variances were found to be unequal.

The objective of the paired t test is to eliminate as many sources of extraneous variation as possible by making the pairs similar with respect to as many variables as possible (Daniel, 1995). The use of the paired t test was necessary for comparison of the phases of the menstrual cycle. From nine cycling women we obtained 9-14 samples over the course of one full menstrual cycle, thus the samples were not independent of one another within the cycle. As a result of the multiple consecutive samples the paired t test was used to control for the extraneous variables helping to determine if the means of various phases of the cycle were different. The data gathered on the females, converted to portion of phase, for one full cycle was used to calculate the p values, data obtained from the second cycle was not used. The statistical software package InStat (GraphPad, San Diego) was used. No correction factor for multiple comparisons within the cycle was used.

2.8.4 Multiple Regression

Multiple regression used to analyze the results was done by using the statistical

software SPSS (Version 6.1.3, 1990-1995). The purpose of multiple regression is to predict the response variable when the other independent variables, which influence the response variable, are known. The software first calculates the multiple correlation coefficient and tests whether or not it is significantly different from zero. If a significant difference is found then there is a relationship between the response variable and the independent variables and this correlation is then used to predict the response variable. If the correlation coefficient is between -0.5 - +0.5 the relationship is weak and the resultant prediction is not reliable. In addition to giving the correlation coefficient the computer also generates the coefficient of determination, the square of the correlation coefficient, and this indicates the amount of variation in the response variable that is due to the influence of the independent variables. This test was done using the original data gathered on the female volunteers in one full cycle.

CHAPTER III

RESULTS

In this chapter I will present the data I have gathered on the volunteers. I will discuss the cycling volunteers Phase I data and the hormone results from Phase II. In addition, the comparison done on the male controls will also be presented. Data from the cytokines that were studied will be presented along with the statistical analysis. All original data can be found in Appendix B. The organization of the results will follow the aims presented in the introduction portion of this document.

3.1 Establishment of Stimulation Conditions

The culture conditions for the various cytokines were determined using PHA stimulation. We wanted optimal conditions for proliferation based on amount of PHA, amount and type of serum added and optimal time for harvest. To do this we examined the differences in proliferation (measured as counts per minute) at different times to determine the optimum time for harvesting cells when quantitating IL-2, IL-4, and INF-γ. We also wanted to see if there was a difference in the response when different media were used.

In an initial experiment to address this question we stimulated PBMC in RPMI, containing various amounts of FCS or autologous serum. Varying amounts of a PHA solution, at a final concentration of 0.1 mg/ml, were added. This solution was obtained

from the Clinical Diagnostic Immunology Laboratory in St. John's, and was used for the initial experiment. Radioactive thymidine was added 15 hours before the cultures were harvested. The counts per minute results of these experiments are seen in Tables 3.1a-d.

Analysis of the cpm generated at different concentrations of PHA and different lengths of cultures with various media indicated that 39 and 72-hour stimulations were inappropriate because the cells were dividing. Conversely, 15 hours was rejected due to the fact that the cells had not yet begun to divide (were not incorporating the thymidine). The times chosen for potential future work were that of 18 and 24 hours. It was determined that there was little difference in the counts when using either autologous or FCS in the media across all concentrations of PHA tested. Consequently autologous serum was discarded as a possible media supplement. We felt that due to the large amount of blood needed to get PBMCs for the many cultures and serum for cytokine and hormone measurements, it was not possible to obtain the amount of serum needed from each individual to include it in the media. Finally, since it was our intention to try and control for extraneous variables and keep all volunteer cultures as similar as possible, it was thought that the addition of autologous serum would circumvent this aim.

In response to the data obtained from the first set of experiments we purchased new PHA and conducted further experiments using the new supply and various concentrations of FCS. The new PHA (20 mg) (Boehringer Mannheim) was reconstituted in 4 ml of sterile water to give a stock solution of 5 mg/ml. The results of this experiment demonstrated that the new PHA did induce lymphocyte stimulation.

Table 3.1a. Time study measuring incorporation of ^{3}H-thymidine. Cells cultured in RPMI with varying concentrations of serum. No PHA added. Values are seen in cpm.

Time Hrs	10% FCS	1% FCS	10% O.S.*	1% O.S.
15	95	83	147	83
18	86	61	95	85
24	258/139[a]	135	215/132	91/196
39	92	93	103	88
72	168	108/275	131	98/404

[a] These values indicate the inability to average due to the marked difference in parallels.
* Autologous serum

Table 3.1b. Time study measuring incorporation of ^{3}H-thymidine at 25 µg/ml PHA. Values are seen in cpm.

Time Hrs	10% FCS	1% FCS	10% O.S.*	1% O.S.
15	-	-	-	-
18	131	95	128	8
24	282	136/275[a]	164/409	184
39	4913	1394	9126	7736
72	13972	7806	14133	19802

[a] These values indicate the inability to average due to the marked difference in parallels.
* Autologous serum

Table 3.1c. Time study measuring incorporation of ^{3}H-thymidine at 50 µg/ml PHA. Values are seen in cpm.

Time Hrs	10% FCS	1% FCS	10% O.S.*	1% O.S.
15	107	103	109	83
18	137	111	126	94
24	170/324[a]	128	215	105/203
39	6319	1135	11776/7362	7021
72	10845/8010	9156	16789	18008

[a] These values indicate the inability to average due to the marked difference in parallels.
* Autologous serum

Table 3.1d. Time study measuring incorporation of ^{3}H-thymidine with 75 µg/ml PHA. Values are seen in cpm.

Time Hrs	10% FCS	1% FCS	10% O.S.*	1% O.S.
15	-	-	-	-
18	126	98	131	97
24	196	117	178/279[a]	162
39	1838	721	9028	6207
72	14620	9808	18560	17993

[a] These values indicate the inability to average due to the marked difference in parallels.
* Autologous serum

It was decided that future cultures would be stimulated for 24 hours.

We then focused on the amounts of cytokine produced in culture when stimulated for 24 hours with various amounts of PHA and FCS in the media. In the first experiment we incubated PBMC with two concentrations of PHA (10 and 50 μg/ml) in media containing 1 and 10% FCS for 24 hours. IL-2, IL-4 and IFNγ were then assayed using the harvested culture supernatants. These results are presented in Table 3.2 In short, IFNγ was not found to be present in any of the supernatants, while IL-2 and IL-4 were found in varying concentrations. It is apparent that stimulation with 10 μg/ml of PHA at either concentration of FCS results in very low amounts of IL-2 and no IL-4. When considering cytokine values for the 50 μg/ml PHA stimulation, 1% FCS gives greater values for IL-2 and IL-4 50% of the time, whereas 10% FCS gives higher values 35% of the time.

A followup experiment was conducted to confirm this and the results are given in Table 3.3. In this experiment we wanted to explore stimulating at higher concentrations of PHA, 75 and 100 μg/ml. It was decided that the cytokine concentration that resulted after such stimulations were too high.

From all these results we concluded that for IL-2 and IL-4 we would use 1%FCS in the media and stimulate at 50 μg/ml for 24 hours.

The last experiment that was carried out concentrated on IFNγ. We had observed in publications that stimulating for 48 or 72 hours resulted in measurable amounts of IFNγ. Based on this information we then proceeded to test this for our study.

Table 3.2. Cytokine concentrations in culture supernatants using various stimulation conditions for 24 hours.

Subject	Cytokine	1% FCS		10% FCS	
	pg/ml	10*	50*	10	50
1	IL-2	1.5	208.0	10.3	29.0
	IL-4	-	18.4	-	-
2	IL-2	-	33.9	2.7	45.0
	IL-4	-	-	-	20.6
3	IL-2	9.7	345	15.7	105.1
	IL-4	-	184.5	-	95.8
4	IL-2	17.8	132.5	16.7	196.1
	IL-4	-	23.9	-	59.1

* µg/ml PHA concentration.

In this experiment we stimulated PBMC for 48 and 72 hours with the two concentrations of PHA, 10, and 50 μg/ml in media supplemented with 1% FCS. The results, in Table 3.4, show that optimal conditions for IFNγ production were cultures with 50 μg/ml of PHA for 48 hours. Thus we decided that in order to obtain IFNγ we would stimulate with 50 μg/ml PHA for 48 hours in 1% FCS in this study.

Table 3.3. Cytokines in culture supernatants after stimulation of PBMC with three different concentrations of PHA for 24 hours in media supplemented with 1% FCS .

Cytokine pg/ml	50 μg/ml PHA	75 μg/ml PHA	100 μg/ml PHA
IL-2	97.5	114.4	178.3
IL-4	45.0	53.1	57.9

Table 3.4. IFNγ concentrations in culture supernatants when PBMC are stimulated for 48 and 72 hours with two different concentrations of PHA in media supplemented with 1% FCS.

48 hours		72 hours	
10 μg/ml PHA	50 μg/ml PHA	10 μg/ml PHA	50 μg/ml PHA
60.3 pg/ml	196.8 pg/ml	60.3pg/ml	150.6 pg/ml

3.2 Creation of a Sample Set from Female Volunteers

Volunteers were chosen based on the conditions described in 2.11. Time of ovulation and length of cycle were recorded for each of the 21 females.

Table 3.5 shows that only 8 females were eligible for Phase II based on the conditions set in the beginning of the study. Some of the volunteers did not ovulate whereas others were not suitable due to the length of the cycle or inconsistent ovulation days.

The volunteers that participated in Phase II of the study were chosen based on the results in Table 3.5. The data indicates that volunteers 21.04.70, 01.04.75, 22.09.75, 15.12.73, 06.09.72, 29.08.71, 22.11.75, and 30.04.65 had relatively stable menstrual cycles and ovulated around the same time of the cycle with respect to their individual cycles. Volunteer 09.10.64 does not have any data available as she was recruited after Phase II was started.

This group of volunteers were fairly uniform in that they were all healthy females who adhered to the stringent set of criteria established at the beginning of the study. They were between the ages of 19-29, they were not taking birth controls pills, they had no major medical problems, they were of normal height and weight, they were not taking any medications and they had regular menstrual cycles.

This group was very cooperative in providing the samples needed for the study. The only case where we encountered some problems with obtaining samples was when 30.04.65 went on vacation with her husband and two children, preventing us from

Table 3.5. Ovulation data and length of cycle of female volunteers participating in Phase I.

	Cycle 1		Cycle 2		Cycle 3	
Vol	Length[a]	Ovul[b]	Length	Ovul	Length	Ovul
21.04.70[c]	28	16	28	14	27	14
13.08.69	33	-	32	21	32	-
01.04.75[c]	28	17	27	14	32	15
22.09.75[c]	32	-	27	15	31	19
15.11.73	26	-	28	18	-	-
22.01.75	-	-	-	-	-	-
06.11.75	27	-	28	13	-	15
15.12.73[c]	29	15	26	13	32	16
03.01.72	31	-	-	-	-	-
06.09.72[c]	26	14	29	17	25	15
29.08.71[c]	27	15	26	14	27	14
06.03.68	29	15	26	14	-	14
22.11.75[c]	31	19	30	19	29	18
04.10.75	34	-	-	-	-	-
04.10.75	-	-	-	-	-	-
30.08.71	35	-	14	-	-	-
30.04.65[c]	30	17	28	17	29	17
25.01.67	-	15	-	-	-	-
18.02.75	29	16	-	17	-	-

[a] Total length of menstrual cycle
[b] Day of ovulation
[c] Volunteer in Phase II

obtaining samples for a week. It was unfortunate that this volunteer was absent for the study at this time as we lost data on her cycle at the time surrounding ovulation.

In Phase II of the study, the magnitude of symptom severity was measured and daily symptoms were monitored with the aid of the Menstrual Cycle Diary (Appendix A). The symptom severity, calculated from the data the volunteers gave on their diary's, was divided into 2 mean ratings, one for the Intermenstrual phase and one for the Luteal phase. As shown by the results obtained from the calender completed by the volunteers there is no significant difference ($p = 0.4375$) between the Intermenstrual mean and Luteal mean for symptoms using the Wilcoxan signed rank test (Table 3.6). This indicates that the volunteers as a group were not experiencing premenstrual syndrome. This method is similar to comparisons others have used to determine if the volunteers were experiencing PMS (Thys-Jacobs and Alvir, 1995).

The length of menstrual cycle for the volunteers in Phase II ranged from 26-31 days and the time of ovulation ranged from day 13 to 19. The mean cycle length was 28.7 with a standard deviation of 1.66 and the mean day for ovulation was day 15.8 $\pm$ 1.99 (Table 3.6).

3.2.1 Sampling Days:

Each of the nine women who participated in the study was bled between 9-15 times during the course of their cycle. Specific sampling days are seen in Figure 3.1. In this figure the cycle is presented in proportion of phase whereby each phase, the follicular

Table 3.6. Cycle data during Phase II for 9 females.

Volunteer	Cycle Length	Day of Ovulation	Mean Symptom Severity	
			Inter-Menstrual[a]	Luteal[b]
21.04.70	29	17	0.857	0
01.04.70	27	16	-	-
22.09.75	26	14	3.85	6.43
15.12.73	29	14	2.14	2.14
06.09.72	28	16	-	-
29.08.71	29	15	1.86	1.29
22.11.75	31	19	2.14	3.14
30.04.65	31	18	0	1.29
09.10.64	28	13	0.857	0.571
Mean	28.7	15.8	1.56*	2.12*
S.D	1.66	1.99	1.36	2.16

[a] Intermenstrual phase mean is defined as the mean symptom score during the 7 days after menstruation had begun.

[b] Luteal phase mean is defined as the mean symptom score of the 7 days before menstruation.

* Wilcoxan signed Rank test p=0.4375.

and luteal, is designated a value of 1, and further subdivided according to the proportion of one. A more in depth explanation of how this was done is seen later in the text (p.117). It is apparent from the figure that the number of samples obtained from the volunteers varies; more samples were collected in the luteal phase of the cycle. The points seen after the luteal phase constitute those samples obtained at the beginning of the second follicular phase and were calculated assuming ovulation occurred. In addition, the red line indicates time of ovulation with three individuals bled at this time.

3.3 Creation of a Sample Set from Male Controls

A control group was established that met the same criteria as the females with the exception of the cycle criteria. This group consisted of four males between the ages of 20-25 who were healthy. Males were chosen as controls since they are not known to cycle with respect to the gonadal hormones examined in this study. These males were sampled between 9 and 12 times over the course of 4-5 weeks, the same weeks that the females were sampled.

In addition to providing serum and cells, the volunteers were asked to complete a self-assessment questionnaire each time they were bled. This questionnaire is similar to the one that the cycling women had to complete and can be found in Appendix A. Based on these questionnaires it was apparent that the group was healthy throughout the study. It is important to note however that one of the males, 17.02.75, contracted a cold during the study and as a result of this we refrained from sampling him during this time.

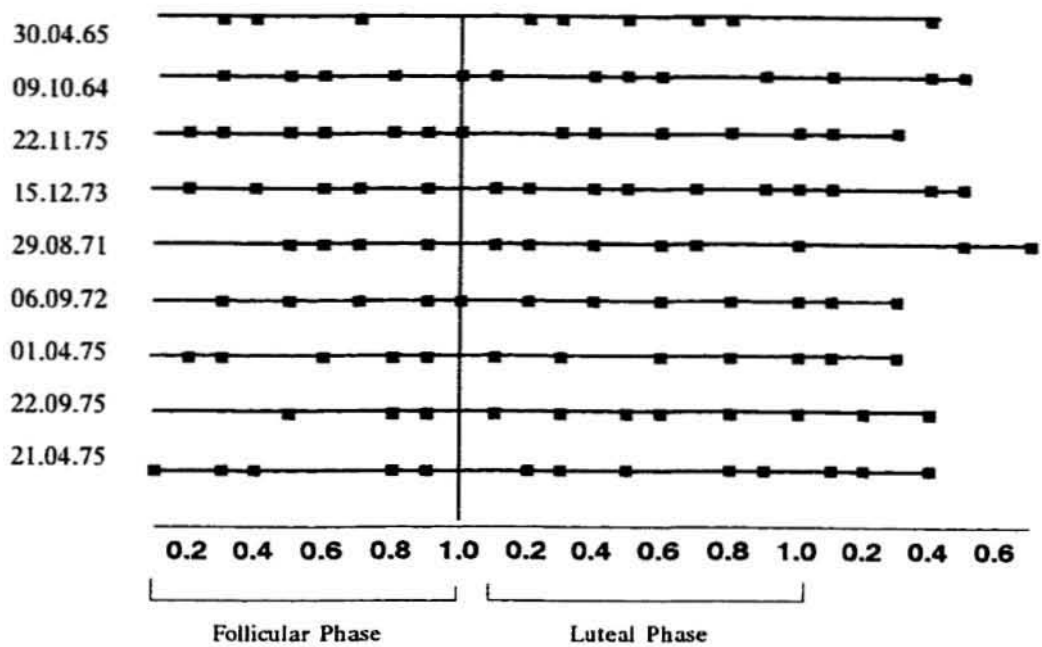

Figure 3.1. Day of sampling for each of the nine volunteers expressed as proportion of phase. Menses commenced on proportionate day 0.1 of the follicular phase; ovulation occurred at proportionate day 1.0 of the follicular phase. For definitions of proportionate day, see text, p.116.

3.4 Hormone Profiles

Serum progesterone, luteinizing hormone and estrogen were measured on the cycling volunteers throughout Phase II of the study. The hormone testosterone was measured on each of the samples obtained from the males.

These hormones were then graphed and can be seen in Figures 3.2-3.10. It is apparent from the data that the women were all normal cycling females with characteristic high estrogen in the follicular and luteal phases, LH surge just prior to ovulation and a progesterone peak in the luteal phase. The actual values can be seen in Appendix B.

Based on these values and patterns one may conclude that the females participating in the study were a sample of the normal population.

Volunteers 21.04.70 and 01.04.75 each have a visible LH peak that occurred on day 16 and day 15 respectively. Estrogen is seen to peak in the follicular phases and then again in the luteal phase of each woman. Progesterone characteristically peaks in the luteal phase.

Volunteer 22.11.75 is missing the LH peak most likely due to the fact that she was sampled on day 17 and day 19, day 19 being the day of ovulation, thus the LH peak was missed. Other hormones exhibit the textbook pattern.

Volunteer 15.12.73 and 29.08.71 have the characteristic textbook hormone pattern.

Volunteers 22.09.75, 06.09.72 and 09.10.64 are all missing the LH peak due to the schedule of bleeding days. Aside from that the hormone patterns look normal.

Volunteer 30.04.65 went away for holidays in the middle of the study and thus

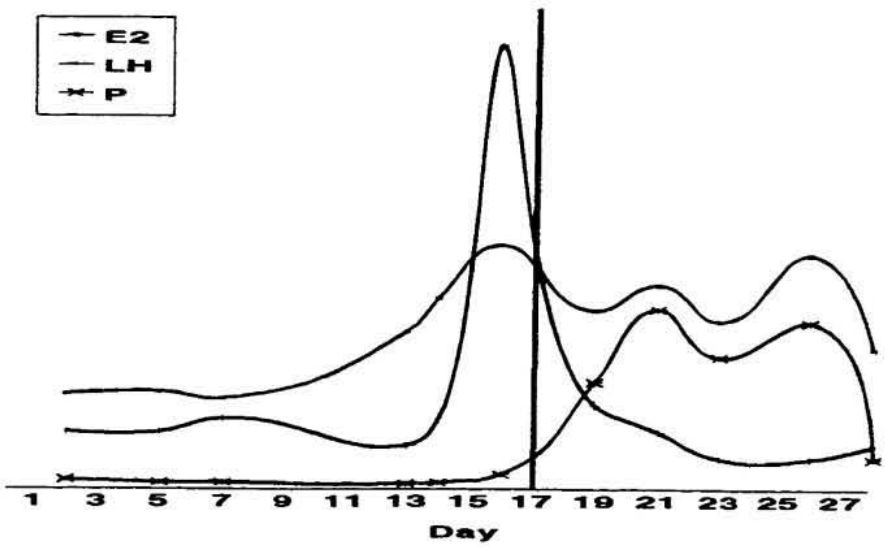

Figure 3.2. Hormone Profile of volunteer 21.04.70.
The changes in serum concentration of estrogen, progesterone and luteinizing hormone were measured using the radioimmunoassay procedure described in the materials and methods, section 2.5. All measurements were made in serum which had been isolated from a clot tube and then stored frozen at -70°C.

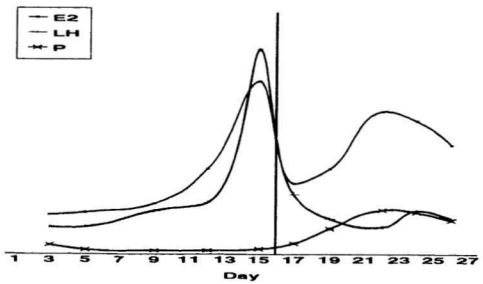

Figure 3.3. Hormone profile of volunteer 01.04.75.
The changes in serum concentration of estrogen, progesterone and luteinizing hormone were measured using the radioimmunoassay procedure described in the materials and methods section 2.5. All measurements were made in serum which had been isolated from a clot tube and then stored frozen at -70°C.

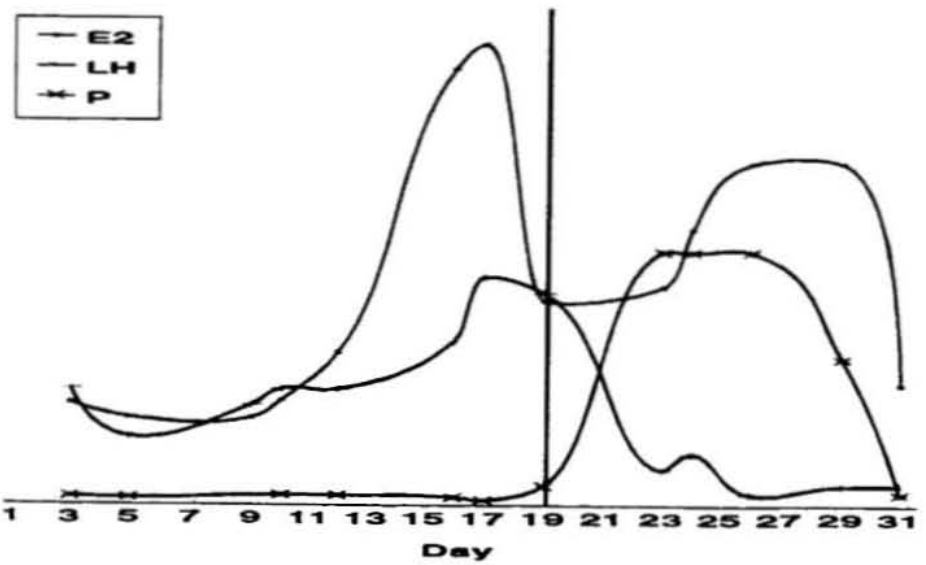

Figure 3.4. Hormone profile of volunteer 22.11.75.
The changes in serum concentration of estrogen, progesterone and luteinizing hormone were measured using the radioimmunoassay procedure described in the materials and methods, section 2.5. All measurements were made in serum which had been isolated from a clot tube and then stored frozen at -70°C.

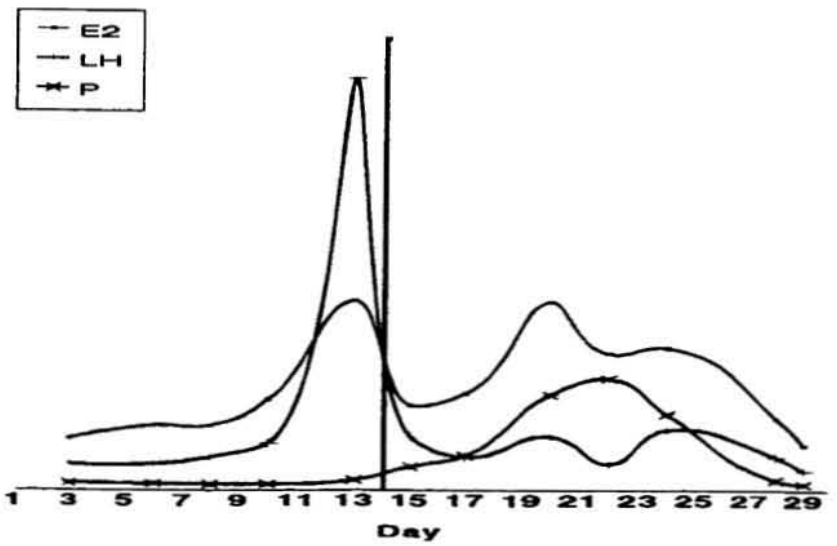

Figure 3.5. Hormone profile of volunteer 15.12.73.
The changes in serum concentration of estrogen, progesterone and luteinizing hormone were measured using the radioimmunoassay procedure described in the materials and methods, section 2.5. All measurements were made in serum which had been isolated from a clot tube and then stored frozen at -70°C.

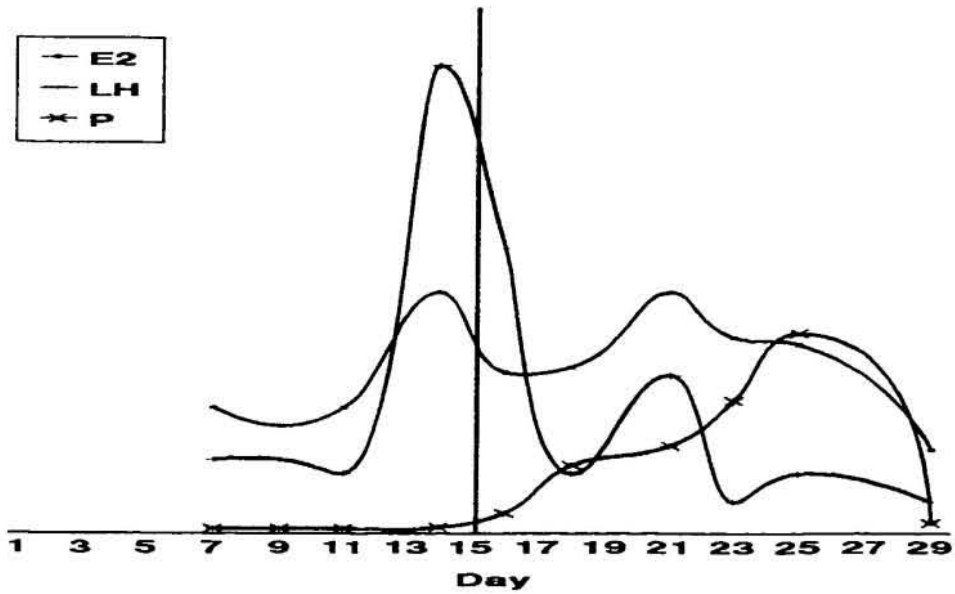

Figure 3.6 . Hormone profile of volunteer 29.08.71.
The changes in serum concentration of estrogen, progesterone and luteinizing hormones were measured using the radioimmunoassay procedure described in the materials and methods, section 2.5. All measurements were made in serum which had been isolated from a clot tube and then stored frozen at -70°C.

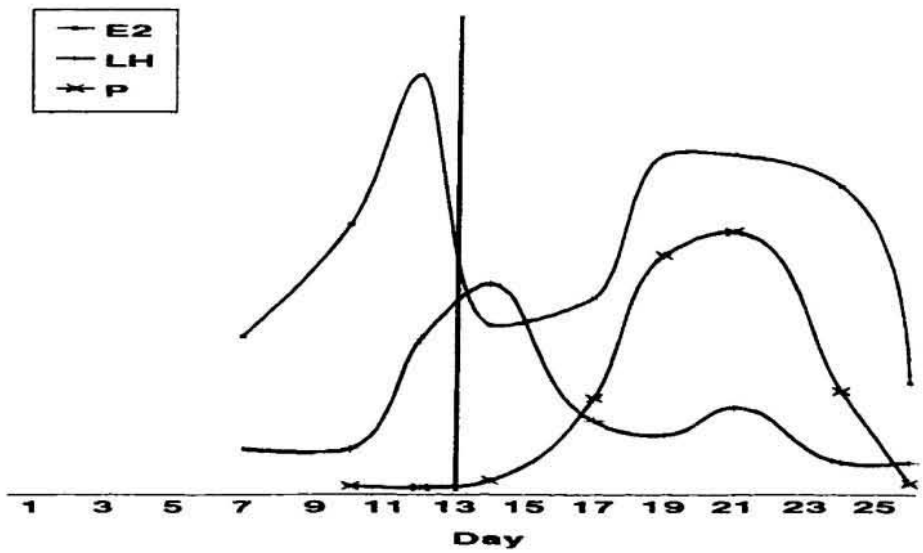

Figure 3.7. Hormone profile of volunteer 22.09.75.
The changes in serum concentration of estrogen, progesterone and luteinizing hormone were measured using the radioimmunoassay procedure described in the materials and methods, section 2.5. All measurements were made in serum which had been isolated from a clot tube and then stored frozen at -70°C.

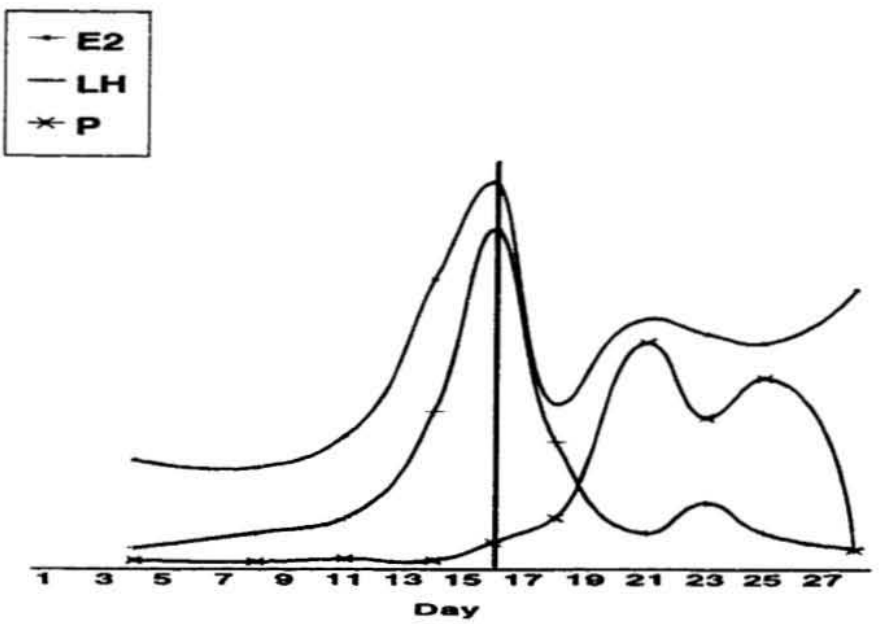

Figure 3.8. Hormone profile of volunteer 06.09.72.
The changes in serum concentration of estrogen, progesterone and luteinizing hormone were measured using the radioimmunoassay procedure described in the materials and methods, section 2.5. All measurements were made in serum which had been isolated from a clot tube and then stored frozen at -70°C.

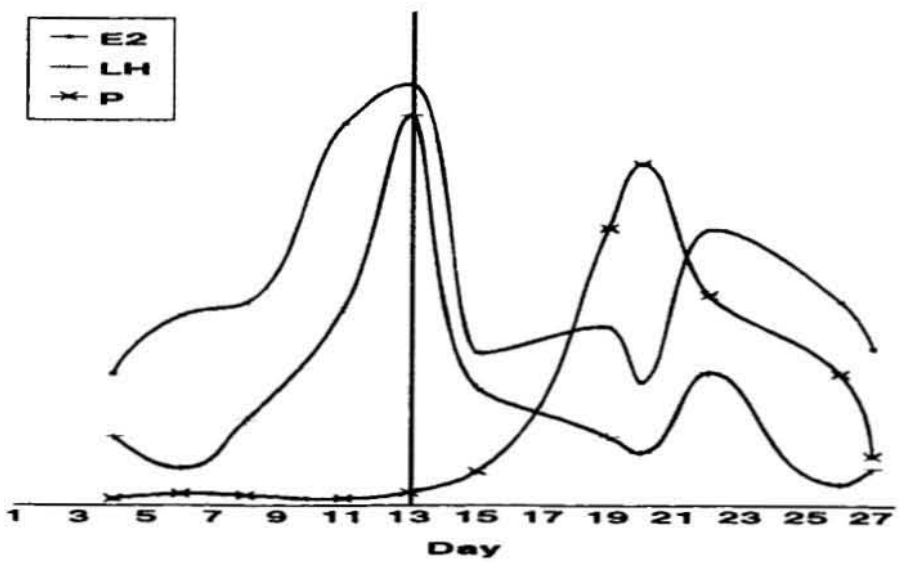

Figure 3.9. Hormone profile of volunteer 09.10.64.
The changes in serum concentration of estrogen, progesterone and luteinizing hormone were measured using the radioimmunoassay procedure described in the materials and methods, section 2.5. All measurements were made in serum which had been isolated from a clot tube and then stored frozen at -70°C.

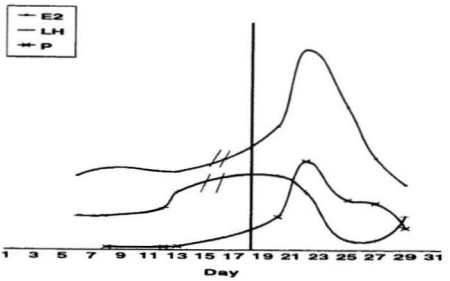

Figure 3.10. Hormone profile of volunteer 30.04.65.
The changes in serum concentration of estrogen, progesterone and luteinizing hormone were measured using the radioimmunoassay procedure described in the materials and methods, section 2.5. All measurements were made in serum which had been isolated from a clot tube and then stored frozen at -70°C.

several days in her cycle were missed (she was sampled on day 13 and then on day 20). The pattern that is displayed in Figure 3.6 is lacking several key data points.

It is important to note that, as shown in Table 3.2, all the volunteers that participated in the study did ovulate even though, as the following hormone profiles show, the LH peak was missed in analysis of the LH serum values using the RIA assay. We do know that this peak did occur since all volunteers showed the urine LH peak when using the OvuQuick kits.

3.4.1 Group Means

The group means and ranges for the hormones measured can be seen in Table 3.7 with the expected values and ranges that are provided with each RIA assay. In examining the data on the hormone levels and the expected values it is possible to say that the female volunteers in this study were all normal with respect to gonadal hormone levels. In the table it is apparent that not all the women fall exactly within the expected ranges, though this is to be expected when dealing with any human population. The expected values are estimates only and our subjects can be considered normal though they do show some deviation from the textbook estimates. This was also found to be true in the previous section when the length of the menstrual cycles were analyzed.

The male testosterone data gathered on each of the control subjects is also included in this table and it is evident that this group is normal with respect to their testosterone measurements.

Table 3.7. Expected and actual ranges, and group means for each of the gonadal hormones measured in this study.

Hormone	n	Expected Range	Actual	
			Range	Mean ± S. D.
17β-Estradiol pg/ml				
Follicular	46	40-500	52-302	124 ± 73
Luteal	50	120-350	57-270	143 ± 52
Progesterone ng/ml				
Follicular	43	0.2-0.9	0.0.3-1.4	0.29 ± 0.28
Luteal	50	3.0-35	0.1-21.0	7.7 ± 5.9
Luteinizing Hormone U/L				
Follicular	46	0.5-100	1-60	9.0 ± 10.8
Luteal	50	not given	1-20	4.6 ± 3.6
Testosterone ng/ml		3-10	3-13	7.6 ± 3.1

3.4.2 Coefficient of Variation

The standard curves and values used for the calculation of the coefficient of variation for the hormone assays are in Appendix C. Table 3.8 shows the coefficients of variation for each of the RIA assays.

This coefficient of variation is obtained by running the same serum sample on each assay (see section 2.9). The C.V. value for the progesterone assay is quite high due to the fact that 3 of the 4 samples gave a value of 0.3 ng/ml while the fourth was 0.6 ng/ml, 50 % higher (see Table C.2 in Appendix C). The lack of a C.V. value for the LH assay is due to the fact that two of the values obtained from the serum samples were below detection of the assay and the other was 0.8 U/L, which is considered not different from zero, according to the manual.

3.4.3 Summary

The conclusions obtained from the hormone analysis are that (i) the cycling volunteers had normal stable menstrual cycles that displayed all the general characteristics known and (ii) there were problems associated with the use of these volunteers and the ability to sample them at the appropriate days.

Table 3.8. Coefficients of Variation for the RIAs.

Hormone	Coefficient of Variation
17β Estradiol	10.9%
Progesterone	40.0%
Luteinizing hormone(LH)	na[a]
Testosterone	8.0%

[a] not available. All the values for the serum sample run on each assay were below detection of the RIA.

3.5 Variability between Cycling Females and Males

3.5.1 Coefficient of Variation

Before presenting the results of the analysis of males and females it is important to examine the coefficient of variation values for each of the ELISA assays used in measuring the cytokines.

The C.V. values calculated for each of the assays are presented in Table 3.9. All the original values used to calculate this inter-assay variation and the standard curves from each assay can be found in Appendix C.

It is apparent from the table that the inter-assay variation is dramatically high for some of the assays we performed. The C.V. values for IL-2, IL-4 and IFNγ are very low but those of IL-1 both serum and culture, were higher. These differences may be due to the fact that the ELISA's used for measuring IL-2, IL-4, and IFNγ were from a different commercial source than the IL-1β ELISA. R&D Systems supplied the ELISA kits for IL-2, IL-4, IFNγ, and IL-1ra, whereas Biosource was the source for IL-1β and some of the IL-4 kits. In addition, on the IL-4 Biosource ELISA assays, the sample that was used for calculation of the CV value was off the scale of the plate, contributing to the inability to calculate a CV value. Finally, the fact that many of the samples used to measure IL-1 had to be diluted contributed to the high variability reflected in the CV values.

Table 3.9. Coefficients of variation for the ELISA assays.

Cytokine	Coefficient of Variation
IL-2[c]	3.8%
IL-4[ac]	7.6%
IL-4[bc]	na[e]
IFNγ[c]	4.6%
IL-1β[c]	35.6%
IL-1β[d]	na
IL-1ra[c]	28.8%
IL-1ra[d]	27.9%

[a] IL-4 R&D Systems ELISA.
[b] IL-4 Biosource ELISA.
[c] Value for culture supernatants.
[d] Value for serum .
[e] not available.

3.5.2 Precision of Cell Counts

An experiment was done to determine the accuracy of cell counting. A suspension of PBMCs was counted 10 times and the results indicate a precision of 98.3%.

3.5.3 The F test

Having determined that the women had normal menstrual cycles it was possible to test whether there was more variation in successive samples taken from the women than from the men for the cytokines.

The statistical F test was used to determine this with α=0.05. A full explanation of the F Test is given in section 2.10.2. All the individual parameter measurements are given in Appendix B.

Table 3.10 shows the results of the analysis with the degrees of freedom and the p value for each individual cytokine. The data in the table show that the cytokines that had significantly more variation over time in the cycling females as compared to the males were IFN-γ and IL-4 induced by stimulating cells in cultures, and IL-1ra and IL-1β detected in serum. The others were not significantly different. That is to say, that if the F value is not large enough to be found significant, then we have no evidence against the hypothesis that the two groups have equal variances.

Table 3.10. F test data on each of the cytokines examined in the study.

Parameter	Ratio F/M	Degrees of Freedom	p value
IL-2[ad]	1.38	106/47	ns[e]
IL-4[ad]	1.72	105/47	<0.05
IL-1ra[a] ns[c]	0.97	82/28	ns
IL-1ra[a] s[d]	0.89	80/24	ns
IL-1β[a] ns[c]	1.25	74/23	ns
IL-1β[a] s[d]	0.316	58/24	ns
IL-1ra[b]	5.07	103/31	<0.01
IL-1β[b]	1.79	92/27	<0.05
IFN-γ[ad]	11.68	104/34	<<0.01

[a] values obtained from culture supernatant.
[b] values obtained from serum aliquot.
[c] nonstimulated culture supernatant.
[d] stimulated culture supernatant.
[e] not significant.

3.5.4 Comparison of Means between Cycling Females and Males

For each cytokine examined the means and ranges for the men and women, and the p values, are given in Table 3.11 and 3.12. It is apparent from this analysis that several of the mean cytokine values are different for males and females suggesting that the sexes vary in the amount of cytokine they produce, both in culture stimulation experiments and circulating levels in serum. This is most striking for IFNγ in stimulated cultures and the IL-1 ratio in serum; in both cases the men produce greater amount of cytokine. This analysis was done using the t test and the data obtained from the females for one full cycle. The samples taken in the second cycle were not used as we did not have an entire sample set and inclusion of the values from the follicular phase would only distort the mean as luteal phase samples are absent from the second cycle.

3.6 Cytokine Levels During the Menstrual Cycle

One of the aims of the study was to monitor serum cytokine levels and cytokine production by unstimulated and stimulated cells over the course of the menstrual cycle. This was successfully completed and the data can be found in Tables B.1-B.30 in Appendix B at the back of this document. In these tables the day in the cycle the sample was obtained and the corresponding cytokine value is presented.

The cytokine IL-1 was measured both in serum and in culture supernatants. The rationale was that the serum values would reflect the *in vivo* situation and the circulating levels of cytokine at the time the sample was taken. Cell cultures were also used to yield

Table 3.11. The means, standard error, ranges and p values for males and females for cytokines assayed from culture supernatants. The t test for independent samples of groups was used, α=0.05.

Cytokine	Sex	n*	mean	SE	Range	p value
IL-2[b]	F	95	477.5	34.5	44.8-1594.0	0.945
	M	39	474.4	28.8	171.2-847.8	
IL-4[b]	F	93	71.6	9.7	2.1-506.4	0.398
	M	39	58.2	7.5	0.0-195.2	
IFN-γ[b]	F	93	276.8	22.4	11.3-1262.0	0.055
	M	38	421.9	70.3	16.9-1669.0	
IL-1ra[a]	F	80	7.54	0.74	0.47-29.50	0.022
	M	31	4.73	0.94	1.05-23.53	
IL-1ra[b]	F	78	44.61	1.44	19.91-76.53	0.146
	M	29	48.69	2.43	21.70-71.86	
IL-1β[a]	F	71	108.7	17.09	0.50-605.30	0.283
	M	27	75.0	23.24	0.20-442.2	
IL-1β[b]	F	62	9724	513	3501-18400	0.982
	M	26	9702	851	3724-17720	
IL-1	F	71	1.81	0.4	0.01-25.8	0.162
Ratio[a]	M	27	1.04	0.3	0.01-5.8	
IL-1	F	62	23.4	1.3	8.6-54.9	0.212
Ratio[b]	M	24	20.1	1.6	8.5-34.5	

* number of samples used in the calculation.
[a] measured in nonstimulated culture supernatant.
[b] measured in stimulated culture supernatant.

Table 3.12. The means, standard error, ranges and p values for each group for the cytokine IL-1 assayed in serum from males and females. The t test for independent samples of groups was used, α=0.05.

Cytokine	Sex	n*	Mean	SE	Range	p value
IL-1ra	F	93	265.19	25.94	13.8-1036.0	0.338
	M	40	208.78	66.24	18.5-2578.0	
IL-1β	F	61	3.80	0.62	0.09-14.80	0.577
	M	30	4.19	0.33	1.06-8.67	
IL-1 ratio	F	60	2.27	0.39	0.01-13.92	0.000
	M	30	7.29	1.14	0.04-33.74	

* number of samples used in the calculation.

information on IL-1 levels, in particular it was felt that the supernatants from cell cultures would indicate what the particular supopulations of cells are capable of producing when stimulated. In addition to measuring cytokine levels in supernatants from stimulated cells we were also interested in levels of cytokines produced by unstimulated cells as these cells, in a sense, reflect the *in vivo* situation. One of the questions we wanted to investigate was whether or not the levels of IL-1 in circulation were related to levels spontaneously secreted in unstimulated cell cultures. If this were true then it was postulated that the curves generated from both conditions would look similar. Though this would assume that the cells that produce IL-1 in circulation are the same cells that produce IL-1 in culture conditions.

It is important to note here that there were some problems with obtaining values for some of the IL-1 measured. In particular there were several values that were either above or below the sensitivity of the assays. These values are indicated in the tables of raw data in Appendix B. In these cases the samples that were above or below the sensitivity of the ELISA were not included in the following statistical calculations. It was not appropriate to estimate the values and thus they were excluded. An alternate method of dealing with this would have been to designate a numerical value to each of those samples. This is dealt with again in the discussion of IL-1 in Chapter IV.

3.7 Cyclical Variation in Females

Our next aim was to determine if there were cyclical variations of cytokine levels

in serum or cytokines produced by cultured cells. In order to determine this we had to devise a method of standardizing the data obtained from the cycle so that each woman was comparable to the next. Even though we chose individuals with normal menstrual cycles and ovulation times each women is different, they do not all ovulate on the same day in their cycle and have the same length of cycle.

3.7.1 Proportions of the Menstrual Cycle:

It is apparent in the hormone graphs that although the cycle lengths were fairly similar, they were not identical. In order to compare the results of one women with those of the others we converted all sampling days to "proportion of phase". Thus a sample taken on day 6 when the women had a cycle length of 28 days with ovulation on day 14 would be 6:14 = 0.4 of the follicular phase; a sample taken on day 21 would be (21-14) : (28-14) = 0.5 of the luteal phase. If an individual had more than one value at a proportionate day then those values were averaged and the resultant mean value was used as the value of that proportionate day. By doing this we could graph the results from the nine cycling women so that they had the same time of ovulation and menses.

To further simplify the comparisons we divided the cycle into eight stages. This was done so that the majority of women would contribute a value to each stage because when we converted to proportionate day of the cycle we were still left with an incomplete data set simply due to the sampling day schedule. The eight stages are as follows:

1. Early follicular (EF) = proportionate days 0.2 and 0.3 of the follicular phase.

2. Midfollicular (MF) = proportionate days 0.4, 0.5 and 0.6 of the follicular phase.

3. Late follicular (LF) = proportionate days 0.7 and 0.8 of the follicular phase.

4. Ovulation (O) = proportionate days 0.9 and 1.0 of the follicular phase and 0.1 of the luteal phase.

5. Early luteal (EL) = proportionate days 0.2 and 0.3 of luteal phase.

6. Midluteal (ML) = proportionate days 0.4, 0.5 and 0.6 of the luteal phase.

7. Late Luteal (LL) = proportionate days 0.7 and 0.8 of the luteal phase.

8. Menses (M) = proportionate days 0.9 and 1.0 of the luteal phase and 0.1 of second follicular phase.

Initially we attempted to create spline graphs with the use of the program S plus. These spline graphs used all the original data and attempted to smooth the curves to create a clear curve absent of any noise. This was difficult to do as the values used to spline are arbitrary and different for each curve. In addition, this method did not indicate the peaks in the data over the course of the cycle. This was most dramatic in the creation of the LH graph where the point just prior to ovulation is the most important point and the computer factored it out. Thus we decided against using such a system.

An alternate method that could have been used to standardize the data was proposed by Doty, 1979. This procedure converts the cycle into 11 phases; 2 menses phases, 3 follicular phases, one ovulation phases, and 5 luteal phases. Doty, 1979 treats

ovulation in much the same way we do in that he uses the 0 (LH peak day) and +1 (the day after LH peak) to stabilize the data and increase the likelihood of encompassing the actual ovulation event. In contrast to this we used only one menses phase, 3 phases for each of the follicular and luteal phase of the cycle and one for ovulation. Our method designates the ovulation phase as encompassing days -1, 0 and +1, which Doty suggests can be done. In addition, the common method of LH-centring of the data and using only 2 phases , that of the follicular and luteal , was not used because it does not allow for the possible differences within these two phases.

3.7.2 Composite Hormone Profile

Figure 3.11 displays the composite hormone graph as proportion of the phase of the menstrual cycle. This figure shows the hormonal changes that occur in the cycling females that participated in Phase II of the study.

As in the individual figures, there is an LH peak at ovulation, an estrogen peak in the follicular and luteal phases and a progesterone peak in the luteal phase.

3.7.3 Cytokine Analysis

Each of the cytokines that were measured by ELISA were graphed (Fig. 3.12-3.20) as proportion of phase following the procedure described in section 3.7.1 and analyzed with the paired t test to determine if there were any differences between the lows and highs indicated by the data. Note that the number of paired comparisons in each of the

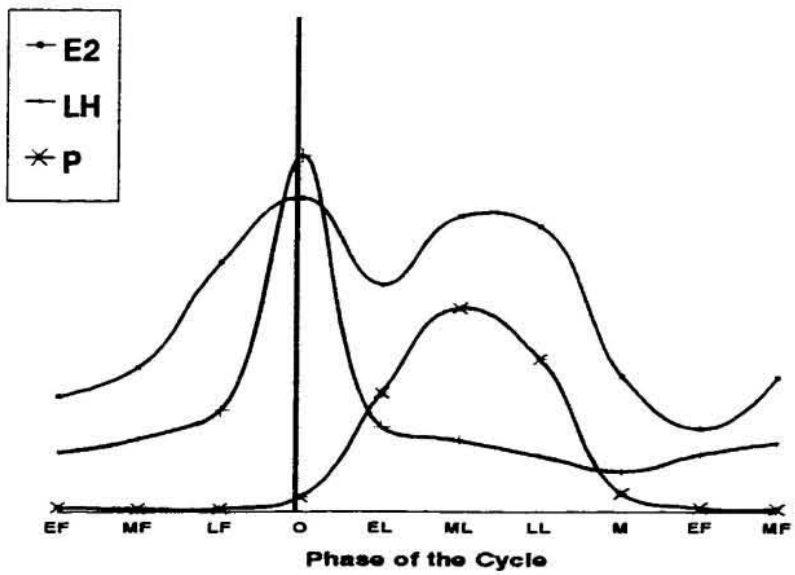

Figure 3.11. Composite hormone profile as seen when the cycle is converted into proportion of phase.

following analyses is designated "n". The individual results (raw data) for the 15 volunteers are presented in Appendix B, Tables B.1-B.30.

In addition, Appendix B, Table B.31 gives the actual converted values as proportion of phase used in the graphs and the standard deviation of each of the points in the graphs. This table can be referred to for standard deviations. It was not practical to include the standard deviations in the graphs which follow due to the fact that these are very large and in some cases extend outside the graphs. This large standard deviation is due to the very large inter-individual variation that is found among the sample population. As the cycling volunteers were bled for approximately 5 weeks the results include the second early follicular phase. It is apparent in the following graphs that for some cytokines there is a discrepancy between the two EF phases of the cycles. There are several possible explanations for this. It is not an uncommon phenomenon in that other investigators have found the same thing when looking at the phases of the cycle in inbred strains of mice (Gallichan and Rosenthal, 1996). Even in genetically similar animals there still is intra-individual variation suggesting that this is not a surprising finding in genetically diverse humans. In addition, due to the way that the sampling days were organized some of the time, the discrepancy could be due to the fact that not the same women contribute to the first and second EF phases in the graphs. Another important point to make is that while we obtained samples from the second EF phases, we did not follow these women during this cycle and thus do not know the total length of their cycle or if the cycle was normal, i.e. if the volunteer ovulated. As a result of not knowing the

total cycle length the points taken in the beginning of the second cycle were classified as EF based on the previous cycle length. Thus we don't know for certain that these points are in the EF phases of the cycle. Finally, it was noted that the levels of certain cells in circulation were significantly different at the end of the study. In other words, there were differences between the first bleed levels of certain cells and the final sample levels (Table A.1, Appendix A). This change in cell populations could very possibly have contributed to the varying levels between the two EF phases we see in this study.

Each of the individual cytokine results will be considered in the following sections.

3.7.3.1 Interleukin 2

IL-2 was measured by ELISA in culture supernatant obtained as described in section 2.3.2.1. IL-2 is expressed in pg/ml and the changes that occur over the menstrual cycle can be seen in Figure 3.12.

The trend of the graph shows a low at ovulation and a high in the midluteal and late luteal phase when estrogen and progesterone are high. Statistical analysis of the graph using the paired t test at α=0.05 confirms the lows and highs seen. The point at ovulation vs late luteal (n=7) gives a p value of 0.0147. Ovulation vs midluteal (n=8) gives a p value of 0.0088.

3.7.3.2 Interleukin 4:

IL-4 was measured by ELISA in culture supernatants obtained as described in

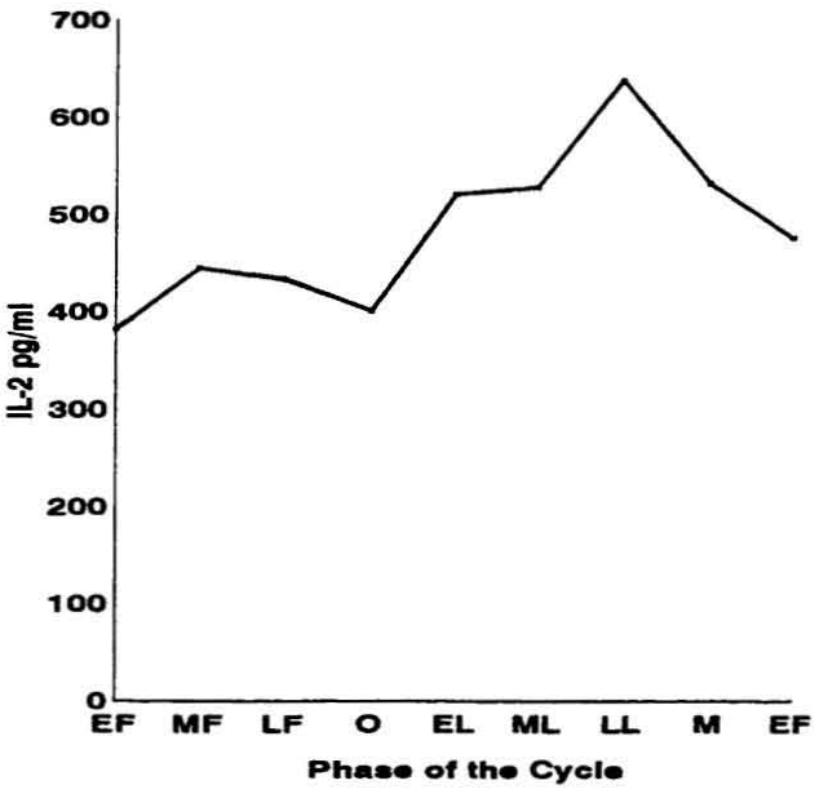

Figure 3.12. The IL-2 cytokine profile in relation to phases of the menstrual cycle. Each point represents the mean value for the volunteers tested at that time of their cycle, see Figure 3.1. IL-2 was measured in supernatants of PHA stimulated mononuclear cells and is shown in pg/ml.

section 2.3.2.1. IL-4 is measured in pg/ml and the changes that occur over the menstrual cycle can be seen in Figure 3.13. Note: results from volunteer 09.10.64 are not included in the calculation of the graph as her values are twice as high as those of the other volunteers.

The trend of the graph shows a low at ovulation and a high at menses. Statistical analysis of the graph using the paired t test at α=0.05 (one tailed) does not statistically confirm this trend. The point at ovulation vs menses (n=7) gives a p value of 0.1350.

3.7.3.3 Interferon γ

IFNγ was measured by ELISA in culture supernatants obtained as described in section 2.3.2.3. IFNγ is measured in pg/ml and the changes that occur over the menstrual cycle can be seen in Figure 3.14.

The trend of the graph shows peaks at midfollicular and late luteal, a low at ovulation and at early luteal. Statistical analysis of the graph using the paired t test at α=0.05 (one tailed) confirms the low at ovulation and the high in the late luteal phase, p=0.01 (n=7). Midfollicular versus ovulation gave a p value of 0.151 (n=7); midfollicular versus early luteal a p value of 0.224 (n=7); ovulation vs menses (n=7) gives a p value of 0.143.

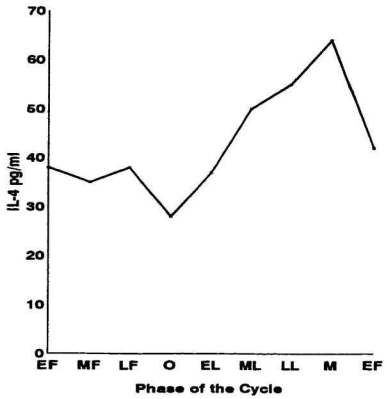

Figure 3.13. The IL-4 cytokine profile in relation to phases of the menstrual cycle. Each point represents the mean value for the volunteers tested at that time of their cycle, see Figure 3.1. IL-4 was measured in supernatants of PHA stimulated mononuclear cells and is shown in pg/ml.

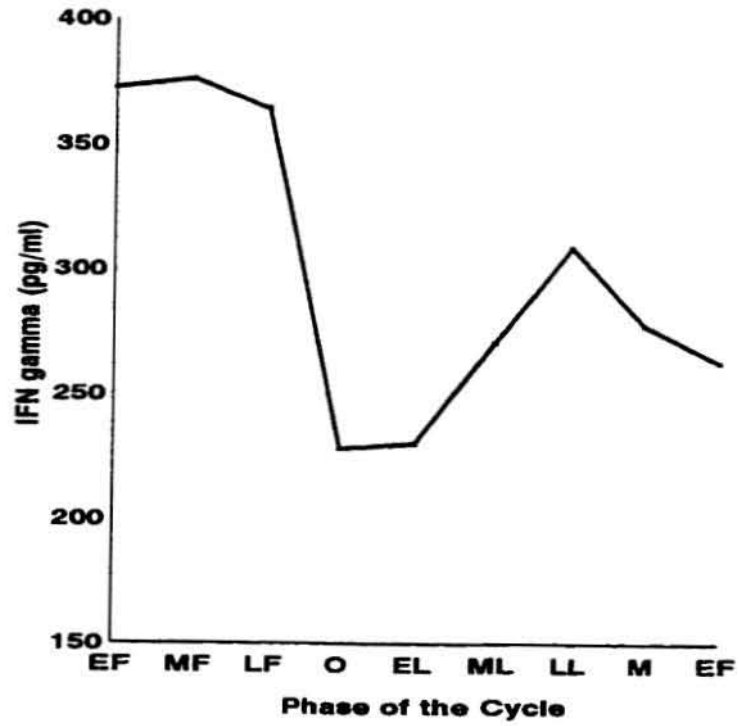

Figure 3.14. The IFNγ cytokine profile in relation to phases of the menstrual cycle. Each point represents the mean value for the volunteers tested at that time of their cycle, see Figure 3.1. IFNγ was measured in supernatants of PHA stimulated mononuclear cells and is shown in pg/ml.

3.7.3.4 Interleukin 1ra in nonstimulated cultures:

IL-1ra was measured by ELISA in culture supernatants obtained as described in section 2.3.2.2. IL-1ra is measured in ng/ml and the changes that occur over the menstrual cycle can be seen in Figure 3.15.

The trend of the graph shows a high at MF, ML and M, a low at O and EL. Statistical analysis of the graph using the paired t test at α=0.05 (one tailed) does not confirm the low at ovulation and the high in midfollicular p value 0..3063 (n=7); ovulation versus midluteal gives a p value of 0.1230 (n=6); ovulation vs menses (n=7) gives a p value of 0.0594.

3.7.3.5 Interleukin 1ra in stimulated cultures:

IL-1ra was measured by ELISA in culture supernatants obtained as described in section 2.3.2.2. IL-1ra is measured in ng/ml and the changes that occur over the menstrual cycle can be seen in Figure 3.15.

The trend of the graph shows a high at O and ML and a low at EF, EL, and M. Statistical analysis of the graph using the paired t test at α=0.05 (one tailed) does not confirm the lows and highs seen. Early follicular versus ovulation gave a p value of 0.100 (n=4); ovulation versus early luteal a p value of 0.300 (n=7); ovulation vs menses (n=7) gives a p value of 0.273.

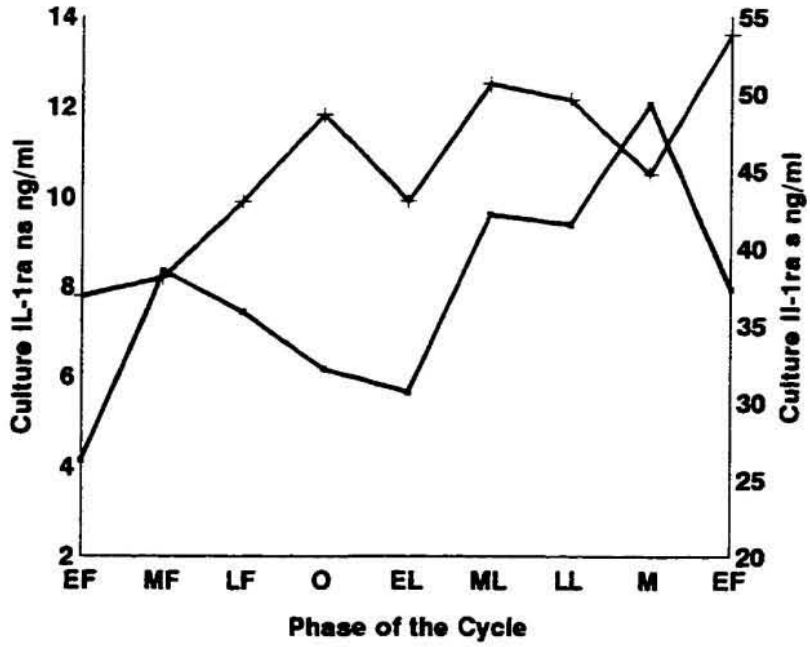

Figure 3.15. The IL-1ra culture cytokine profile in relation to phases of the menstrual cycle. Each point represents the mean value for the volunteers tested at that time in their cycle, see Figure 3.1. IL-1ra was measured in supernatants of LPS non stimulated (o) (NS) and stimulated (+) (S) mononuclear cells.

3.7.3.6 Interleukin 1β in nonstimulated cultures:

IL-1β was measured by ELISA in culture supernatants obtained as described in section 2.3.2.2. IL-1β is measured in pg/ml and the changes that occur over the menstrual cycle can be seen in Figure 3.16.

The trend of the graph shows a high at late follicular and menses and lows at ovulation, early luteal, and late luteal. Statistical analysis of the graph using the paired t test at α=0.05 (one tailed) does confirm the low at ovulation and the high at LF, p=0.0471 (n=8). Ovulation versus late luteal gave a p value of 0.1633 (n=6) and ovulation vs menses (n=7) gives a p value of 0.0170.

3.7.3.7 Interleukin 1β in stimulated cultures:

IL-1β was measured by ELISA in culture supernatants obtained as described in section 2.3.2.2. IL-1β is measured in pg/ml and the changes that occur over the menstrual cycle can be seen in Figure 3.16. Note: data from volunteer 22.09.75 was excluded because there are only two values in the cycle and both are in the luteal phase.

The trend of the graph shows a high at ovulation and menses and a low at midfollicular and midluteal. Statistical analysis of the graph using the paired t test at α=0.05 (one tailed) does not confirm the lows and highs seen. Midfollicular versus ovulation gave a p value of 0.1274 (n=6) and ovulation versus midluteal a p value of 0.2221 (n=5).

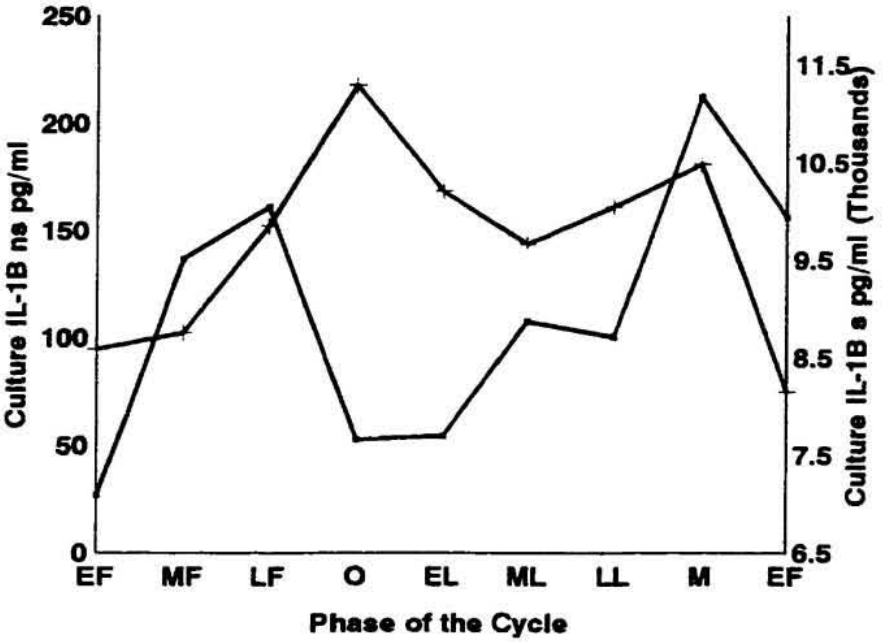

Figure 3.16. The IL-1β culture cytokine profile in relation to phases of the menstrual cycle. Each point represents the mean value for the volunteers tested at that time in their cycle, see Figure 3.1. IL-1β was measured in supernatants of LPS non stimulated (o) (NS) and stimulated (+) (S) mononuclear cells.

3.7.3.8 Interleukin 1:

IL-1 is expressed as 100x the ratio of IL-1β to IL-1ra and the changes that occur both in the nonstimulated and stimulated cultures over the menstrual cycle can be seen in Figure 3.17. The IL-1 Ratio graph for supernatants of stimulated cells excludes the data from volunteer 22.09.75 as there are only two values for her and both are in the luteal phase of the cycle.

As a result of the problems in determining the correct dilution factor for some of the samples we had a loss of some data thus there were some cases where the IL-1β/IL-1ra was not available.

The trend of the graph for stimulated cultures shows a high at ovulation and at menses with a lows at the late follicular and midluteal phase. Statistical analysis of the graph using the paired t test at α=0.05 confirms the lows and highs seen. The point at midluteal vs menses in the stimulated graph gives a p value of 0.0057 with n=4. and O vs ML p=0.1549 (n=5). The trend in the nonstimulated graph shows a high at LF and M, with a low at time of O. Differences between means were not significant.

3.7.3.9 Problems with IL-1 Data

It is important to note here that there was some difficulty in assaying the culture supernatants for IL-1β and IL-1ra. A dilution had to be used in order to determine the concentrations of each of the cytokines. When assaying IL-1β, nonstimulated culture supernatants were assayed undiluted, whereas it was necessary to dilute stimulated

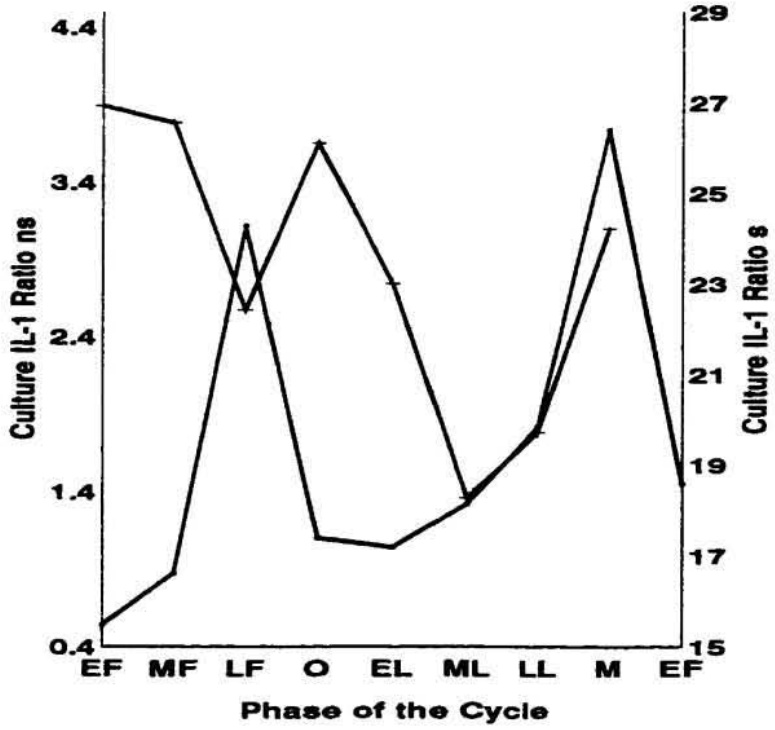

Figure 3.17. The IL-1 ratio cytokine profile in relation to phases of the menstrual cycle. Each point represents the mean value for the volunteers tested at that time of their cycle, see Figure 3.1. IL-1 was measured in supernatants of LPS non stimulated (.)(NS) and stimulated (+) (S) mononuclear cells and is shown as a ratio of IL-1β/IL-1ra x 100.

supernatants 1:10 or 1:20 (See Appendix B data tables for specific information). For the IL-1ra assay, nonstimulated culture supernatants were assayed at a dilution of 1:10 and stimulated supernatants at 1:20. Even with the dilutions some of the samples still had values above O.D. 3.0. Out of 96 samples there are 21 missing IL-1β values. The loss of 22% of the data makes the trends we are seeing less reliable and we may be missing other important trends.

3.7.3.10 Serum Interleukin 1ra:

IL-1ra was measured by ELISA in serum obtained as described in section 2.3.3.2. IL-1ra was measured in pg/ml and the changes that occur over the menstrual cycle can be seen in Figure 3.18.

The trend of the graph shows an increase in IL-1ra from MF to M. Statistical analysis of the graph using the paired t test at α=0.05 looking at the point at early follicular vs ovulation in the graph gives a p value of 0.0208 with n=5, and ovulation vs midfollicular gives a p value of 0.0075 with n=7.

3.7.3.11 Serum Interleukin 1β:

IL-1β was measured by ELISA in serum obtained as described in section 2.3.3.2. IL-1β was measured in pg/ml and the changes that occur over the menstrual cycle can be seen in Figure 3.19.

The trend of the graph show highs at EF and ML with lows at LF, O and

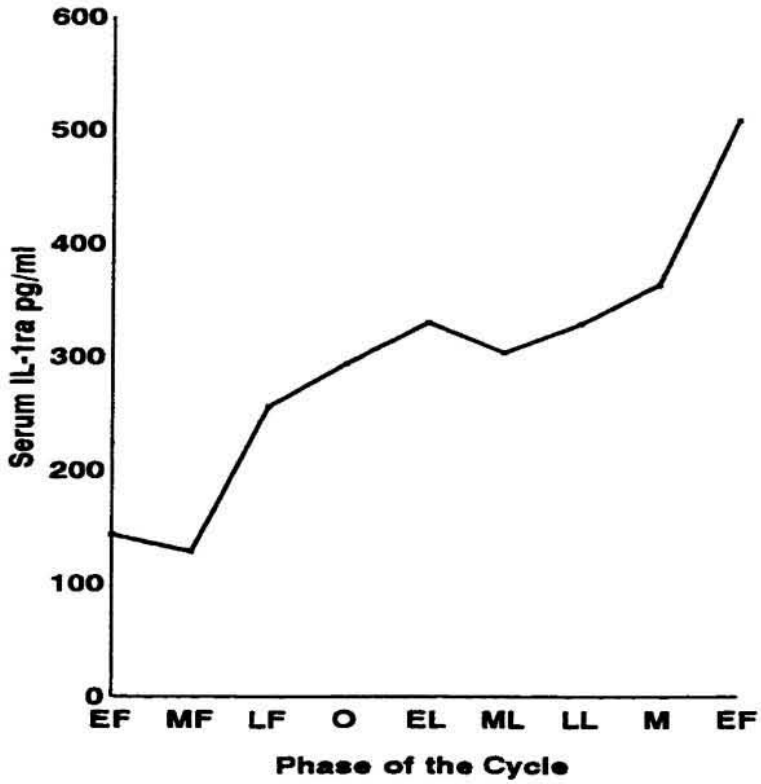

Figure 3.18. Serum IL-1ra cytokine profile in relation to phases of the menstrual cycle. Each point represents the mean value for the volunteers tested at that time in their cycle, see Figure 3.1.

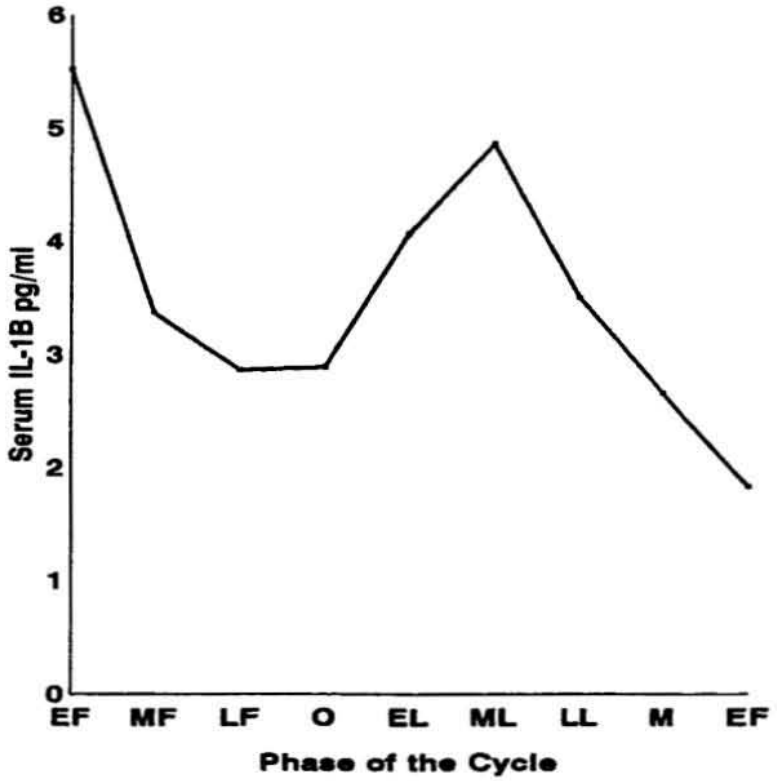

Figure 3.19. The serum IL-1β cytokine profile in relation to phases of the menstrual cycle. Each point represents the mean value for the volunteers tested at that time of their cycle, see Figure 3.1.

menses. Statistical analysis of the graph using the paired t test at α=0.05 looking at the point at EF vs O in the graph gives a p value of 0.2872 with n=3; O vs ML gives a p value of 0.1327 with n=4, and EF vs M, p=0.2825 with n=3.

3.7.3.12 Serum IL-1 Ratio:

IL-1 is expressed as 100x the ratio of IL-1β to IL-1ra and the changes that occur over the menstrual cycle can be seen in Figure 3.20. Note: volunteers 30.04.65 and 01.04.75 were excluded from the graph as they only had one value each during the cycle.

The trend of the graph shows a decline to time of ovulation and a rise to peak in the luteal phase. Statistical analysis of the graph using the paired t test at α=0.05 looking at the point at early follicular vs ovulation in the graph gives a p value of 0.1450 with n=3, and ovulation vs late luteal gives a p value of 0.1709 with n=4.

3.7.3.13 IL-6 and TNFα

There were problems obtaining data for IL-6 and TNFα. There were approximately 75 samples assayed and all were below detection of the ELISA. Thus we have no data for these cytokines.

3.7.4 Summary

A summary of the cyclical changes that occur during the course of the menstrual cycle can be viewed in Table 3.13. A feature common to most of the cytokines is a low

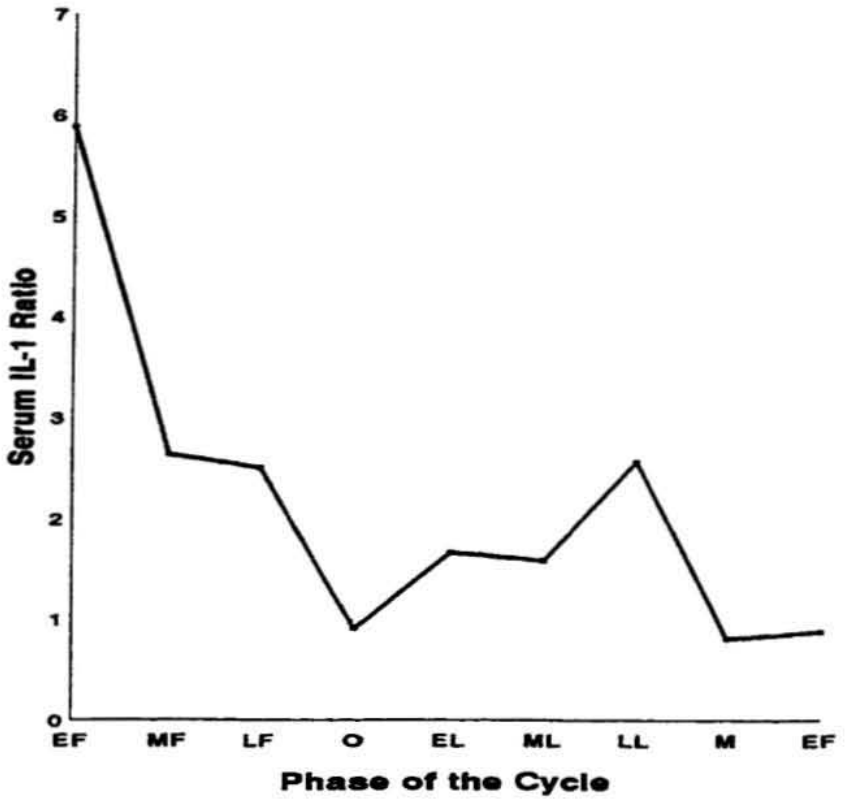

Figure 3.20. The IL-1 ratio cytokine profile in relation to phases of the menstrual cycle. Each point represents the mean value for the volunteers tested at that time of their cycle, see Figure 3.1. IL-1 was measured in serum and is shown as a ratio of IL-1β/IL-1ra x 100.

Table 3.13. A summary of the changes in relative levels of cytokines that were found to occur during the course of the menstrual cycle.

Cytokine	Phase							
	Follicular			Ovul	Luteal			Menses
	E	M	L		E	M	L	
IL-2[b]				Low[1]		High	High	
IL-4[b]				Low				High
IFNγ[b]				Low[1]			High	
IL-1ra[a]		High		Low		High		High
IL-1ra[b]	Low			High				Low
IL-1β[a]			High	Low[1]				High
IL-1β[b]		Low		High		Low		High
IL-1ra[c]	Low	Low		High[1]				
IL-1β[c]	High			Low		High		
IL-1R[a]			High	Low				High
IL-1R[b]				High		Low		High[1]
IL-1R[c]	High			Low			High	

[a] measured in nonstimulated culture supernatant.
[b] measured in stimulated culture supernatant.
[c] measured in serum.
[1] significant $p<0.05$

at ovulation, IL-1 (IL-1β/IL-1ra) in the stimulated cultures is the exception.

3.8 Regressions and Correlations

Once we had established that there were cyclical variations in cytokine expression the next step was to determine if the variation was linked to cyclical changes in the hormones estrogen, progesterone and luteinizing hormone. This was accomplished by using multiple regression. The following results indicate what independent variables influence the cytokines. Each cytokine is examined separately. All the comparisons made in this section were generated with the original raw data obtained for one full cycle.

3.8.1 IL-2

Multiple stepwise regression of IL-2 with age of the volunteer, day in the cycle the sample was taken, day of ovulation, length of the cycle, estrogen, LH, and progesterone values gives a correlation coefficient of 0.59801 and a coefficient of determination of 0.35761. These values indicate a relationship between day of ovulation, length of cycle, age and IL-2. The variables and the confidence intervals for the equation are given in Table 3.14.

This correlation indicates that if the day in the cycle that ovulation occurs is closer to day 1 then IL-2 will be increased by 30.20 pg/ml per day, that the longer the total length of cycle the greater the amount of IL-2 produced by 173.31 pg/ml per day and that the older the individual is the lower the production of IL-2 by stimulated cells by 158.14

Table 3.14. Variables in the predictor equation for IL-2 with the 95% confidence intervals and the p values.

Variable	B	95% Confidence Interval B		p value
Day of ovulation	-30.20	-47.35	-13.06	0.0007
Length of cycle	173.31	112.36	234.26	0.0000
Age	-158.14	-203.13	-113.16	0.0000
Constant	-1331.10	-2497.32	-164.89	0.0258

pg/ml per year. These approximate amounts by which IL-2 will change are on the condition that everything else that has an effect on IL-2 be held constant. In addition, the coefficient of determination indicates that 35% of the variation in IL-2 is due to the influence of these independent variables.

3.8.2 IL-4

Multiple stepwise regression of IL-4 with age of the volunteer, day in the cycle the sample was taken, day of ovulation, length of the cycle, estrogen, LH, and progesterone gives a correlation coefficient of 0.63416 and a coefficient of determination of 0.40215. These values indicate a very good relationship between age, day of ovulation and IL-4. The variables and the confidence intervals for the equation are given in Table 3.15.

This correlation indicates that if the day in the cycle that ovulation occurs is closer to day 1 then IL-4 will be increased by 13.44 pg/ml per day, and that the older the individual is the greater the amount of IL-4 that will be produced by stimulated PBMC, IL-4 will increase by 13.33 pg/ml for every 1 year increase in age. These approximate amounts by which IL-4 will change are on the condition that everything else that has an effect on IL-4 be held constant. In addition, the coefficient of determination indicates that 40% of the variation in IL-4 is due to the influence of these independent variables.

Table 3.15. Variables in the predictor equation for IL-4 with the 95% confidence intervals and the p values.

Variable	B	95% Confidence Interval B		p value
Age	13.33	9.13	17.54	0.0000
Day of Ovulation	-13.44	-21.29	-5.60	0.0010
Constant	-20.64	-187.82	146.54	0.8067

3.8.3 IFNγ

Multiple stepwise regression of IFNγ with age of the volunteer, day in the cycle the sample was taken, day of ovulation, length of the cycle, estrogen, LH, and progesterone gives a correlation coefficient of 0.21940 and a coefficient of determination of 0.04814. These values indicate a weak relationship between age and IFNγ. The variables and the confidence intervals for the equation are given in Table 3.16.

This analysis is not reliable in that the correlation coefficient is only .21 and the amount of variation in IFNγ due to the influence of these variables is only 5%. Thus it is not possible to say that if age increases by 1 year then IFNγ will increases by 12.73 pg/ml. This prediction is not reliable do to the low correlation.

3.8.4 Culture IL-1ra

Multiple stepwise regression of IL-1ra with age of the volunteer, day in the cycle the sample was taken, day of ovulation, length of the cycle, estrogen, LH, and progesterone tested gives a correlation coefficient of 0.43696 and a coefficient of determination of 0.19093 for unstimulated cultures and a correlation coefficient of 0.36596 and a coefficient of determination of 0.13392 for stimulated cultures. These values indicate a relationship between day in the cycle that the sample was taken, age, and IL-1ra in nonstimulated cultures and day in the cycle the sample was taken and IL-1ra in stimulated cultures. The variables and the confidence intervals for the equation are given in Table 3.17 and 3.18.

Table 3.16. Variables in the predictor equation for IFNγ with the 95% confidence intervals and the p values.

Variable	B	95% Confidence Interval B		p value
Age	12.73	0.73	24.72	0.0377
Constant	-12.86	-289.62	263.90	0.9266

Table 3.17. Variables in the predictor equation for IL-1ra in nonstimulated cultures with the 95% confidence intervals and the p values.

Variable	B	95% Confidence Intervals B		p value
Day*	0.1971	0.0055	0.3887	0.0439
Age	0.7463	0.3569	1.136	0.0003
Constant	-12.56	-22.26	-2.863	0.0118

*Day in the cycle that the sample was taken.

Table 3.18. Variables in the predictor equation for IL-1ra in stimulated cultures with the 95% confidence intervals and the p values.

Variable	B	95% Confidence Intervals B		p value
Day*	0.6376	0.2620	1.013	0.0011
Constant	34.23	27.48	40.99	0.0000

*Day in the cycle that the sample was taken.

This regression indicates that for both nonstimulated and stimulated cultures IL-1ra production will increases by 0.1971 and 0.6376 ng/ml, respectively, the later in the cycle the sample is taken. Thus IL-1ra increases in the luteal phases of the cycle which corresponds to the cycle data results. In addition, IL-1ra production by nonstimulated cells will increase by 0.7463 ng/ml for every year older a person is.

The coefficient of determination suggests that between 13 and 19% of the variation occurring in IL-1ra production in culture is due to these independent variables and that prediction of IL-1ra is between 37 and 44% accurate. This is not extremely high but it does suggest that the variables indicated do play a role in determining how much IL-1ra will be produced in culture.

3.8.5 Culture IL-1β

Multiple stepwise regression of IL-1β with age of the volunteer, day in the cycle the sample was taken, day of ovulation, length of the cycle, estrogen, LH, and progesterone tested gives a correlation coefficient of 0.34083 and 0.4277, and a coefficient of determination of 0.11616 and 0.17874, for nonstimulated and stimulated cultures respectively. These values indicate a relationship between cycle length and IL-1β in nonstimulated cultures and day of ovulation in stimulated cultures. The variables and the confidence intervals for the equation are given in Table 3.19 and 3.20.

These correlations indicate that IL-1β production in nonstimulated cultures will be greater the longer the total cycle length is; it will increases by 32.84 pg/ml for every

Table 3.19. Variables in the predictor equation for IL-1β in nonstimulated cultures with the 95% confidence intervals and the p values.

Variable	B	95% Confidence Intervals B		p value
Length of cycle	32.84	10.92	54.76	0.0039
Constant	-830.6	-1458.68	-202.47	0.0103

Table 3.20. Variables in the predictor equation for IL-1β in stimulated cultures with the 95% confidence intervals and the p values.

Variable	B	95% Confidence Intervals B		p value
Day of Ovulation	972.38	424.53	1520.23	0.0008
Constant	-5733.25	-14533.57	3067.07	0.1974

1 day longer. In addition, the analysis indicates that IL-1β production in stimulated cultures will be larger, by 972.38 pg/ml per day, the further in the cycle ovulation occurs. Finally, the suggestion is that between 12 and 18% of the variation for IL-1β is due to influence of the independent variables. The low dependency implies that only a small portion of IL-1β variation can be explained using our independent variables and that other more significant variables are responsible for the changes in IL-1β.

3.8.6 Serum IL-1

Multiple stepwise regression of IL-1ra with age of the volunteer, day in the cycle the sample was taken, day of ovulation, length of the cycle, estrogen, LH, and progesterone gives a correlation coefficient of 0.58700 and a coefficient of determination of 0.34456. These values indicate a good relationship between length of cycle, age, the hormone estrogen and IL-1ra in serum. The variables and the confidence intervals for the equation are given in Table 3.21.

Multiple stepwise regression of IL-1β with the same variables used for IL-1ra gives a correlation coefficient of 0.75567 and a coefficient of determination of 0.57103. These values indicate a very good relationship between length of cycle, age, day of ovulation and IL-1β in serum. The variables and the confidence intervals for the equation are given in Table 3.22.

The statistical analyses is indicating that for IL-1ra, if every other influence on IL-1ra is held constant, then, the older the individual is the lower the

Table 3.21. Variables in the predictor equation for IL-1ra in serum sample with the 95% confidence intervals and the p values.

Variable	B	95% Confidence Intervals B		p value
Length of cycle	-58.58	-88.18	-28.98	0.0002
Age	-16.34	-28.37	-4.32	0.0083
Estrogen	1.22	0.52	1.91	0.0008
Constant	2153.40	1309.79	29997.06	0.0000

Table 3.22. Variables in the predictor equation for IL-1β in serum sample with the 95% confidence intervals and the p values.

Variable	B	95% Confidence Interval B		p value
Age	-0.79	-.98	-0.60	0.0000
Length of cycle	3.27	2.44	4.10	0.0000
Day of Ovulation	-2.36	-2.93	-1.80	0.0000
Constant	-36.43	-52.84	-20.03	0.0000

level of IL-1ra will be in circulation, that it will decrease by 16.34 pg/ml for every 1 year and that the longer the length of the cycle, IL-1ra levels will decrease by 58.58 pg/ml for every 1 day. Also, we know that 34% of the variation in IL-1ra is due to these independent variables.

IL-1β also has a very good correlation with, 0.76, length of cycle, age and day of ovulation. Specifically, the older the individual is the lower the amount of IL-1β in circulation; that it will decrease by 0.79 pg/ml for every 1 year and the further away day of ovulation is from day 1, the lower the level of IL-1β by 2.36 pg/ml per day. In contrast, if the length of the cycle is increased more IL-1β will be present when individuals are the same age and have the same day of ovulation. These two variables are connected in that in most cases, as stated in the Introduction, it is the follicular phase that is more variable in length than the luteal phase and day of ovulation is considered a part of the follicular phase, thus if the day of ovulation is further away from day 1 then the follicular phases will be longer resulting in the probability that the total cycle length will be longer.

It can also be said that 58% of the variation in circulating levels of IL-1β is due to the independent variables mentioned above.

3.8.7 Serum Levels and Nonstimulated Production of IL-1

As discussed in section 3.6 of this chapter we measured both circulating levels of IL-1 and IL-1 production by cultured cells because they give information on different

phenomenon, both *in vivo* and *in vitro*. In addition we wanted to determine if the circulating levels in serum were comparable to the level of IL-1 produced in nonstimulated cells. The results suggest that the two measures are not similar at all. The proportion of phase graphs presented in the subsections of 3.7 indicate that there is no relationship. Regression analysis comparing the two shows no relationship at all. The correlation coefficient is 0.07234 and coefficient of determination 0.00523, for IL-1ra. IL-1β is similar to IL-1ra in that the correlation coefficient is very low, 0.07383 and the coefficient of determination is 0.00614. In this study we can find no relationship between the circulating levels of IL-1 and IL-1 production by nonstimulated cells in culture. This phenomenon could be due to several factors, first, that there is truly no relationship between the levels of IL-1 in circulation and the levels spontaneously secreted in culture in the absence of any intentional stimulation. Second, hormones affect the levels and production of IL-1 and the fact that FCS is known to contain low levels of the hormone estrogen could have altered the secretion of IL-1 from the cultures. Third, circulating hormones could alter the catabolism thus altering the circulating half time but not affecting production or secretion. Finally, although we did not intentionally stimulate the cultures it is possible that low levels of endotoxin present in the equipment used to set up the cultures and in the media could have resulted in stimulation. This unintentional stimulation would have been low since that our results show significant differences between the amount of IL-1 produced in stimulated cultures as compared to nonstimulated cultures.

3.8.8 Summary

A summary of the relevant variables associated with the cytokine measurements during the course of the menstrual cycle is presented in Table 3.23.

3.9 Justification of Statistical Methods

A description of the various statistical tests used to analyze the data were presented in the Materials and Methods chapter of this text in section 2.10. In this section I will briefly explain the reasons for doing these tests as they are fundamental to the tests themselves and are already mentioned in the previous section. In addition, I will propose alternate tests and why they were not used.

The F test was used to determine if there was more variation in the males than in the females. This test gives an idea of which group has more amplitudes, or peaks and troughs than the other. This test was chosen because it would distinguish between a sample set that varied more or less than another, in this case the sample sets were males and females. There is no other test that can be done in place of this.

The t test was used to compare the means of samples taken from one full cycle in females to the means over a similar length of time in males. This test was used to distinguish differences in levels for the cytokines and gave us some indication if females produced more or less of one cytokine than males. This test was used because the two samples are independent of each other and we must assume normal distribution of the sample sets due to the central limit theorem. This theorem states that if the number of

Table 3.23. Variables, determined by multiple regression, that significantly influence, positively (+) or negatively (-), the cytokines measured in females.

Cytokine	E2	Day[1] of S	Day[2] of O	Length of Cycle	Age
IL-2[b]			-	+	-
IL-4[b]			-		+
IFNγ[b]					+ w[d]
IL-1ra[a]		+			+
IL-1ra[b]		+			
IL-1β[a]				+	
IL-1β[b]			+		
IL-1ra[c]	+			-	-
IL-1β[c]			-	+	-

[1] Day in the Cycle the sample was taken.
[2] Day of Ovulation.
[a] measured in nonstimulated culture supernatant.
[b] measured in stimulated culture supernatant.
[c] measured in serum.
[d] weak.

samples is greater than 30, normal distribution can be assumed. As we combined all the cycle data for all the nine volunteers the result was greater than 90 points, similarly the males had greater than 30 points. Nonparametric tests could be used in place of this test, but the nonparametric tests are less stringent than parametric tests. Also, because nonparametric tests do not use the original values, but convert them to ranks, the comparisons are more prone to error.

The paired t test was used to compare within the cycle as the samples are not independent from each other. Within the cycle, the samples are dependent on each other, or are successive. We wanted to determine if the highs and lows that the data indicated, when it was converted to proportion of phase, were statistically the same or different. In order to do this we had to use a paired test that attempts to control for extraneous variables. Again nonparametric tests were not substituted for t tests for the reasons above.

Finally, ANOVA is often used to analyze data such as those obtained in this study though it should not always be used. Usually a sample set that contains the same number of observations is needed to perform such a test. This was not the case in this project as we obtained a different number of samples from each of the females. In addition, it should only be used when the samples are obtained on the same days for every volunteer throughout the cycle. A final point, there have been studies that use ANOVA to compare males to females in different phases of the menstrual cycle for a particular observation. This is not appropriate as males have not been shown to cycle in the same way women

have with respect to estrogen, progesterone and luteinizing hormone. Males do not have a menstrual cycle thus the two are incompatible.

3.10 Technical problems

There were several problems that were encountered during the course of the study. To begin with it was very difficult to obtain females who were of the correct age and not taking oral contraceptives. The university population is a difficult place to try and obtain young women who are not taking oral contraceptives. Several hundred students were presented with an outline of the study and very few conformed to all the criteria needed to gain entrance to the study. In addition, some of the women who were eligible to participate did not want to donate what they perceived as large amounts of blood over a 5-week period of time. The level of commitment to this project also resulted in individuals declining to participate.

There was also a problem with the timing of samples in Phase II of the study. The women were scheduled to be bled three times a week, Monday, Wednesday, and Friday. During the course of this phase there were several Monday holidays thus that day was missed; in that case some of the volunteers were bled Tuesday, Wednesday, and Friday. This contributed to missed days in the cycle and loss of samples. We also encountered difficulty in getting the volunteers to notify us immediately when they began menstruating resulting in loss of samples in the early follicular phase. In addition, as the study was voluntary we could not prevent the volunteers from going on vacation during the study.

This occurred with one of the cycling volunteers, 30.04.65;as a result we missed several key days in her cycle.

The problem of frequent sampling days (3x a week) also contributed to the inability to obtain samples on some days. At times the subject could donate only a small amount of blood before the vein "collapsed".

The ELISA method contributed to some of the problems encountered. To cut costs we purchased ELISAs from Biosource, and as can be seen from the C.V. values, the results were not as reliable as those purchased from R&D. It is well known that different commercial ELISA assays give different results with the same sample tested (Ledur *et al.*, 1995). This is discussed further in chapter IV.

In addition, the assays for the culture supernatants of IL-1β and IL-1ra stimulations had to be diluted due to the high concentrations of the cytokines present. This was unknown when the first assay was done and thus there was a loss of materials and results. The subsequent assays were performed by diluting the samples as described in the Materials and Methods section. This dilution was high and added an additional variable in the system leading to inaccuracies, and, even with the dilution, some of the values were too high to be measured. In this case we put a greater than sign (>) in the table and did not use that sample day for calculation.

Finally, we had intended to measure TNFα and IL-6 in serum using a commercial ELISA but when tested both cytokines were below the sensitivity of the assays. This was disappointing as we had intended to collect information on all three inflammatory

cytokines (IL-1, TNFα, and IL-6). The information we obtained from the manufacturer (Biosource) indicated that we should have found measurable amounts of TNFα and IL-6 in approximately 75% of the samples.

CHAPTER IV

DISCUSSION

4.1 Creation of Sample Set

In the introduction of this thesis I have outlined the many aims and objectives of this project. The following sections of the discussion will discuss the creation of the sample set of serum and cells from healthy normal females at known points in their cycle and a similar sample set created of controls using normal healthy males. In addition, it was also our intention to monitor serum gonadal hormone levels in females and males.

4.1.1 Female Sample Set

Nineteen women participated in Phase I of the study. This phase was designed to determine if the volunteers had stable menstrual cycles with respect to time of ovulation and length of cycle. Eight women were chosen to proceed to Phase II where the immune parameters were measured.

There were 9 women, ages 19-29, who participated in Phase II, one of whom was not studied during Phase I but was demonstrated to have a normal menstrual cycle. These women were required to complete a daily assessment of any symptoms experienced over the course of their menstrual cycle in the form of a Menstrual Cycle Diary. The results indicated that there were no significant differences between the symptoms during the course of the cycle suggesting that they were not experiencing premenstrual syndrome

CHAPTER IV

DISCUSSION

4.1 Creation of Sample Set

In the introduction of this thesis I have outlined the many aims and objectives of this project. The following sections of the discussion will discuss the creation of the sample set of serum and cells from healthy normal females at known points in their cycle and a similar sample set created of controls using normal healthy males. In addition, it was also our intention to monitor serum gonadal hormone levels in females and males.

4.1.1 Female Sample Set

Nineteen women participated in Phase I of the study. This phase was designed to determine if the volunteers had stable menstrual cycles with respect to time of ovulation and length of cycle. Eight women were chosen to proceed to Phase II where the immune parameters were measured.

There were 9 women, ages 19-29, who participated in Phase II, one of whom was not studied during Phase I but was demonstrated to have a normal menstrual cycle. These women were required to complete a daily assessment of any symptoms experienced over the course of their menstrual cycle in the form of a Menstrual Cycle Diary. The results indicated that there were no significant differences between the symptoms during the course of the cycle suggesting that they were not experiencing premenstrual syndrome

(PMS). This is important to consider because it is believed that women who have PMS do not have normal menstrual cycles (Thys-Jacobs and Alvir, 1995).

In addition to not displaying any symptoms of PMS we were also able to establish that the volunteers were healthy as any medications and/or sickness they experienced were recorded. Examination of the diary from all the volunteers indicated that while some of the volunteers did periodically take analgesics they were generally in good health during the study. This is confirmed by the self-assessment questionnaires the volunteers completed each day they were sampled. This questionnaire monitored any medications consumed, sexual activity within 12 hours of sampling, and exercise since the last day they were bled. Examination of these questionnaires indicate that the volunteers did not partake of any excessive exercise that could alter hormone levels. Sexual intercourse has also been known to alter hormone levels for up to 12 hours and although it would have been ideal to have the volunteers abstain from sexual intercourse it was felt, considering the intense involvement in the study already, that abstinence was not an appropriate request. Some of the volunteers did engage in sexual intercourse during the study though there appears to be no effect on the results obtained as no hormone value is 50% different from the previous or subsequent sample.

To control for any influence of season on the menstrual cycle in our volunteers we also restricted the study to the summer months, that of May to August. All the volunteers that participated in the study, including the controls, were sampled during these months to minimize the variability between the groups and individual volunteers.

It is apparent from the data collected on length of cycle and day of ovulation that the female cycling volunteers could be considered to have normal stable menstrual cycles. It is generally accepted that the average cycle length is 29.5 days with a range of 26-34 days (Asso, 1983) and based on our data the females that participated in Phase II of the study fall within this range.

The time of ovulation was measured using urinary LH kits (OvuQuick). A recent study evaluated the usefulness of this home urine LH assay in women with regular ovulatory menstrual cycles without any obvious confounding variables. The group found that urine LH testing using this kit is a reliable method of predicting ovulation within the next 48 hours (Miller *et al.*, 1996).

During the course of Phase II the nine cycling female volunteers were bled between 9-15 times during the course of their menstrual cycles. The volunteers of the study were all sampled between 8:30-10:00 a.m. on all sampling days to reduce variability due to diurnal changes in number or function of circulating leukocytes. Variability in cytokine production and numbers of immune cells has been demonstrated by Born *et al.*, 1997, in relation to circadian rhythm. There were some problems with sampling days as illustrated in Figure 3.1. It is apparent from the figure that the number of samples obtained from the volunteers vary and that the goal of three samples per week was reached for most donors in the luteal phase of the cycle. Some early samples were missed since we were not always immediately notified when a volunteer first started her cycle. In addition, 50 ml of blood was not always obtained from the donors every sample day

leading to missing data.

4.1.2 Male Control Sample Set

In addition to the experimental group a control group consisting of four males between the ages of 20-25 were chosen. Males were chosen as a control group due to the fact that they do not cycle with respect to the gonadal hormones examined as females do. Thus the group would constitute a sample that did not have the influence of regular monthly hormonal changes contributing to the changes seen in the cytokine levels.

The males were required to meet previously set standards (section 2.12) before entering into the study. During the course of the study these individuals were also required to complete a self-assessment questionnaire each time they were sampled that was similar to the assessment the females completed. Based on these questionnaires it is apparent that the control group was healthy throughout the study; one male contracted a cold during the study and thus was excluded until after symptoms had disappeared. The men did not participate in extreme exercise and all sexual activity was reported as in the females. Examination of the data indicated that sexual intercourse 12 hours before time of bleeding did not result in the testosterone levels measured exceeding the given normal range.

4.1.3 Hormone Levels

The levels of some of the gonadal hormones were measured via radioimmunoassay. 17β-estradiol, progesterone, and luteinizing hormone were measured each time the volunteers were bled. The continuous hormone measurement gave us information on the actual levels of the hormones throughout the menstrual cycle and was used to determine if the females had the normal levels of the hormones at different phases of the menstrual cycle. In addition, the males were bled between 9-12 times over the course of 4 weeks and testosterone levels were measured on each sample.

The diagnostic RIA kits included the expected physiological ranges. The expected and actual ranges of these hormones are found in Table 3.7 in the results section of this paper. As is apparent from the levels of these hormones during the course of the study, the females had normal physiological levels of 17β-estradiol, progesterone and LH during the cycle that was studied. The hormone profile figures 3.2-3.10 can be viewed and demonstrate the normality of the individual volunteers when compared to the textbook profile seen in Figure 1.1.

Five of the volunteers are missing the LH peak due to the pattern of sampling days. This peak was not found in the LH hormone assays for the five women but was known to occur as indicated by the OvuQuick assay kit that the women used. Thus we do know that even though sampling days resulted in missing the serum LH peak it did occur indicating normal ovulatory cycles.

Based on all the information gathered on the cycling volunteers it is reasonable to

say that according to conventional terms the volunteers were healthy normal females with regular ovulatory cycles.

Thus, considering all the data gathered on the volunteers it is apparent that to the best of our knowledge both the females and the males were normal healthy subjects that would have fewer extraneous variables influencing the data gathered than a group less stringently screened.

4.2 Variability between Cycling females and Males

One of the aims of this study was to compare the variability in cytokine production in circulation and in supernatants of stimulated and nonstimulated cells in culture, in males and females. In this section the cytokines will be grouped and discussed as a group.

We examined the variability in cytokine production of all the cytokines measured in both the cycling (9 women) and noncycling (4 males) volunteers with the aid of the F test. Simplified, this test determines if the females have more variable cytokine production in successive samples than the males, i.e. if the females have more peaks and troughs than the males.

In addition, this section will discuss the means comparisons between the males and the females for all the cytokines measured. This statistical comparison provides information on whether one group actually produces more of one cytokine than the other.

4.2.1 IL-2, IL-4, and IFNγ

Statistical analysis showed that there was more variation over time in the females for IL-4 and IFNγ, thus supporting our hypothesis that the cyclical nature of gonadal hormones in the female system influences the immune system.

When we looked at the mean values of IL-2, IL-4 and IFNγ for the two groups we found that there was no statistical difference (p=0.945,0.398, and 0.055, respectively) between the amount of IL-2, IL-4 and IFNγ produced between the noncycling and cycling groups. Though we did note that the ranges for the two groups were quite different for IL-2 and IL-4. The difference between the minimum and maximum values for the cycling volunteers was 1549 pg/ml whereas for the men it was only 677 pg/ml for IL-2. IL-4 showed a difference of 504 pg/ml and 195 pg/ml, respectively. These differences were primarily due to a greater difference between ranges for the individual women, i.e. we had "high producers" and "low producers" among the women. This difference in ranges indicates that females, although they do not as a group produce more IL-2 or IL-4 they do appear to have a greater individual differences in the amounts produced.

4.2.2 Culture IL-1

The results indicate that, although there are differences in group means between the cycling and noncycling volunteers, there was no significant difference in variability in successive samples for any of the isoforms of IL-1 measured in either the stimulated or nonstimulated culture supernatants.

For the groups means, our results indicated the following in nonstimulated cultures: women had higher levels of IL-1ra than did men. While IL-1β, and the agonist/antagonist ratio, IL-1β/IL-1ra, showed no differences between gender.

In cell cultures stimulated with LPS, no differences were found in IL-1β or IL-1ra production from stimulated cultures between males and females, similarly, the IL-1β/IL-1ra ratio analysis indicates that no gender differences exist based on the data from this study. However, 2 of the 29 stimulated samples from the men were lost because they were above the sensitivity of the plate while 17 of the 81 samples from the women were lost, thus weakening our conclusions.

Lynch *et al.*, 1994 looked at IL-1α, IL-1β and IL-1ra production in nonstimulated and LPS stimulated cultures. This group found that cells from women isolated during the follicular phase, secrete more IL-1ra in nonstimulated cultures than cells isolated from men. In addition, they found that women secrete more IL-1β than men, in contrast to this we found no difference. In stimulated cultures Lynch and associates determined that there were no significant gender related concentration differences in any isoform of IL-1 tested which is consistent with the results of this study. Furthermore, Lynch and associates found that the ratio of IL-1α + IL-1β to IL-1ra was higher in the luteal phase for unstimulated cultures but higher in the follicular phase for stimulated cultures. Our results agree with these observations if we assume that Lynch *et al.*, sampled their volunteers during the MF and ML phases of the cycle.

The differences observed in the results from the Lynch study, more IL-1ra and

IL-1β produced by women than men in unstimulated cultures, and this study could be due to a number of factors. The Lynch paper analyzed cytokine concentration by RIA, whereas in this study cytokine concentration was measured by ELISA. In addition to this, Lynch *et al.*, cultured PBMC in the presence of 1% autologous serum and stimulated with 1 ng/ml of LPS. In this study, 1% FCS was used to supplement the media and stimulation was with a much larger concentration of LPS, that of 8 ug/ml. Our values for IL-1ra production were much higher, particularly in the stimulated cultures, than those found in the study by Lynch *et al.* Our values for IL-1β in stimulated cultures were also much higher than theirs. This difference in the amount of LPS used to stimulate the cultured cells in the two studies could have contributed greatly to the differences in the data. It is known that LPS activates monocytes to secrete IL-1 by binding to specific receptors on the cell surface. The differences in the amounts of IL-1 secreted under stimulation with different concentrations of LPS may be due to differences in the number or quality of receptor being bound by LPS, thus possibly influencing the levels of IL-1 produced by the cells in culture.

Not only are there differences in the methods used but there is a large difference when considering results using the agonist/antagonist ratio. Lynch *et al.*, had IL-1β and IL-1α as agonists, whereas we only measured IL-1β. Perhaps the most important difference is that Lynch *et al.* did not determine exactly where in the menstrual cycle their donors were when they were bled (days after first day of menses was known).

4.2.3 Serum IL-1

Changes in IL-1β and IL-1ra in circulation over time in both the cycling females and the noncycling males were examined. Statistical analysis demonstrated that both IL-1β and IL-1ra in serum showed more variation over time, $p=<0.01$ and <0.05, respectively, in the females as compared to the males, the females had significantly larger variances in the successive samples than did the males. This lends support to the influence of cyclical hormonal changes on cytokine levels supporting the possibility that the variation is influenced by the fluctuation in hormones in females and that this difference might be partly due to these hormones.

Comparing the mean values for the noncycling and cycling groups we found that there was no significant difference for IL-1β or IL-1ra between the two groups.

The finding that the females have more variation in the samples obtained than the males but the lack of any significant difference between the overall means in serum IL-1 suggest that women do not produce more total IL-1 than men but that they do have many more lows and highs in IL-1 production than do the males.

4.3 Cytokines and The Menstrual Cycle

A significant portion of the objectives of this study was to monitor serum cytokine and cytokine production by cultured cells over the course of the menstrual cycle and then determine if there was cyclical variation in cytokine levels in females.

4.3.1 IL-2, IL-4 and IFNγ

We found that the amounts of IL-2, IL-4 and IFNγ produced by cultured PBMC stimulated with PHA varied during the course of the menstrual cycle. IL-2 and IFNγ production is low at time of ovulation and high at the late luteal phase whereas IL-4 is low at ovulation and high at menses.

It is obvious from inspection of Figures 3.12- 3.15 regarding production of IL-2, IL-4 and IFNγ that the levels of IFNγ from the EF phase of the first menstrual cycle are not comparable with the EF phase of the second menstrual cycle. There are several possible explanations for this which are discussed in section 3.7.3 and will be briefly mentioned here. First, in the second cycle the female volunteers did not measure their time of ovulation, or the length of the cycle, thus the time of a volunteer's second EF phase is assumed rather than actually determined. It is true that all the volunteers had normal cycles but that does not mean that they will always ovulate or that all their cycle lengths will be the same. It is common for females to periodically have annovulatory cycles (Prior *et al.*, 1990) and, as is obvious from our Phase I results, every individual varies in the total length of cycle, from cycle to cycle. Second, not all nine volunteers were bled in both the first and the second EF phase. Third, it has been noted by other groups that the intra-individual variation of the response of human lymphocytes to mitogenic stimulation can be quite great in humans (Herrera *et al.*, 1992) and in production of IgA and IgG antibodies in inbred strains of mice (Gallichan and Rosenthal, 1996). Thus, it is apparent that continuous sampling of a small number of females over

several cycles is an important experiment that needs to be done.

No detectable levels of IL-2, IL-4, and IFNγ were observed in supernatants of unstimulated cultures. This is consistent with results of a study done by Candore *et al.*, 1994, using PHA stimulated PBMC and harvest times similar to ours.

It is apparent from the statistical analysis that, for all three cytokines measured in culture, production is low at ovulation and high in the luteal phase of the cycle. This suggests a dampening of the ability of the T cells to produce these cytokines at time of ovulation when estrogen and LH is very high. Estrogen has been shown at high physiological levels to inhibit proliferation of PHA stimulated lymphocyte from females (Herrera *et al.*, 1992).

In addition, in male rats, Atherya *et al.*, 1993, demonstrated that *in vivo* administration of E2 and PHA caused a decrease in the percentage of $CD4^+$ T cells. Since IL-2, IL-4 and IFNγ are produced by $CD4^+$ T cells this could explain the lows we see at ovulation and the resultant higher levels in the late luteal phase and menses when estrogen levels are lower.

Studies done on cyclical variations in white cell populations from females and males (Mathur *et al.*, 1979) also show that lymphocyte counts are negatively correlated with E2 (decreased at pre-ovulatory E2 peak in the follicular phase) and monocyte counts were positively correlated with P. No cyclical variation in WBC populations were seen in males.

These data suggest that the variation observed by us in cytokine production is not

necessarily due to a change in amount of cytokine produced per cell but rather due to a change in relative numbers of mononuclear cell subpopulations present in the circulation and therefore in culture. The suggestion is that our results could reflect *in vivo* changes in relative numbers of mononuclear cells, controlled by hormones.

It is important to consider the T_H1/T_H2 paradigm in the immune system. The T_H1 and T_H2 patterns of cytokine production are well characterized in mice. T_H1 $CD4^+$ T cells produce IL-2 and IFNγ, whereas T_H2 $CD4^+$ T cells produce IL-4, IL-5, IL-10 and IL-13 (Mossmann and Coffman, 1989; Romagnani, 1991, 1995). T_H1 cells promote cell-mediated immune responses and macrophage activation. In contrast T_H2 cells promote humoral immune responses (Finkelman and Holmes., 1990).

The type of effector cell, either T_H1 or T_H2 , that develops from the naive T_H0 $CD4^+$ T cells depend largely on the conditions of antigen exposure. It has been shown that the cytokines that T cells are exposed to influence the development of a particular subset. Activation of naive T cells in the presence of IL-4 promotes T_H2 cells, whereas IFNγ and IL-12 promote T_H1 development (Swain *et al.*, 1990; Maggi *et al.*, 1992; Seder *et al.*, 1992; Hsieh *et al.*, 1992; McKnight *et al.*, 1994).

It is also known that the cytokine products of TH_1 and TH_2 cells are mutually inhibitory. IFNγ selectively inhibits proliferation of TH_2 cells and IL-4 inhibits proliferation of TH_1 cells (Mosmann and Sad, 1996).

The terms T_H1 or T_H2 are often used to refer to responses dominated by IFNγ and IL-4, respectively (Mosmann and Sad, 1996). In this study we saw that both IL-4 and

IFNγ were low at ovulation but that levels of IL-4 were very high at menses and of IFNγ were high at MF and LL. This suggests a tendency toward a T_H1 response in the MF and LL phase of the cycle with a T_H2 response during menses. Thus it would be reasonable to suggest that the cycling of these cytokines could reflect the effect of hormones on the relative numbers of circulating T_H1 and T_H2 T cells. To confirm this it would be necessary to analyze the T cell populations present at different times in the menstrual cycle. Such an analysis is now possible with the advent of a new technique that allows detection of a cell-surface marker that can distinguish T_H1 from T_H2 $CD4^+$ T cells. It has been found that IL-12R is not present on unstimulated T cells but that after stimulation with antigen there is low level expression of the αβ chains of the receptor. T_H1 cells express both receptor chains but in T_H2 cells there is selective loss of β2 chain expression (Rogge *et al.*, 1997; Gallagher, 1997).

In future studies it would be possible to tag T cells and determine numbers of T_H1 and T_H2 cells present at different times throughout the cycle thus supplying more information on what is actually occurring.

Marzi *et al.*, 1996, looked at antigen and mitogen stimulated cytokine production by PBMC of healthy pregnant and nonpregnant controls (with normal menstrual cycles). The data showed that as gestation advanced from the 1st to the 3rd trimester of pregnancy, production of IFNγ by PBMC stimulated *in vitro* with PHA decreases and the T_H2 cytokines IL-4 and IL-10 increase in successful pregnancy with a T_H1 to T_H2 shift characterising the third trimester. The progesterone levels in the second and third

trimester are similar to the physiological levels of P present in the luteal phase of the cycle (Polan *et al.*, 1994).

In addition to finding that IL-4 is high at menses we also observed a steady increase in IL-4 production from ovulation to menses. In contrast, IFNγ levels in the LL phase are much lower than IFNγ levels in the MF phase, though still higher than those at ovulation. The data seem to parallel the trend seen in pregnancy with decreased IFNγ production and increased IL-4 production as progesterone levels rise in the luteal phase.

4.3.2 Culture IL-1

The results of this study indicate that over the course of the menstrual cycle there are significant changes in both the production by mononuclear cells and in the circulating levels of IL-1ra and IL-1β. This section deals with the isoforms of IL-1 produced in culture. The next section will deal with the results seen in the circulating levels of the cytokines.

We have measured by ELISA the production of IL-1ra and IL-1β by cultured mononuclear cells unstimulated and stimulated with LPS. From the data graphed (Figures 3.15-3.17) as phase of the cycle, it is apparent that there are fluctuations in the production of these cytokines. Specifically, in nonstimulated cultures representing the baseline production, IL-1ra fluctuation is characterised by highs in the MF, ML and at menses with lows at ovulation and EL. Statistical analysis of these lows and highs does not confirm the trend observed in the cycling women. IL-1ra production in LPS stimulated cultures

indicates a pattern of highs seen during ovulation and ML with lows at EF and M, opposite to that seen in the nonstimulated cultures, though these changes are not confirmed by statistical analysis.

When considering IL-1β production in the supernatants of nonstimulated cultures the data suggests a trend of a high in the LF phase and at menses and a low at the time of ovulation, p=0.0471 and p=0.0170. In the stimulated culture supernatants there is a high at ovulation, as seen in IL-1ra, and during menses with low levels at MF and ML though these were not significant. It is very difficult to deduce anything from the graph due to the fact that many of the values for IL-1β are above the sensitivity of the plate and thus are not known. Even though the samples were diluted 1:10 or 1:20 (see Appendix B) there are still many points missing in the data. Out of 96 values there are 21 missing data points contributing to the uncertainty in dealing with the data; only 4 points of these missing points occur in the follicular phase suggesting that IL-1β values in the luteal phase are actually higher than seen in the graph. One way to deal with this would be to convert all values to percent and estimate the high values as possibly 150%, thus allowing for their use in the calculations and contribution to the graphs.

As a measure of functional IL-1, the biological activities attributed to IL-1 depend in part on the ratio of agonist (IL-1α and IL-1β) to antagonist (IL-1ra) (Lynch *et al.*, 1994). We therefore calculated the ratio of IL-1β/IL-1ra. When considering this agonist/antagonist ratio in unstimulated cultures we see at high at LF and M with a low at ovulation, however these differences did not reach significance.

In cell cultures stimulated with LPS, the agonist/antagonist ratio shows a high at ovulation and menses, with a low at ML. ML versus menses, p=0.0057. In addition, comparing with the Lynch *et al.*, paper, if we consider the points of MF vs ML we find that our results correlate with those of the Lynch study. Both studies indicate that the IL-1 ratio is higher in the follicular phase than in the luteal phase. The problem with comparing the two studies is that Lynch *et al.*, does not indicate the exact days in the follicular and luteal phase the samples were obtained and as is indicated in this study there are fluctuations in the production of cytokines during the stages of the two phases as well as differences between the two phases.

It is apparent from the data gathered on the cyclical changes in IL-1 that the picture is still hazy but is different in nonstimulated versus stimulated culture supernatants.

4.3.3 Serum IL-1

The amounts of IL-1β and IL-1ra in serum were measured by ELISA.

We found that both IL-1ra and IL-1β levels in circulation changed during the menstrual cycle, (Figures 3.19 and 3.20). IL-1ra levels were found to increase from the MF phase to menses. Statistical analysis of the graph confirmed that the EF and MF phases were low compared to the levels seen at ovulation p=0.0208 and 0.0075, respectively. This trend implies that during the course of the menstrual cycle the concentration of IL-1ra in circulation increases.

IL-1β on the other hand, has a different trend. The data from IL-1β levels were graphed and there appears to be a high at the EF phase, a decline to time of ovulation, with the levels rising again to peak at the ML phase. Analysis of the graph does not confirm this trend though it is suggested that perhaps with a larger sample size, as only 3-4 pairs were available, the change would be confirmed.

We also calculated the agonist/antagonist ratio as IL-1β/IL-1ra. This gives a measure of the functional IL-1 although, as in the culture, we did not measure IL-1α. When the IL-1 ratio was graphed as phase of the cycle, (Figure 3.18), we see that there is a high at the EF phase and a low at time of ovulation, p=0.1450. Though this change is not found to be significant, again we suggest that with a larger sample size it could prove to become significant. The levels in the luteal phase are lower than that of the follicular phase but higher than the levels at ovulation. This apparent high in the follicular phase is in contrast to the results of a study by Cannon and Dinarello in 1985 where they found IL-1 activity in plasma, as measured by a bioassay (D10 thymocyte proliferation assay) to be greater in the luteal phase compared to the follicular phase.

Differences in the results could be due to the fact that we measured actual IL-1β and IL-1ra concentration in serum whereas they measured IL-1 bioactivity which would include IL-1α, IL-1β and IL-1ra in combination. The inclusion of IL-1α in an analysis of IL-1 activity or concentration appears to be important for the entire IL-1 picture. They judged their five donors to be in the luteal phase when serum progesterone was 7.4-12.9 ng/ml and in the follicular phase when serum progesterone was 0.7-0.9 ng/ml/. In our

study only two women had progesterone values between 0.7 and 0.9 ng/ml in the follicular phase (the other values were lower). The mean IL-1 ratio for the corresponding two samples was 0.03. Eight women had luteal progesterone values between 7.4-12.9 ng/ml. The mean IL-1 ratio for the corresponding samples was 1.49 which is similar to Cannon and Dinarello's findings of higher IL-1 values in the luteal phase.

Future studies would have to include a method of measuring IL-1α; we could not find an ultrasensitive commercial ELISA assay needed for measuring this isoform of IL-1 in serum. Perhaps using a bioassay for IL-1 would be more appropriate for serum levels and perhaps more biologically relevant.

From the data gathered on serum IL-1 levels it is evident that there is a change in circulating IL-1 during the menstrual cycle. This is consistent with the other cytokines examined and implies that hormones do affect the production of cytokines supporting the hypothesis that hormones affect the immune response in humans.

4.4 Correlations and Regressions

A final aim of this study was to determine if the variation we observed in cytokine expression is linked or correlated with the gonadal hormones estrogen, progesterone and luteinizing hormone as well as several other variables we had gathered information on.

The various results of the regression analysis are presented in section 3.8 of this thesis.

4.4.1 IL-2, IL-4 and IFNγ

Multiple regression of the data to determine if any of the variables measured had an influence on the production of the cytokines indicates that IL-2 is related to day of ovulation, length of cycle and age of the volunteer r=0.59801, p=0.0007, 0.0000 and 0.0000, respectively. IL-4 had a relationship with day of ovulation and age of the volunteer, r=0.63416, p=0.0010, and 0.0000, respectively. IFNγ was found to have a weak relationship with age, r=0.21940, p=0.0377.

Specifically the regression illustrated that IL-2 decreased with age, though the production of IL-4 and possibly IFNγ increased with age. Similarly, Engwerda *et al.*, 1996, demonstrated the same phenomenon in male mice T cells stimulated with immobilized anti-CD3ε chain monoclonal Ab and soluble anti-CD28 mAb for 24 hrs. In addition, both IL-2 and IL-4 production was found to decrease as the follicular phase becomes longer, meaning that the further away ovulation day is from the beginning of the cycle the lower the amount of IL-2 and IL-4 will be produced in culture. Unfortunately it is impossible to deduce information from the IFNγ regression due to the low coefficient of correlation demonstrating a weak relationship, if one at all.

4.4.2 Culture IL-1

Multiple regression analysis indicated that IL-1ra levels in nonstimulated cultures had a correlation of r=0.43696 with day in the cycle the sample was taken (p=0.0439) and age of the volunteer (p=0.0003). IL-1ra in stimulated cultures showed a significant

correlation with day in the cycle the samples were taken, r=0.36596, p=0.0011.

These results indicate the IL-1ra production from both nonstimulated and stimulated cultures are related to the day in the menstrual cycle the sample was taken further supporting the hypothesis that IL-1ra levels change during the cycle. Specifically, these results indicate that the later in the cycle the sample is taken the greater the amount of IL-1ra produced in culture. This corresponds to the cycle data we have that indicates that IL-1ra production is higher during the later half of the cycle, the luteal phase. As with IL-4 and IFNγ, IL-1ra production in culture also seems to increase with age of the volunteer. This finding is not obvious when the raw data are examined; the coefficient of determination for this regression was between 13 and 19%, suggesting that only a relatively small portion of the variance in IL-1ra can be attributed to these factors.

Regression of IL-1β indicates a correlation with cycle length in nonstimulated cultures, r=0.34083, p=0.0039, and in stimulated cultures with day in the cycle ovulation occurred, r=0.42277, p=0.0008, suggesting that changes during the menstrual cycle do influence the production of IL-1β. Both cycle length and day of ovulation are controlled by hormonal factors in the body. It is these hormones that determine when in the cycle a person will ovulate and how long the total length the cycle will be thus contributing to the hypothesis that IL-1β production in cultures are influenced by hormonal variation. IL-1β production in nonstimulated cultures was found to be greater the longer the total cycle length, and, in stimulated cultures, the further into the cycle ovulation occurs. Both of these variables are intimately related in that the further into the cycle ovulation occurs

the longer the cycle will be. Examining the mean values in Tables B.16-B.30 in Appendix B shows that 3 of 5 volunteers who had longer cycles had much higher amounts of IL-1β produced in stimulated cultures.

4.4.3 Serum IL-1

Multiple regression was also performed on the data to determine if any of the variables measured had an influence on the levels of the IL-1 isoforms in serum. IL-1ra was found to have a strong correlation r=0.58700 with the length of the cycle, p=0.002, age of the volunteer, p=0.0083, and with the concentration of estrogen during the cycle, p=0.0008. IL-1β was found to correlate, r=0.75567, with the length of the cycle, the day of ovulation, and age, p=0.0000 for all variables. This correlation further supports that changes during the menstrual cycle influence the level of IL-1β and IL-1ra in circulation.

To clarify, both isoforms of IL-1 are found to have lower levels in circulation the older the individual is. In addition, IL-1 is also related to the total length of the cycle. The regression indicates that IL-1ra decreases as cycle length increases but IL-1β increases as cycle length increases. Examining Tables B1-B15 in Appendix B, indicates approximately that the four individuals who were the youngest of the group had a mean IL-1ra level of 435.4 pg/ml compared to the five older individuals who had a mean of 159.2 pg/ml. Examination of IL-1β levels shows that the younger individuals have a mean of greater than 6.03 pg/ml, whereas the older subjects had a mean of less than 0.66 pg/ml, consistent with the results of the regression analysis. In addition, the data shows

that individuals who had cycle lengths between 29 and 31 days had a mean IL-1ra level of 212.3 pg/ml whereas those with cycle lengths between 26 and 28 days had a mean of 369.2 pg/ml; IL-1β levels were approximately 4.4 pg/ml and 0.5 pg/ml, respectively.

Finally, it is important to note that these r values are very high indicating strong correlations with the independent variables and that the coefficient of determinations for IL-1 are 34 and 57%, respectively, suggesting that much of the variation we see in the levels of IL-1 in circulation is due to theses variables.

4.5 C3 and C'H50

These components were measured by two other students using the sample set created in this project. The same analyses were done on the results as on the cytokines measured in this project. Briefly, it was found that C3 concentrations and hemolytic activity, C'H50, changed during the menstrual cycle with peaks in early to midfollicular and midluteal phase and lows at time of ovulation. Thus the complement components exhibit the same general pattern of decreased levels in circulation at time of ovulation as many of the cytokines we measured. In addition, C'H50 was found to be more variable in the females than in the males in successive samples. Finally, men were found to have significantly higher C3 concentrations than females. This is similar to the findings of Agostini *et al.*, 1968.

4.6 Receptors

Significant differences between the immune systems of males and females have been demonstrated in this study. It is known that females have a) greater incidence of autoimmune diseases than males; b) higher levels of immunoglobulin; c) stronger immune responses; and d) greater resistance to bacterial and parasitic infections than do males (see Section 1.2 and 1.4). The implication is that sex hormones or other products of the gonads play an important role in these differences.

Both estrogen and progesterone are thought to influence the immune response. These effects have been demonstrated in a variety of studies. Estrogen has been shown to increase production of IL-1 *in vivo* by mouse peritoneal (Flynn, 1986) and *in vitro* by rat peritoneal macrophages (Hu *et al.*, 1988).

The effects of sex hormones could be indirect, as a combination of factors that influence the immune response. Alternately, the influence of sex hormones could be due to a direct effect of these hormones on the various arms of the immune response. A direct effect could possibly be facilitated by the action of the sex hormones on the immune cells via specific receptors. Recent evidence has demonstrated the presence of specific hormone receptors on various cells of the immune system and supports a possible direct effect.

Gulshan *et al.*, 1990, using whole cell assays, investigated the possibility of 17β-Estradiol receptors on male rat peritoneal macrophages and on a human monocytic leukaemia cell line, JIII, established from a female patient. They found that both cell

types expressed high affinity binding receptors for estrogen. The results also indicated that there were two different estrogen binding receptors.

Similarly, Cutolo *et al.*, 1996, investigated human synovial macrophages obtained from RA and healthy subjects for the presence of estrogen receptors. Specific monoclonal Ab revealed immunostaining for estrogen receptors in cultures of macrophages obtained from both groups of subjects. Two types of estrogen receptors were demonstrated on macrophages, a type I (high affinity, low capacity) and type II (lower affinity, higher capacity) receptor. In addition, they found increased levels of estrogen receptors of both types on cells from rheumatoid samples. The authors suggests that these results correlate with a stimulatory effect by physiological levels of estrogen implicating estrogen in the pathophysiology of RA.

Both of these studies provide evidence for the possibility of a direct action of the sex hormone, estrogen, via specific receptors on immune cells. IL-1, TNFα, and IL-6, are principally produced by macrophages. The changes we found in this study, in both secretion of IL-1β and IL-1ra from cultured cells and in circulation could be due in part to the direct effect of estrogen during the cycle on the macrophages that produce these cytokines.

Estrogen receptors have also been identified on human blood mononuclear cells and thymocytes. Weusten *et al.*, 1986, investigated the estrogen binding sites in mononuclear cells from healthy donors, patients with leukaemia or SLE and in thymocytes, using the dextran-coated charcoal assay. The results indicated that estrogen

receptors were present on MNC isolated from both sexes, and from female patients who had leukaemia or SLE, and on thymocytes.

Not only have estrogen receptors been identified on thymocytes, Cohen *et al.*, 1993, demonstrated the presence of estrogen receptors on human peripheral T cells. The peripheral T cells were obtained from thoracic duct drainage performed before renal transplantation. The results suggested that estrogen receptors are present on $CD8^+$ T cells but not on $CD4^+$ T cells. Thus the authors suggest that the immunoregulatory effects of estrogen could be modulated by $CD8^+$ cells but not by $CD4^+$ cells. Similarly, Stimson, 1988, found the same results for human T lymphocytes.

Thus it is apparent that many studies have shown the presence of estrogen receptors on various cells of the immune system lending support to the theory that sex hormones directly influence the immune response.

In addition to the evidence of estrogen receptors on immune cells, a study done by Fox *et al.*, 1991, indicates that estrogen regulates IFNγ cytokine gene expression. In this study male mice spleen cells obtained by macerating the spleens were stimulated with Con A in the presence or absence of estrogen. Exposure to physiological concentrations of estrogen increased IFNγ expression in mitogen stimulated spleen cells. In addition, they demonstrated that estrogen has a direct stimulatory effect on IFNγ promoter activity. Thus the authors suggest that the IFNγ gene is subject to direct hormonal control.

IFNγ is produced by both $CD4^+$ and $CD8^+$ T cells as are IL-2 and IL-4. Based on the published evidence of specific estrogen receptors on CD8+ T cells, and the finding

that IFNγ gene expression is regulated by estrogen, we suggest, that the cytokine changes we see during the menstrual cycle in humans, may in part be due to direct hormonal regulation of these cytokines via specific receptors.

In addition, Vik *et al.*, 1991, described structural features of the human C3 gene and found a number of regions that share homology with known regulatory sequences including IFNγ, IL-6 and NF-κB responsive elements and also the estrogen response element suggesting that C3 production may be influenced by estrogen. However, there is clearly no simple relationship between estrogen and serum C3 level, since estrogen in our cycling women peaked at pre-ovulation and again at midluteal, with the lowest values at early follicular. One would have to postulate an inhibitory effect of high concentrations of estrogen and possibly a stimulatory effect of progesterone in order to explain changes in serum C3 concentration as a direct result of changes in hormone levels.

4.7 Conclusions

This study of changes in immune parameters during the menstrual cycle has indicated that there are cyclical fluctuations in the level of cytokines as well as C3 and C'H50, over the course of the menstrual cycle in females. The intriguing finding in this study was that the majority of the parameters examined were low at time of ovulation. Why this is so is not known, though we know that estrogen is very high just prior to ovulation and perhaps this has an inhibitory effect on the immune system. The presence of estrogen receptors on various cells makes possible a direct effect of estrogen on the immune system. Whether or not progesterone or luteinizing hormone also has an effect is unknown. The literature has very little evidence of the effect of these hormones and further investigations into this are needed.

This study suggests a T_H1 to T_H2 shift over the course of the menstrual cycle, from the follicular to the luteal phase, which is consistent with the finding in pregnancy. This observation is interesting and could have relevance to selecting the optimal time of vaccination in women.

The changes in the inflammatory cytokine IL-1 over the menstrual cycle indicates that there are times during the cycle when women are more likely to produce a larger amount of IL-1 in response to antigenic exposure.

We demonstrated that cycling females, as compared to males, are more variable over time for IL-4 and IFNγ, produced by cultured cells, and IL-1ra and IL-1β in circulation. In addition, nonstimulated cultured cells from females had significantly higher

mean values for IL-1ra than males though females showed lower mean IL-1 ratio levels in serum than males.

Taken together, these results indicate that there are cyclical changes in the concentration of the cytokines suggesting that regulation of cytokine production are fundamentally different in women than men and that, in females, the immune response is influenced by changes in hormone status.

4.8 Future Studies

This project had several aims and based on the results obtained it is apparent that those aims have been met. Although we have found that cyclical changes do occur in the immune parameters measured in serum and in culture, there are still a number of questions left unanswered.

Future projects investigating the influence of hormones on the immune response should involve culturing of PBMC with various concentrations of estradiol and progesterone as have been done in some other studies but also with various concentrations of luteinizing hormone since the majority of the cytokines appear to be low at ovulation when LH is very high.

Any future study would also have to involve a larger sample size to allow for better statistical outcomes. Timing of samples would have to be more rigorous as we failed to obtain blood during the EF phase from many of the volunteers and missed the time around ovulation. As ovulation appears to be a time of "immunosuppression" it would be imperative to have more samples around this time.

Our data suggests a T_H1 to T_H2 shift in cytokine production from the follicular to the luteal phase of the menstrual cycle, based on cytokine production patterns. A new technique published this year allows separation of the T_H1 and T_H2 phenotypes by surface markers thus it would be possible to confirm this trend during the cycle by quantifying T cells.

The present investigation focused on healthy cycling female responses during the

menstrual cycle in order to establish a baseline against which results of women with certain diseases could be compared. An investigation of women with autoimmune diseases like SLE or RA would be interesting. Do such females follow a similar or different pattern of expression than the normal female population?

A potential future project would be to examine healthy females taking oral contraceptives as they do not have the same hormonal changes as women who are not using them. How do the cytokine levels vary in this group? Do they vary at all, if as we suspect, hormonal changes are influencing immune response? Are their immune systems depressed or enhanced by the oral contraceptive and do they return to "normal" after they cease using the oral contraceptives? Prior *et al.*, 1995, has shown that females taking medroxyprogesterone acetate experience increased basal body temperature, which suggest changes in cytokines.

There are a large number of questions to be asked about regulation of cytokine production and it appears that as more work is done more questions arise. Research in immune-endocrine interaction may provide information about basic biology and also be relevant to such areas as autoimmunity, oncology, and vaccines.

REFERENCES

Abbas, A. K., A. H. Lichtman, & J. S. Pober. 1994. Cellular and Molecular Immunology, 2nd ed. W.B. Saunders Company, Philadelphia, Pennsylvania.

Agostini, A., C. Vergani, & R. Stabilini. 1968. Immunochemical quantitation of serum β_{1A}-globulin in health and disease. *Z Klin Chem Klin Biochem* **6**:446-448.

Ahmed, S. A., & W. J. Penhale. 1982. The influence of testosterone on the development of autoimmune thyroiditis in thymectomized and irradiated rats. *Clin Exp Immunol* **48**:367-374.

Ahmed, S. A., M. J. Dauphinee, & N. Talal. 1985. Effects of short-term administration of sex hormones on normal and autoimmune mice. *J Immunol* **134**:204-210.

Ainbender, E., R. B. Weisinger, M. Hevizy, & H. L. Hodes. 1968. Difference in the immunoglobulin class of polioantibody in the serum of men and women. *J Immunol* **101**:92-98.

Asso, D.. 1983. The Real Menstrual Cycle. John Wiley & Sons, Toronto, Ontario.

Athreya, B. H., J. Pletcher, F. Zulian, D. B. Weiner, & W. V. Williams. 1993. Subset-specific effects of sex hormones and pituitary gonadotropin on human lymphocyte proliferation *in vitro*. *Clin Immunol and Immunopathol* **66**:201-211.

Baral, E., E. Nagy, & I. Berczi. 1996. Modulation of lymphokine-activated killer cell-mediated cytotoxicity by estradiol and tamoxifen. *Int J Cancer* **66**:214-218.

Barnes, E. W., A. C. MacCuish, N. B. Loudon, J. Jordan, & W. J. Irvine. 1974. Phytohaemagglutinin-induced lymphocyte transformation and circulating autoantibodies in women taking oral contraceptives. *Lancet* **1**:898-900.

Bjune, G.. 1976. *In vitro* lymphocyte responses to PHA show covariation with the menstrual cycle. Tenth meeting of the Scandinavian Society for Immunology. *Scand J Immunol* p.362.

Blakemore, A. I. F., S. Laird, M. Okon, T. C. Li, & J. K. Maskall. 1996. Serum interleukin-10 levels do not alter during the menstrual cycle. *Immunol, Suppl 1*. **89**:70.

Born, J., T. Lange, K. Hansen, M. Molle, & H. Fehm. 1997. Effects of sleep and circadian rhythm on human circulating immune cells. *J Immunol* **158**:4454-4464.

Butterworth, M., B. McClellan, & M. Allansmith. 1967. Influence of sex on immunoglobulin levels. *Nature* **214**:1224-1225.

Caggiula, A. R., C. M. Stoney, K. A. Matthews, J. F. Owens, M. C. Davis, & B. S. Rabin. 1990. T-lymphocyte reactivity during the menstrual cycle in women. *Clin Immunol and Immunopathoi* **56:**130-134

Candore, G., D. Cicgna, F. Gervasi, A. T. Colucci, M. A. Modica, & C. Caruso. 1994. *In vitro* cytokine production by HLA-B8, DR3 positive subjects. *Autoimmun* **18**:121-132.

Cannon, C. A., & J. G. Dinarello. 1985. increased plasma interleukin-1 activity in women after ovulation. *Science* **227**:1247-1249.

Chao, T., P. J. Van Alten, J. A. Greager, & R. J. Walters. 1995. Steroid sex hormones regulate the release of tumour necrosis factor by macrophages. *Cellular Immunol* **160**:43-49.

Clarke, I. J.. 1995. The preovulatory LH surge: A case of a neuroendocrine switch. *Trends Endocrinol Metab* **6**:241-247.

Cohen, J. H. M., L. Danel, G. Cordier, S. Saez, & J. Revillard. 1993. Sex steroid receptors in peripheral T cells: absence of androgen receptors and restriction of estrogen receptors to OKT8-positive cells. *J Immunol* **131**:2767-2771.

Cutolo, M., S. Accardo, B. Villaggio, A. Barbone, A. Sulli, D.A. Coviello, C. Carabbio, L. Felli, D. Miceli, R. Farruggio, G. Carruba, & L. Castagnetta. 1996. Androgen and estrogen receptors are present in primary cultures of human synovial macrophages. *J Clin Endocrinol Metab* **81**:820-827.

Danel, L., G. Souweine, J.C. Monier, & S. Saez. 1983. Specific estrogen binding sites in human lymphoid cells and thymic cells. *J Steroid Biochem* **18:**559-563.

Daniel, W. W. 1995. Biostatistics: a foundation for analysis in the health sciences, 6th ed. John Wiley & Sons, Inc., New York, New York.

Daynes, R. A., D. J. Dudley, & B. A. Araneo. 1990. Regulation of murine lymphokine production *in vivo*. *Eur J Immunol* **20**:793-802.

Detlefsen, M. A., B. C. Smith, & H. W. Dickerman. 1977. A high-affinity, low capacity receptor for estradiol in normal and anemic mouse spleen cytosols. *Biochem and Biophysic Res Communic* **76**:1151-1158.

Doty, R. L.. 1979. A procedure for combining menstrual cycle data. *J Clin Endocrinol* **48**:912-918.

Engwerda, C. R., B. S. fox, & B. S. Handwerger. 1996. Cytokine production by T lymphocytes from young and aged mice. *J Immunol* **156**:3621-3630.

Erbach, G. T., & J. M. Bahr. 1988. Effect of chronic or cyclic exposure to estradiol on the humoral immune response and the thymus. *Immunopharma* **16**:45-51.

Erbach, G. T., & J. M. Bahr. 1991. Enhancement of *in vivo* humoral immunity by estrogen: permissive effect of a thymic factor. *Endocrinol* **128**:1352-1358.

Finkelman, F. D., & J. Holmes. 1990. Lymphokine control of *in vivo* immunoglobulin isotype selection. *Ann Rev Immunol* **8**:303-333.

Flynn, A. 1984. Stimulation of interleukin-1 production from placental monocytes. *Lymphokine Res* **3**:1-5.

Flynn, A. 1986. Expression of Ia and the production of interleukin 1 by peritoneal exudate macrophages activated in vitro by steroids. *Life Sci* **38**:2455-2461.

Fox, H. S., B. L. Bond, & T. G. Parslow. 1991. Estrogen regulates the IFN-γ promoter. *J Immunol* **146**:4362-4367.

Gallager, R.. 1997. Tagging T cells: T_H1 or T_H2. *Science* **275**:1615.

Gallichan, W. S., & K. Rosenthal. 1996. Effects of the estrous cycle on local humoral immune responses and protection of intranasally immunized female mice against herpes simplex virus type 2 infection in the genital tract. *Virol* **224**:487-497.

Gershwin, M. E., Y. Ohsugi, A. Ahmed, J. J. Castles, R. Scibienski, & R. M. Ikeda. 1980. Studies of congenitally immunologically mutant New Zealand Mice. *J Immunol* **125:**1189-1195.

Gilbody, J. S., M. J. Wheeler, R. Wolstencroft, & B. D. Greenstein. 1992. Dose related effects of oestradiol on rat thymic and splenic T-lymphocyte responsiveness to mitogens. *Int J Immunopharma* **14:**167-172.

Graff, R. J., M. A. Lappe, & G. D. Snell. 1969. The influence of the gonads and adrenal glands on the immune response to skin grafts. *Transplantation* **7**:105-111.

Grossman, C. J.. 1984. Regulation of the immune system by sex steroids. *Endocrine Rev* **5**:435-455.

Grossman, C. J.. 1985. Interactions between the gonadal steroids and the immune system. *Science* **722**:257-261.

Gulshan, S., A. B. McCruden, & W. H. Stimson. 1990. Oestrogen receptors in macrophages. *Scand J Immunol* **31**:691-697.

Herrera, L. A., R. Montero, J. M. Leon-Cazares, E. Rojas, M. E. Gonsebatt, & P. Ostrosky-Wegman. 1992. Effects of progesterone and estradiol on the proliferation of phytohemagglutinin-stimulated human lymphocytes. *Mutation Res* **270:**211-218.

Holdstock, G., B. F. Chastenay, & E. L. Krawitt. 1982. Effects of testosterone, oestradiol and progesterone on immune regulation. *Clin Exp Immunol* **47**:449-456.

Hsieh, C., A. B. Heimberger, J. S. Gold, & A. O'Garra. 1992. Differential regulation of T helper phenotype development by interleukins 4 and 10 in an αβ T-cell-receptor transgenic system. *Immunol* **89**:6065-6069.

Hsueh, A. J. W., & H. Billig. 1995. Ovarian hormone synthesis and mechanism of action, pp. 2019-2030. In L. J. DeGroot (ed.), *Endocrinology*, Volume 3, 3rd ed. W. B. Saunders Company, Philadelphia, Pennsylvania.

Hu S., Y. L. Mitcho, & N. C. Rath. 1988. Effect of estradiol on interleukin 1 synthesis by macrophages. *J Immunopharmal* **10**:247-252.

Huber, S. A., L. P. Job, K. R. Auld, & J. F. Woodruff. 1981. Sex-related differences in the rapid production of cytotoxic spleen cells active against uninfected myofibres during coxsackievirus b-3 infection. *J Immunol* **126:**1336-1340.

Inman, R. D.. 1978. Immunologic sex differences and the female predominance in systemic lupus erythematosus. *Arthrit Rheum* **21**:849-852.

Jarvinen, L. S., S. Pyhonene, K. J. A. Kairemo, & T. Paavonen. 1996. The effect of anti-oestrogens on cytokine production *in vitro*. *Scand J Immunol* **44**:15-20.

Kabat, E., & M. Mayer. 1971. Kabat and Mayer's experimental immunochemistry. Charles C. Thomas, Springfield. pp 135-139.

Kenny, J. F., & J. A. Gray. 1971. Sex differences in immunologic response: studies of antibody production by individual spleen cells after stimulus with Escherichia coli antigen. *Pediat Res* **5**:246-255.

Kenny, J. F., P. C. Pangburn, & G. Trail. 1976. Effect of estradiol on immune competence: in vivo and in vitro studies. *Infect Immun* **13**:448-456.

Kimura, M., H. Wantanabe, S. Sato, & T. Abo. 1994. Female predominance of extrathymic t cells in mice: statistical analysis. *Immunol Letters* **39**:259-267.

Konstadoulakis, M. M., K. N. Syrigos, C. N. Baxevanis, E. I. Syrigou, M. Papamichail, P. Peveretos, M. Anapliotour, & B. C. Golematis. 1995. Effect of testosterone administration, pre- and postnatally on the immune system of rats. *Horm Metab Res* **27**:275-278.

Kunkel, S. L. 1988. The importance of arachidonate metabolism by immune and nonimmune cells. *Lab Invest* **58**:119-121.

Kutteh, W. H., & D. L. Yetman. 1996. Characterization of interleukin-6 (IL-6) levels in human cervical mucus: influence of endogenous hormones. *Fertil Steril* **66**, abstracts.

Ledur, A., C. Fitting, B. David, C. Hamberger, & J. Cavaillon. 1995. Variable estimates of cytokine levels produced by commercial ELISA kits: results using international cytokine standards. *J Immunol Met* **186**:171-179.

Leslie, C. A., W. A. Gonnerman, & E. S. Carthcart. 1987. Gender differences in eiscanoid production from macrophages of arthritis-susceptible mice. *J Immunol* **138**:413-416.

Leslie, C. A., & D. P. Dubey. 1994. Increased PGE_2 from human monocytes isolated in the luteal phase of the menstrual cycle. Implications for immunity? *Prostaglandins* **47**:41-53.

Lillehoj, H. S., K. Beisel, & N. R. Rose. 1981. Genetic factors controlling the susceptibility to experimental autoimmune thyroiditis in inbred rat strains. *J Immunol* **127**:654-659.

Loy, R. A., J. Loukides, & M. L. Polan. 1992. Ovarian steroids modulate human monocyte tumour necrosis factor alpha ribonucleic acid levels in cultured human peripheral monocytes. *Fertil Steril* **58**:733-739.

Lynch, E. A., C. A. Dinarello, & J. G. Cannon. 1994. Gender differences in IL-1α, IL-1β, and IL-1 receptor antagonist secretion from mononuclear cells and urinary excretion. *J Immunol* **153**:300-306.

Maggi, E., P. Parronchi, R. Manetti, C. Simonelli, M. Piccinni, F. S. Rugiu, M. De Carli, M. Ricci, & S. Romagnani. 1992. Reciprocal regulatory effects of IFN-γ and IL-4 on the in vitro development of human Th1 and Th2 clones. *J Immunol* **148**:2142-2147.

Marshall, J. C.. 1995. Hormonal regulation of the menstrual cycle and mechanisms of anovulation, pp. 2046-2058. In L. J. DeGroot (ed.), *Endocrinology*, Volume 3, 3rd ed. W. B. Saunders Company, Philadelphia, Pennsylvania.

Marshall, J. C.. 1995. Regulation of gonadotropin secretion, pp. 1993-2007. In L. J. DeGroot (ed.), *Endocrinology*, Volume 3, 3rd ed. W. B. Saunders Company, Philadelphia, Pennsylvania.

Marzi, M., A. Vigano, D. Trabattoni, M. L. Villa, A. Salvaggio, E. Clerici, & M. Clerici. 1996. Characterization of type 1 and type 2 cytokine production profile in physiologic and pathologic pregnancy. *Clin Exp Immunol* **106**:127-133.

Mathur, S., R. S. Mathur, J. M. Goust, H. O. Williamson, & H. H. Fudenberg. 1979. Cyclic variations in white cell subpopulations in the human menstrual cycle: Correlations with progesterone and estradiol. *Clin Immunol Immunopathol* **13**:246-253.

Matsuzaki, N., R. Neki, K. Sawai, K. Shimoya, T. Okada, M. Sakata, F. Saji, & Y. Koishihara. 1995. Soluble interleukin-6 (IL-6) receptor in sera of pregnant women forms a complex with IL-6 and augments human chorionic gonadotropin production by normal human trophoblast through binding to the IL-6 signal transducer. *J Clin Endocrinol Metab* **80**:2912-2917.

McDermott, M. R., D. A. Clark, & J. Bienenstock. 1980. Evidence for a common mucosal immunologic system. *J Immunol* **124**:2536-2539.

McKnight, A. J., G. J. Zimmer, I. Fogelman, S. F. Wolf, & A. K. Abbas. 1994. Effects of IL-12 on helper T cell-dependent immune responses *in vivo*. *J Immunol* **152**:2172-2179.

Mendelsohn, J., M. M. Multer, & J. L Bernheim. 1977. Inhibition of human lymphocyte stimulation by steroid hormones: cytokinetic mechanisms. *Clin Exp Immunol* **27:**127-134.

Miller, B. B., & M. S. Soules. 1996. The usefulness of a urinary LH kit for ovulation prediction during the menstrual cycles of normal women. *Obstet Gynecol* **87**:13-17.

Miller, C. J., D. W. Kang, M. Marthas, Z. Moldoveanu, H. Kiyono, P. Marx, J. H. Eldridge, J. Mestecky, & J. R.McGhee. 1992. Genital secretory immune response to chronic simian immunodeficiency virus (SIV) infection: a comparison between intravenously and genitally inoculated rhesus macaques. *Clin Exp Immunol* **88:**520-526.

Milligan, G. N., & D. I. Bernstein. 1995. Generation of humoral immune responses against herpes simplex virus type 2 in the murine female genital tract. *Virol* **206:**234-241.

Mossmann, T. R., & R. L. Coffman. 1989. TH1 and TH2 cells: Different patterns of lymphokine secretion lead to different functional properties. *Ann Rev Immunol* **7**:145-173.

Mossmann, T. R., & S. Sad. 1996. The expanding universe of T cell subsets: Th1, Th2 and more. *Immunol Today* **17**:138-146.

Muster, T., B. Ferko, A. Klima, M. Purtscher, A. Trkola, P. Schulz, A. Grassaeur, O. G. Engelhardt, A. Garcia-Sastre, P. Palase, & H. Katinger. 1995. Mucosal model of immunization against human immunodeficiency virus type 1 with a chimeric influenza virus. *J Virology* **69:**6678-6686.

Myers, M. J., & B. H. Peterson. 1985. Estradiol induced alterations of the immune system. *Int J Immunopharma* **7:**207-213.

Nikolaevich, K. N., S. J. Ivanivich, & S. S. Victorvich. 1991. Major reproduction hormones as regulators of cell-to-cell interactions in humoral immune responses. *Brain, Behaviour, and Immunity* **5**:149-161.

Oppenheim, J. J., F. W. Ruscetti, & C. Falttnek. 1994. Cytokines, pp. 105-123. In D. P. Stites, A. I. Terr, T. G. Parslow (eds.), Basic & Clinical Immunology, 8th ed. Appleton and Lange Paramount Publishing Business and Professional Group, Norwalk, Connecticut.

Ogra, Pearay L., Swantarta S. Ogra. 1973. Local antibody response to poliovaccine in the human female genital tract. *J Immunol* **110:**1307-1311.

Paavonen, T., L. C. Andersson, & H. Aldercreutz. 1981. Sex hormone regulation of *in vitro* immune response, estradiol enhances human B cell maturation via inhibition of suppressor T cells in pokeweed mitogen-stimulated cultures. *J Exp Med* **154**:1935-1945.

Pacifici, R., L. Rifas, R. McCracken, I. Vered, C. McMurty, L. V. Avioli, & W. A. Peck. 1989. Ovarian steroid treatment blocks a postmenopausal increase in blood monocyte interleukin 1 release. *Proc Natl Acad Sci USA* **86**:2398-2402.

Pacifici, R., C. Brown, E. Puscheck, E. Freirich, E. Slatopolsky, D. Maggio, R. McCracken, & L. V. Avioli. 1991. Effect of surgical menopause and estrogen replacement on cytokine release from human blood mononuclear cells. *Proc Natl Acad Sci USA* **88**:5134-5138.

Parr, E. L., & M. B. Parr. 1990. A comparison of antibody titres in mouse uterine fluid after immunization by several routes, and the effect of the uterus on antibody titres in vaginal fluid. *J Reprod Fert* **89**:619-625.

Parr, M. B., L. Kepple, M. R. McDermott, M. D. Drew, J. J. Bozzola, & E. L. Parr. 1994. A mouse model for studies of mucosal immunity to vaginal infection by herpes simplex virus type 2. *Lab Invest* **70**:369-380.

Polan, M. L., A. Daniele, & A. Kuo. 1988a. Gonadal steroids modulate human monocyte interleukin-1 (IL-1) activity. *Fertil Steril* **49**:964-968.

Polan, M. L., S. Carding, & J. Loukides. 1988b. Progesterone modulates interleukin-1 (IL-1) mRNA production by human pelvic macrophages. Proc of the 44th annual Meeting of the American Fertility Society. Abstract 9.

Polan, M. L., J. Loukides, P. Nelson, S. Carding, M. Diamond, A. Walsh, & K. Bottomly. 1989. Progesterone and estradiol modulate interleukin-1β messenger ribonucleic acid levels in cultured human peripheral monocytes. *J Clin Endocrinol Metab* **69**:1200-1206.

Polan, M. L., A. Kuo, J. Loukides, & K. Bottomly. 1990. Cultured human luteal peripheral monocytes secrete increased levels of interleukin-1. *Endocrinol Metab* **70**:480-484.

Polan, M. L., J. Loukides, & J. Honig. 1994. Interleukin-1 in human ovarian cells and in peripheral blood monocytes increases during the luteal phase: evidence for a midcycle surge in the human. *Am J Obstet Gynecol* **170**: 1000-1007.

Prior, J. C., Y. M. Vigna, M. T. Schechter, & A. E. Burgess. 1990. Spinal bone loss and ovulatory disturbances. *N Engl J Med* **323**:1221-1227.

Prior, J. C., D. W. McKay, Y. M. Vigna, & S. L Barr. 1995. Medroyprogesterone increases basal temperature: a placebo-controlled crossover trial in postmenopausal women. *Fertil Steril* **63**:1222-1226.

Rachman, F., V. Casimiri, A. Psychoyos, & O. Bernard. 1983. Immunoglobulins in the mouse uterus during the oestrous cycle. *J Reprod Fert* **69**:17-21.

Ralston, S. H., R. G. G. Russell, & M. Gowen. 1990. Estrogen inhibits release of tumour necrosis factor from peripheral blood mononuclear cells in postmenopausal women. *J Bone Mineral Res* **5** (9):983-988.

Rogge, L., L. Barberis-Maino, M. Biffi, N. Passini, D. H. Presky, U. Gubler, & F. Sinigaglia. 1997. Selective expression of an interleukin-1 receptor component by human T helper 1 cells. *J Exp Med* **185**:825-831.

Romagnani, S.. 1991. Human T_H1 and T_H2 subsets: doubt no more. *Immunol Today* **12**:256-257.

Romagnani, S.. 1995. Biology of human T_H1 and T_H2 cells. *J Clin Immunol* **15**:121-129.

Schuurs, A. H. W. M., & H. A. M. Verheul. 1990. Effects of gender and sex hormones on the immune response. *J Steroid Biochem* **35**:157-172.

Seder, R. A., W. E. Paul, M. M. Davis, & B. Fazekas de St. Groth. 1992. The presence of Interleukin 4 during *in vitro* priming determines the lymphokine-producing potential of $CD4^+$ T cells from T cell receptor transgenic mice. *J Exp Med* **176**:1091-1098.

Stern, K., & I. Davidson. 1955. Effect of estrogen and cortisone on immune hemoantibodies in mice of inbred strains. *J Immunol* **74**:479-484.

Sthoeger, Z. M., N. Chiorazzi, & R. G. Lahita. 1988. Regulation of the immune response by sex hormones. *J Immunol* **141**:91-98.

Stimson, W. H., & I. C. Hunter. 1976. An investigation into the immunosuppressive properties of oestrogen. *J Endocrinol* **69**:42p-43p.

Stimson, W. H. 1988. Oestrogen and human T lymphocytes: presence of specific receptors in the T-suppressor/cytotoxic subset. *Scand J Immunol* **28**:345-350.

Struve, J., B. Aronsson, B. Frenning, F. Granath, M. Von Sydow, & O. Weiland. 1992. Intramuscular versus intradermal administration of a recombinant hepatitis B vaccine: a comparison of response rates and analysis of factors influencing the antibody response. *Scand J Infect Dis 24:423-429.*

Sullivan, D. A., B. J. Underdown, & C. R. Wira. 1983. Steroid hormone regulation of free secretory component in the rat uterus. *Immunol* **49**:379-386.

Sullivan, D. A., G. S. Richardson, D. T. MacLaughlin, & C. R. Wira. 1984. Variations in the levels of secretory component in human uterine fluid during the menstrual cycle. *J Steroid Biochem.* **20**:509-513.

Swain, S. L., A. D. Weinberg, M. English, & G. Huston. 1990. IL-4 directs the development of Th2-like helper effectors. *J Immunol* **145**:3796-3806.

Thys-Jacob, S., & M. J. Alvir. 1995. Calcium-regulating hormones across the menstrual cycle: Evidence of a secondary hyperparathyroidism in women with PMS. *J Clin Endocrinol Metab* **80**:2227-2232.

Usala, S. J., F. O. Usala, R. Haciski, J. A. Holt, & G. F. B. Schumacher. 1989. IgG and IgA content of vaginal fluid during the menstrual cycle. *J Reprod Med* **34**:292-294.

Vik, D. P., P. Amiguet, G. J. Moffat, M. Fey, F. Amiguet-Barras, R. A. Wetsel, & B. F. Tack. 1991. Structural features of the human C3 gene: Intron/exon organization, transcriptional start site, and promoter region sequence. *Biochem* **30**:1080-1085.

Wang, Y., K. Ben, X. Cao, & Y. Wang. 1996. Transport of anti-sperm monoclonal IgA and IgG into murine male and female genital tracts from blood. *J Immunol* **156**:1014-1019.

Weetman, A.P., A. M. McGregor, B. R. Smith, & R. Hall. 1981. Sex hormones enhance immunoglobulin synthesis by human peripheral blood lymphocytes. *Immunol Letters* **3**:343-346.

Weusten, J. J. A. M., M. A. Blankenstein, F. H. J. Gmelig-Meyling, H. J. Schuurman, L. Kater, & J. H. H. Thijssen. 1986. Presence of oestrogen receptors in human blood mononuclear cells and thymocytes. *Endocrinologica* **112**:409-414.

White, H. D., K. M. Crassi, A. L. Givan, J. E. Stern, J. L. Gonzalez, V. A. Memoli, W. R. Green, & C. R. Wira. 1997. $CD3^+$ $CD8^+$ CTL activity within the human female reproductive tract: Influence of stage of the menstrual cycle and menopause. *J Immunol* **158**:3017-3027.

Wira, C. R., & C. P. Sandoe. 1977. Sex steroid hormone regulation of IgA and IgA in rat uterine secretions. *Nature* **268:**534-536.

Wira, C. R., & C. P. Sandoe. 1980. Hormonal regulation of immunoglobulins: influence of estradiol on immunoglobulins A and G in the rat uterus. *Endocrinol* **106:**1020-1026.

Wira, C. R., & R. M. Rossoll. 1995. Antigen-presenting cells in the female reproductive tract: influence of the estrous cycle on antigen presentation by uterine epithelial and stromal cells. *Endocrinol* **136**:4526-4534.

Wyle, F. A., & J. R. Kent. 1977. Immunosuppression by sex steroid hormones. *Clin Exp Immunol* **27:**407-415.

APPENDIX A

CYTOKINE EXPRESSION AT DIFFERENT PHASES OF THE MENSTRUAL CYCLE

INITIAL QUESTIONNAIRE

CODE _____ ADDRESS _____

AGE _____ _____

HEIGHT _____ PHONE (HOME)_____

WEIGHT _____ (WORK)_____

APPROXIMATELY HOW LONG IS YOUR MENSTRUAL CYCLE?

_____

WOULD YOU DESCRIBE YOUR CYCLE AS REGULAR? YES NO
IF NO, EXPLAIN.

_____
_____
_____

CAN YOU GIVE A MENSTRUAL CYCLE DIARY OF THE PAST COUPLE OF CYCLES?
(i.e. beginnings of your periods)

_____
_____
_____

COULD YOU PLEASE DESCRIBE ANY SYMPTOMS YOU EXPERIENCE IN ASSOCIATION WITH YOUR PERIOD.

(i.e. severity of cramps and pain) YES NO

_____
_____
_____
_____

DO YOU TAKE MEDICATION FOR THESE SYMPTOMS? YES NO
PLEASE INDICATE SPECIFICALLY.

_____
_____
_____

WHEN WAS THE LAST TIME YOU TOOK B.C.P.? N/A

ARE YOU SEEING A DOCTOR FOR ANY MEDICAL PROBLEMS ?
IF YES, PLEASE EXPLAIN. YES NO

_____
_____
_____
_____

WHAT , IF ANY, MEDICATION ARE YOU CURRENTLY TAKING ? NONE
(include over the counter medication)

_____
_____
_____

DO YOU EXERCISE REGULARLY ? PLEASE OUTLINE BRIEFLY
YOUR SCHEDULE.

_____
_____

CYTOKINE EXPRESSION AT DIFFERENT PHASES OF THE MENSTRUAL CYCLE

INITIAL QUESTIONNAIRE CONTROLS

CODE _____ ADDRESS _____

AGE _____ _____

HEIGHT _____ PHONE (HOME)_____

WEIGHT _____ (WORK)_____

ARE YOU SEEING A DOCTOR FOR ANY MEDICAL PROBLEMS ?
IF YES, PLEASE EXPLAIN. YES NO

_____
_____
_____

WHAT , IF ANY, MEDICATION ARE YOU CURRENTLY TAKING ?
(include over the counter medication)

_____
_____
_____

DO YOU EXERCISE REGULARLY ? PLEASE OUTLINE BRIEFLY YOUR SCHEDULE.

_____
_____
_____

Menstrual Cycle Diary

Instructions to use the diary

1. On the first day of menstruation prepare the diary: Considering the first day of bleeding as day 1 of your menstrual cycle enter the corresponding calender day for each day in the space provided below.

2. Each Evening: At about the same time complete the column for that day as described below

Bleeding: Indicate if you have had bleeding by shading the box above that days date ;for spotting use an

Symptoms: If you do not experience any symptoms leave the corresponding square blank. If present indicate severity

MILD: 1 (noticeable but not troublesome)
MODERATE: 2 (interferes with normal activity)
SEVERE: 3 (temporarily incapacitating)

MEDICATION: On the bottom of the sheet indicate the medications you have taken during your cycle, include any (over the counter medications: cough syrup, aspirin, cold tablets, etc). Indicate the days you took them and the dose.

CALENDAR

eeding y of nstrual cycle nth: Date:	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	24	26	27	28	29	30	31	32	33	34
mptoms																																		
ritable																																		
tigue																																		
bile Mood (crying)																																		
pressed																																		
stless																																		
xious																																		
somnia																																		
ema or rings tight																																		
east Tenderness																																		
dominal Bloating																																		
petite: up ↑ down ↓																																		
x Drive: up ↑ down ↓																																		
ills(C) / Sweats(S)																																		
adaches																																		
ave: sweets, salt																																		
usea																																		
nstrual Cramps																																		
me off Work																																		
creased use of Alcohol																																		
gorous Exercise																																		
wels: const.(c), loose																																		

EDICATIONS:

CYTOKINE EXPRESSION AT DIFFERENT PHASES OF THE MENSTRUAL CYCLE

ROUTINE QUESTIONNAIRE

CODE_____ DATE_____

HAVE YOU TAKEN ANY MEDICATION IN THE LAST FEW DAYS ?
(i.e. aspirin , cold tablets , prescriptions, birth control pills) YES NO

_____
_____
_____

DO YOU BELIEVE THAT YOU COULD BE PREGNANT? YES NO

WHEN DID YOU LAST START YOUR PERIOD?

_____

HAVE YOU ENGAGED IN SEXUAL INTERCOURSE IN THE LAST 12 HOURS. YES NO

HOW HAVE YOU BEEN FEELING THESE LAST FEW DAYS ?
ANY NIGHTMARES , RESTLESSNESS , TROUBLE SLEEPING ?

_____
_____
_____
_____
_____
_____

COULD YOU PLEASE OUTLINE YOUR EXERCISE SCHEDULE FOR THE PAST FEW DAYS. INCLUDE ANY WALKING , AEROBICS , WEIGHT LIFTING , SWIMMING , ETC. .

CYTOKINE EXPRESSION AT DIFFERENT PHASES OF THE MENSTRUAL CYCLE

ROUTINE
QUESTIONNAIRE
CONTROLS

CODE_____ DATE_____

HAVE YOU TAKEN ANY MEDICATION IN THE LAST FEW DAYS ?
(i.e. aspirin , cold tablets , prescriptions)

_____
_____
_____

HAVE YOU ENGAGED IN SEXUAL INTERCOURSE IN THE LAST 12 HOURS. YES NO

HOW HAVE YOU BEEN FEELING THESE LAST FEW DAYS ?
ANY NIGHTMARES , RESTLESSNESS , TROUBLE SLEEPING ?

_____
_____
_____
_____
_____

COULD YOU PLEASE OUTLINE YOUR EXERCISE SCHEDULE FOR THE PAST FEW DAYS. INCLUDE ANY WALKING , AEROBICS , WEIGHT LIFTING , SWIMMING , ETC. .

_____
_____
_____

Table A.1. The volunteers hemoglobin levels (g/L) and various cell population numbers (10^9/L)at the beginning (B) of the study and at the end (E). The mean and the p value for the paired comparison. Minimum levels of 90 g/L was required to gain entrance to the study.

Volunteer	Hgb		WBC[1]		Monocytes[1]		Lymphocytes[1]		Neutrophils	
	B	E	B	E	B	E	B	E	B	E
21.04.70	135	126	5.3	3.8	0.5	0.3	1.4	1.1	3.4	2.4
01.04.75	141	131	6.0	4.8	0.6	0.4	1.9	1.7	3.4	2.6
22.09.75	135	126	5.8	6.5	0.4	0.2	1.6	1.6	3.7	4.3
15.12.73	129	116	5.7	4.3	0.3	0.3	1.6	1.4	3.5	2.4
06.09.72	145	136	3.7	3.6	0.4	0.4	1.3	1.0	2.0	2.1
29.08.71	143	na[2]	5.6	na	0.5	na	1.6	na	3.2	na
22.11.75	137	123	4.5	3.7	0.4	0.2	1.6	1.5	2.3	1.6
09.10.64	152	146	5.7	5.0	0.3	0.3	1.7	1.6	3.5	3.0
30.04.65	138	123	5.7	4.6	0.4	0.3	2.3	1.9	2.6	2.2
06.11.71	160	na	5.4	na	0.5	na	1.7	na	3.1	na
24.02.71	160	na	5.1	na	0.3	na	1.3	na	3.5	na
17.02.75	155	156	5.7	5.9	0.5	0.5	1.5	1.5	3.6	3.8
16.05.74	157	na	4.5	na	0.4	na	1.6	na	2.7	na
19.02.36	136	124	4.4	5.0	0.3	0.3	1.1	1.1	2.8	3.4
17.10.38	138	na	5.6	na	0.7	na	2.0	na	2.3	na
Mean	140[3]	131	5.3	4.7	0.4	0.3	1.6[3]	1.4	3.1	2.8

[1] cells at 10^3.
[2] not available.
[3] $p<0.05$, two-tailed, non-parametric.

APPENDIX B

Table B.1-B.15. Original sample data, means, and standard deviations, for all the volunteers in the study. Tables include the parameters measured in serum and day that the sample was taken. For the female volunteers "day" is expressed as day in the cycle the sample was taken and for the males and post-menopausal women "day" is expressed as month and day the sample was obtained. Serum parameters included in the tables are IL-1ra, IL-1β, C'H50, C3 and the hormones, 17β-Estradiol, Progesterone and Luteinizing hormone. For the cycling women cycle length and day of ovulation (O) are given below the volunteer identification code.

Volunteeer 21.04.70
Cycle 29 Days (O=Day 17)

DAY	C3 mg/ml	IL-1ra pg/ml	IL-1β pg/ml	C'H50 U/ml	E2 pg/ml	LH u/l	PRO ng/ml
2	0.95	18.3	0.30	282	68	4	0.4
5	nt†	nt	nt	nt	70	4	0.2
7	0.90	13.8	0.17	262	65	5	0.2
13	0.77	21.7	0.84	nt	115	3	0.1
14	0.78	176.7	<‡	nt	140	5	0.2
16	0.88	155.5	<	nt	180	33	0.8
19	0.78	185.8	2.23	270	131	6	7.7
21	0.79	139.6	1.26	nt	150	4	13.2
23	0.82	50.3	0.27	269	123	2	9.6
26	0.86	29.3	1.50	222	173	2	12.2
28	0.91	63.5	2.10	210	102	3	2.0
1	0.83	206.7	1.63	226	61	3	0.2
4	0.84	nt	0.10	295	55	6	0.1
6	0.86	70.5	<	nt	53	4	0.1
MEAN	0.84	94.3	1.04	255	106	6	3.4
S.D.	0.06	73.1	0.81	31.1	45	8	4.9

† not tested.
‡ < 0.083 pg/ml.

Volunteer 01.04.75
Cycle 27 Days (O=Day 16)

DAY	C3 mg/ml	IL-1ra pg/ml	IL-1β pg/ml	C'H50 units	E2 pg/ml	LH u/l	PRO ng/ml
3	1.06	116.1	4.3	196	45	3	0.8
5	0.94	213.0	>/	171	48	3	0.2
9	1.07	180.3	>	nt	59	5	0.1
10	nt*	141.5	>	163	79	6	0.1
12	1.02	380.2	>	nt	102	6	0.1
15	0.91	230.9	>	136	210	25	0.3
17	0.91	337.2	>	nt	84	7	1.0
19	0.87	266.5	>	144	102	4	2.8
22	0.99	406.6	>	nt	173	3	5.1
24	0.88	399.8	>	nt	161	5	4.8
26	1.05	386.5	>	152	131	4	3.8
2	0.92	507.0	>	140	38	2	0.2
4	0.92	567.2	>	174	40	3	0.2
MEAN	0.97	317.9	-	160	98	6	1.5
S.D.	0.08	139.5	-	20	56	6	1.9

* not tested.
/ > 10 pg/ml.

Volunteer 06.09.72
Cycle 28 Days (O =Day 16)

DAY	C3 mg/ml	IL-1ra pg/ml	IL-1β pg/ml	C'H50 units	E2 pg/ml	LH u/l	PRO ng/ml
4	1.16	44.0	1.61	202	68	1	0.2
8	0.99	164.0	0.45	nt	63	2	0.1
11	1.03	71.3	1.03	179	84	3	0.3
14	0.93	nt[†]	0.50	190	187	10	0.2
16	0.98	133.3	1.26	196	251	22	1.4
18	0.93	179.2	0.28	nt	105	8	3.0
21	1.06	80.3	0.61	nt	161	2	14.5
23	1.02	66.0	2.02	199	151	4	9.6
25	1.04	170.0	0.10	187	145	2	12.2
28	1.01	191.7	0.31	206	180	1	0.9
2	1.13	158.6	0.28	nt	57	3	0.2
4	1.19	332.5	<[‡]	nt	52	2	0.2
MEAN	1.04	144.6	0.77	194	125	5	3.6
S.D.	0.13	81.2	0.63	10	63	6	5.3

[†] not tested.
[‡] < 0.083 pg/ml.

Volunteer 22.09.75
Cycle 26 Days(O=Day 13)

DAY	C3 mg/ml	IL-1ra pg/ml	IL-1β pg/ml	C'H50 units	E2 pg/ml	LH u/l	PRO ng/ml
7	nt†	nt	nt	nt	112	3	nt
10	nt	912.2	0.55	nt	193	3	0.3
12	nt	1029.0	nt	nt	302	11	0.2
14	1.21	1025.0	0.086	nt	120	15	0.7
17	1.23	1007.0	<‡	nt	140	5	6.7
19	1.32	1036.0	<	143	244	4	17.1
21	1.23	877.4	5.82	138	244	6	18.8
24	1.28	972.9	<	144	221	2	7.2
26	1.28	851.9	0.87	163	78	2	0.5
2	1.40	929.6	0.14	171	46	3	0.3
5	1.37	116.1	0.086	158	73	2	0.2
MEAN	1.29	875.7	1.26	153	161	5	5.2
S.D.	0.07	274.9	2.26	13	84	4	7.3

† not tested.
‡ < 0.083 pg/ml.

Volunteer 29.08.71
Cycle 29 Days (O=Day 15)

DAY	C3 mg/ml	IL-1ra pg/ml	IL-1β pg/ml	C'H50 units	E2 pg/ml	LH u/l	PRO ng/ml
7	1.29	49.7	0.10	nt†	87	5	0.1
9	1.11	53.8	<‡	206	74	5	0.1
11	1.18	91.1	0.30	202	87	4	0.1
14	0.97	112.5	0.20	200	169	33	0.2
16	0.96	163.4	0.13	201	112	20	1.2
18	1.12	208.9	0.45	nt	116	4	4.6
21	1.23	162.2	<	243	169	11	6.0
23	1.14	283.0	<	250	137	2	9.2
25	1.12	377.8	0.89	nt	132	4	14.0
29	1.12	291.4	0.19	239	57	2	0.5
8	1.25	267.2	<	249	102	5	0.8
10	1.11	281.6	<	232	95	5	0.2
MEAN	1.13	195.2	0.32	225	111	8	3.1
S.D.	0.09	106.2	0.28	22	35	9	4.5

† not tested.
‡ < 0.083 pg/ml.

Volunteer 15.12.73
Cycle 29 Days (O=Day 14)

DAY	C3 mg/ml	IL-1ra pg/ml	IL-1β pg/ml	C'H50 units	E2 pg/ml	LH u/l	PRO ng/ml
3	0.91	107.6	13.82	nt[†]	68	3	0.2
6	0.93	96.9	13.49	205	87	3	0.1
8	0.97	181.1	14.39	193	87	4	0.03
10	0.93	128.8	14.80	nt	125	6	0.03
13	0.85	236.3	12.49	nt	271	60	0.6
15	0.93	579.9	13.13	153	116	7	2.6
17	0.84	344.9	13.63	nt	134	4	4.2
20	1.04	754.1	14.11	213	270	7	13.2
22	0.98	191.7	11.85	nt	193	3	15.6
24	0.89	201.2	10.04	149	202	8	10.3
28	0.94	310.4	12.08	186	97	4	0.6
29	0.95	408.1	12.43	143	58	2	0.1
2	0.96	576.6	11.59	154	56	3	0.1
6	0.98	276.1	11.33	196	65	4	0.1
7	1.02	316.1	12.20	nt	44	4	0.1
MEAN	0.94	314.0	12.76	177	125	8	3.2
S.D.	0.06	192.7	1.30	27	75	14	5.3

[†] not tested.

Volunteer 09.10.64
Cycle 28 Days (O=Day 13)

DAY	C3 mg/ml	IL-1ra pg/ml	IL-1B pg/ml	C'H50 units	E2 pg/ml	LH u/l	PRO ng/ml
4	0.90	73.0	<‡	nt†	79	4	0.1
6	0.92	91.1	<	187	115	2	0.4
8	0.94	95.9	<	nt	123	5	0.3
11	0.86	84.5	0.93	191	235	12	0.1
13	1.00	347.7	0.58	nt	259	24	0.5
15	0.94	126.3	0.17	nt	92	7	1.8
19	0.98	92.1	<	nt	108	4	17.0
20	0.95	138.7	<	nt	74	3	21.0
22	0.97	100.6	<	nt	169	8	12.8
26	0.90	164.5	0.25	nt	124	1	7.9
27	0.93	110.2	0.17	nt	95	2	2.8
1	0.97	108.2	0.79	210	69	5	0.5
5	0.83	119.7	<	197	90	4	0.2
6	0.96	152.1	<	212	128	5	0.1
MEAN	0.93	128.9	0.48	199	126	6	4.7
S.D.	0.07	68.2	0.33	11	58	6	7.1

† not tested.
‡ < 0.083 pg/ml.

Volunteer 22.11.75
Cycle 31 Days (O=Day 18)

DAY	C3 mg/ml	IL-1ra pg/ml	IL-1β pg/ml	C'H50 units	E2 pg/ml	LH u/l	PRO ng/ml
3	1.20	199.7	2.43	194	61	7	0.3
5	1.17	209.3	2.27	206	52	4	0.2
9	1.14	132.1	2.87	nt†	52	6	nt
10	1.15	143.5	1.59	184	63	7	0.4
12	1.13	176.9	2.06	nt	93	7	0.4
16	1.09	181.6	4.08	197	270	10	0.3
17	1.04	200.7	3.06	185	285	14	0.1
19	1.07	247.4	3.02	170	125	13	1.0
23	1.08	147.3	4.26	165	134	2	15.6
24	1.03	154.0	3.77	177	170	3	15.6
26	1.11	193.1	4.03	nt	211	5	15.6
29	1.17	205.5	4.97	172	211	1	8.9
31	1.09	470.9	3.76	198	73	1	0.5
2	1.08	286.6	3.34	197	33	2	0.3
5	1.12	200.7	3.52	174	51	3	0.4
MEAN	1.11	209.9	3.27	185	126	6	4.0
S.D.	0.05	82.5	0.92	13	84	4	6.5

† not tested.

Volunteer 30.04.65
Cycle 31 Days (O=Day 18)

DAY	C3 mg/ml	IL-1ra pg/ml	IL-1β pg/ml	C'H50 units	E2 pg/ml	LH u/l	PRO ng/ml
6	1.32	262.7	<‡	67	90	4	nt
8	1.29	249.3	<	67	99	4	0.1
12	1.20	368.7	<	60	95	5	0.1
13	nt†	333.3	0.35	62	95	7	0.2
20	1.21	318.1	<	71	152	9	3.9
22	1.36	326.6	<	73	247	7	10.8
25	1.45	261.7	<	73	176	1	6.0
27	1.36	236.0	<	74	112	1	5.6
29	1.32	313.3	<	nt	79	4	2.5
8	1.29	310.4	<	nt	84	5	0.01
MEAN	1.31	298.0	-	68	123	5	3.2
S.D.	0.08	43.0	-	5	54	3	3.7

† not tested.
‡ < 0.083 pg/ml.

Volunteer 24.02.71
Male Control

DATE	C3 mg/ml	IL-1ra pg/ml	IL-1β pg/ml	C'H50 units	TEST ng/ml
M 15	1.32	2578	1.06	nt	7
17	1.27	981.4	<‡	155	4
19	nt†	nt	nt	nt	nt
23	1.45	305.3	<	nt	12
24	1.29	246.7	<	164	7
26	1.29	434.6	<	173	nt
29	1.43	234.9	<	172	13
31	1.46	276.6	<	174	10
J 2	1.36	195.3	<	nt	6
5	1.47	212.9	<	nt	3
7	1.26	223.9	<	167	6
9	1.29	191.6	<	169	6
MEAN	1.35	534.7	-	168	7
SD	0.08	714.8	-	7	3

† not tested.
‡ < 0.083 pg/ml.

Volunteer 06.11.71
Male Control

DATE	C3 mg/ml	IL-1ra pg/ml	IL-1β pg/ml	C'H50 units	E2 pg/ml	TEST ng/ml
M15	1.29	225.6	4.91	169	49	6
17	1.38	302.4	5.28	153	35	10
19	1.33	65.5	5.41	157	27	4
23	1.13	133.1	6.49	158	32	9
24	1.11	36.8	4.75	156	44	13
26	1.32	25.8	2.09	171	48	9
29	1.20	18.5	2.22	156	59	9
31	1.32	46.3	2.06	171	64	7
J 2	1.26	44.8	1.84	nt†	44	8
5	1.21	32.1	2.38	nt	41	7
7	1.19	39.4	2.23	168	34	3
9	1.24	22.8	3.04	162	55	5
MEAN	1.25	82.8	3.56	162	44	8
S.D.	0.08	91.3	1.67	7	11	3

† not tested.

Volunteer 17.02.75
Male Control

DATE	C3 mg/ml	IL-1ra pg/ml	IL-1β pg/ml	C'H50 units	E2 pg/ml	TEST ng/ml
M24	1.28	46.3	4.55	nt†	78	7
26	1.21	108.4	4.33	83	64	7
J 2	1.16	46.3	4.18	82	53	13
5	1.19	42.5	4.05	82	59	10
7	1.24	49.5	7.23	nt	81	12
9	1.15	45.5	3.97	86	71	10
14	1.23	75.9	3.79	86	68	13
16	1.27	91.9	7.97	88	49	9
19	1.27	25.7	8.67	98	46	7
MEAN	1.22	59.1	5.42	86	63	10
S.D.	0.48	26.8	1.95	6	12	2

† not tested.

Volunteer 16.05.74
Male Control

DATE	C3 mg/ml	IL-1ra pg/ml	IL-1β pg/ml	C'H50 units	TEST ng/ml
J14	nt†	nt	nt	nt	nt
19	1.06	192.6	3.93	168	6
21	1.09	160.8	3.59	nt	5
24	1.00	102.4	4.54	174	4
26	1.16	81.72	4.06	186	6
28	1.07	95.55	4.50	188	3
31	nt	91.61	4.56	178	5
A 1	1.07	90.63	2.90	175	13
3	1.02	129.5	5.08	nt	4
MEAN	1.07	118.1	4.15	178	6
S.D.	0.05	39.7	0.68	8	3

† not tested.

Volunteer 19.02.36
PM Control

DATE	C3 mg/ml	IL-1ra pg/ml	IL-1β pg/ml	C'H50 pg/ml	E2 pg/ml	LH u/l	PRO ng/ml
M15	1.19	137.2	0.57	130	29	10	0.1
19	1.43	131.4	0.55	nt[†]	32	10	0.2
23	1.25	135.3	3.79	122	27	10	0.4
24	1.25	146.2	1.28	122	40	10	0.4
26	1.27	217.1	0.50	124	59	8	0.2
29	1.20	86.5	0.37	128	41	9	0.1
J12	1.14	115.0	0.75	127	34	9	0.1
14	1.24	148.2	0.63	138	37	10	0.3
16	1.22	146.2	0.86	nt	51	9	0.4
23	1.36	97.3	0.90	132	46	10	0.2
27	1.29	129.4	0.97	125	46	8	0.1
28	1.16	136.3	1.88	122	28	10	0.1
30	1.21	171.5	0.51	123	44	10	0.3
MEAN	1.25	138.3	1.04	127	40	9	0.2
S.D.	0.08	32.4	0.92	6	10	1	0.1

[†] not tested.

Volunteer 17.10.38
PM Control

DATE	C3 mg/ml	IL-1ra pg/ml	IL-1β pg/ml	C'h50 units	E2 pg/ml	LH u/l	PRO ng/ml
M15	1.31	260.4	>/	nt†	23	29	0.5
17	1.43	276.3	>	nt	28	22	0.3
19	1.31	234.7	>	224	20	24	0.2
23	1.36	302.9	>	nt	13	24	0.1
24	1.21	292.4	7.4	237	13	26	0.3
26	1.24	290.1	>	202	31	25	0.3
29	1.25	245.8	>	nt	31	25	0.2
31	1.20	368.6	>	nt	19	23	0.1
J 2	1.34	299.4	1.9	nt	28	29	0.1
5	1.23	269.5	>	nt	76	28	0.3
7	1.21	272.9	>	nt	44	26	0.3
9	1.12	268.3	>	202	32	24	0.1
MEAN	1.27	281.8	-	216	30	25	0.2
S.D.	0.10	34.2	-	17	17	2	0.1

† not tested.
/ >10 pg/ml.

Table B.16-B.30. Original sample data, means and standard deviations, for all the volunteers in the study. Tables include all the cytokines measured in culture supernatants and day that the sample was taken. For the female volunteers "day" is expressed as day in the cycle the sample was taken and for the males and post-menopausal women "day" is expressed as month and day the sample was obtained. The cytokines included in the tables are IL-2, IL-, IFNγ, IL-1ra and IL-1β. For the cycling women cycle length and day of ovulation (O) are given directly below the volunteer identification code.

Volunteer 21.04.70
Cycle 29 days (O=Day 17)

DAY	IFNγ pg/ml	IL-2 pg/ml	IL-4 pg/ml	IL-1ra ng/ml		Il-1β * pg/ml	
				ns[T]	s[t]	ns	s
2	nt[-]	47.3	8.5	4.57	38.43	nt	11770
5	868.9	55.9	8.7	2.93	24.60	nt	9149
7	1262	67.4	10.5	3.16	25.53	nt	14010
13	293.4	113.7	3.7	2.31	44.88	4.5	13610
14	570.5	94.6	14.7	2.28	50.56	8.1	14850
16	257.2	85.8	7.7	3.56	36.98	5.2	14480
19	205.8	95.6	14.3	2.87	49.08	3.9	16010
21	287.5	44.8	10.7	3.04	34.93	4.3	12300
23	251.1	103.0	3.2	3.34	74.26	8.2	15970
26	395.8	94.6	nt	nt	nt	nt	nt
28	367.1	61.9	7.5	7.47	38.92	254.4	9617
1	676.8	94.1	3.0	nt	nt	nt	nt
4	500.7	176.8	10.9	5.87	60.99	25.7	nt
6	565.5	126.3	16.9	6.57	27.03	50.8	15360
MEAN	500.2	90.1	9.3	4.00	42.18	40.6	13375
S.D.	300.1	34.9	4.2	1.73	14.86	81.7	2391

[T] non stimulated.
[t] stimulated
* Stimulated culture samples were diluted 1:20.
[-] not tested.

Volunteer 01.04.75
Cycle 27 Days (O=Day 16)

DAY	IFNγ pg/ml	IL-2 pg/ml	IL-4 pg/ml	IL-1ra ng/ml		IL-1β* pg/ml	
				ns[T]	s[t]	ns	s
3	48.6	403.5	46.6	nt[†]	nt	nt	nt
5	160.6	205.8	4.0	2.84	49.16	<[a]	7868
9	195.1	211.9	23.9	3.49	44.36	<	>[b]
10	353.0	518.4	20.5	2.76	41.43	nt	8216
12	173.3	171.5	2.1	4.82	50.05	173.9	8444
15	66.7	624.0	23.4	3.34	48.86	<	7117
17	81.0	68.4	38.7	2.87	34.37	2.1	7161
19	344.4	488.0	27.5	2.55	47.83	<	8476
22	153.9	386.5	28.4	3.60	48.03	3.5	6840
24	384.2	399.8	32.6	3.78	37.48	10.5	7341
26	347.4	368.5	66.5	3.39	32.32	3.8	8765
2	202.5	507.0	49.7	2.36	35.58	1.5	6458
4	437.8	567.2	74.4	4.78	40.80	40.3	8153
MEAN	226.8	378.5	33.7	3.38	42.46	33.7	7713
S.D.	131.1	168.8	21.4	0.79	6.42	63.3	763

[T] non stimulated.
[t] stimulated
* Stimulated culture samples were diluted 1:10.
[†] not tested.
[a] < 1 pg/ml.
[b] > 10,000 pg/ml.

Volunteer 06.09.72
Cycle 28 Days (O =Day 16)

DAY	IFNγ pg/ml	IL-2 pg/ml	IL-4 pg/ml	IL-1ra ng/ml		IL-Iβ* pg/ml	
				ns[f]	s[t]	ns	s
4	148.2	245.9	48.5	nt[†]	nt	nt	nt
8	186.2	545.4	67.1	2.62	22.89	2.8	5545
11	392.9	619.4	27.5	2.69	30.62	5.6	6131
14	161.1	332.8	22.8	1.39	28.78	0.5	6494
16	247.1	311.3	33.0	2.37	37.61	1.0	8460
18	236.7	727.2	21.9	3.86	51.81	0.7	8294
21	299.3	693.2	nt	13.7	57.45	493.1	6234
23	370.3	328.9	29.4	3.66	32.26	5.4	7733
25	409.5	881.1	24.4	12.66	63.54	118	>[b]
28	172.1	710.9	23.3	6.11	45.93	360.8	8665
2	283.6	768.1	30.3	8.66	48.06	247.7	>
4	211.3	876.9	36.7	nt	nt	nt	nt
MEAN	259.9	586.8	33.2	5.77	41.90	123.6	7195
S.D.	91.9	228.8	13.6	4.44	13.48	181.4	1226

[f] non stimulated.
[t] stimulated
* Stimulated culture samples were diluted 1:10.
[†] not tested.
[b] > 10,000 pg/ml.

Volunteer 22.09.75
Cycle 26 Days (O=Day 13)

DAY	IFNγ pg/ml	IL-2 pg/ml	IL-4 pg/ml	Il-1ra ng /ml		IL-1β* pg/ml	
				ns[F]	s[t]	ns	s
7	nt[†]	nt	29.9	nt	nt	nt	nt
10	207.0	166.3	38.9	3.70	26.92	5.5	>[b]
12	119.1	253.8	20.1	5.46	>[c]	108	>
14	51.4	353.2	5.4	6.26	57.41	30.1	>
17	61.6	414.0	21.0	6.62	46.26	46.1	>
19	139.2	451.4	29.9	5.87	32.47	14.9	>
21	176.9	173.4	43.1	7.15	36.12	62.2	5744
24	199.8	766.7	49.0	4.28	28.88	12.1	5783
26	276.5	510.9	11.2	23.62	39.32	132	>
2	44.0	487.1	38.6	13.18	59.74	403	>
5	80.3	566.2	57.8	8.56	57.33	51.9	>
MEAN	135.6	414.3	31.4	8.47	42.72	86.6	5764
S.D.	78.2	186.3	16.1	5.94	12.91	118.6	27.6

[F] non stimulated.
[t] stimulated
* Stimulated culture samples were diluted 1:10.
[†] not tested.
[b] > 10,000 pg/ml.
[c] > 3.0 O.D.

Volunteer 29.08.71
Cycle 29 Days (O=Day 15)

DAY	IFNγ pg/ml	IL-2 pg/ml	IL-4 pg/ml	IL-1ra ng/ml		IL-1β* pg/ml	
				ns[F]	s[t]	ns	s
7	126.4	673.4	40.1	15.58	41.16	170.0	15930
9	128.3	670.6	40.2	6.82	35.62	44.9	11210
11	341.1	775.5	103.6	21.73	47.06	274.6	15460
14	131.9	778.3	27.5	13.05	56.71	149.6	16370
16	206.8	773.2	39.0	17.34	37.44	177.7	16710
18	186.0	747.3	61.5	16.32	50.23	22.7	>[b]
21	173.5	1102.0	63.0	11.79	57.37	28.7	>
23	135.0	460.3	87.7	17.00	76.53	290.8	>
25	214.6	1021.0	61.1	17.59	61.22	224.1	16430
29	264.0	880.9	245.8	12.07	59.22	128.9	18400
8	144.9	999.3	90.4	18.41	48.75	260.2	18200
10	104.7	893.1	34.9	14.85	67.92	61.1	>
MEAN	179.7	814.6	74.6	15.21	53.27	152.8	16089
S.D.	68.6	177.1	59.2	3.86	12.28	97.4	2223

[F] non stimulated.
[t] stimulated
* Stimulated culture samples were diluted 1:20
[b] > 20,000 pg/ml

Volunteer 15.12.73
Cycle 29 Days(O=Day 14)

DAY	IFNγ pg/ml	IL-2 pg/ml	IL-4 pg/ml	IL-1ra ng/ml		IL-1β* pg/ml	
				ns[T]	s[t]	ns	s
3	206.4	675.3	26.9	nt	nt	nt	nt
6	nt[†]	635.3	nt	10.13	39.65	51.6	7578
8	426.8	584.0	14.3	0.61	27.81	28.0	6576
10	1213.0	889.8	25.2	1.37	61.79	212.1	6699
13	512.8	1105.0	26.4	0.86	>[c]	12.8	>[b]
15	499.3	894.0	47.4	2.25	60.08	51.8	5648
17	509.2	1109.0	36.1	1.28	19.91	16.3	>
20	587.7	1594.0	66.8	0.47	45.29	9.8	8057
22	256.9	921.3	61.8	1.21	>	13.4	>
24	508.9	1035.0	38.4	1.77	39.82	152.5	8851
28	430.9	1240.0	85.7	1.43	34.12	369.0	8133
29	338.7	1348.0	48.1	nt	nt	nt	nt
2	153.9	834.3	41.8	1.37	45.60	63.5	>
6	124.1	652.3	35.5	1.68	41.42	67.9	7679
7	226.7	1091.0	47.7	nt	nt	nt	nt
MEAN	428.2	973.9	43.0	2.04	41.55	87.4	7403
S.D.	272.0	285.7	18.8	2.60	12.92	108.0	1030

[T] non stimulated.
[t] stimulated
[†] not tested.
* Stimulated culture samples were diluted 1:20.
[b] > 20,000 pg/ml.
[c] > 3.0 O.D.

Volunteer 09.10.64
Cycle 28 Days (O=Day 13)

DAY	IFNγ pg/ml	IL-2 pg/ml	IL-4 pg/ml	IL-1ra ng/ml		IL-1β* pg/ml	
				ns[T]	s[t]	ns	s
4	586.1	649.2	506.4	5.51	24.69	19.6	3501
6	384.7	942.6	327.0	10.08	55.21	16	4886
8	362.8	280.7	227.7	10.03	28.09	33.8	5867
11	307.7	362.5	246.2	7.36	46.03	36.0	5547
13	124.5	85.3	123.0	nt[†]	nt	nt	nt
15	592.9	407.5	469.1	10.52	59.82	64.9	>[b]
19	299.4	196.2	289.6	29.07	28.53	>[d]	4221
20	517.0	518.4	324.7	nt	nt	nt	nt
22	247.7	297.8	53.8	nt	nt	nt	nt
26	201.6	135.6	183.1	29.50	55.19	446.7	>
27	259.1	375.2	253.4	nt	nt	nt	nt
1	330.3	424.6	276.4	nt	nt	nt	nt
5	333.3	224.2	253.1	nt	nt	nt	nt
6	509.3	380.1	449.8	nt	nt	nt	nt
MEAN	361.1	377.1	284.6	14.58	42.51	102.8	4804
S.D.	142.6	220.5	127.2	10.20	15.03	169.3	965

[T] non stimulated.
[t] stimulated
[†] not tested.
* Stimulated culture samples were diluted 1:10.
[b] > 20,000 pg/ml.
[d] > 500 pg/ml

Volunteer 22.11.75
Cycle 31. Days (O=Day 19)

DAY	IFNγ pg/ml	IL-2 pg/ml	IL-4 pg/ml	IL-1ra ng/ml		IL-1β* pg/ml	
				ns[T]	s[t]	ns	s
3	73.7	116.1	39.5	4.30	41.27	20.8	>[b]
5	555.3	219.0	112.3	3.88	29.39	21.0	16160
9	89.9	159.5	101.4	4.24	30.14	40.6	9091
10	59.1	221.8	48.1	nt[†]	nt	nt	nt
12	101.8	282.1	41.5	nt	48.59	nt	>
16	11.3	100.2	34.1	12.51	44.71	286.8	14670
17	76.2	209.9	37.6	7.66	52.86	83.1	16450
19	51.3	149.6	38.3	nt	nt	nt	nt
23	62.5	152.7	41.3	5.73	43.55	254.7	12980
24	167.7	242.9	62.9	9.82	55.79	282.7	15910
26	57.0	652.6	92.3	nt	nt	nt	nt
29	70.9	432.1	43.2	9.18	59.56	39.9	12730
31	70.1	126.8	20.0	nt	nt	nt	nt
2	103.4	180.3	60.8	nt	nt	nt	nt
5	122.8	270.6	49.6	nt	nt	nt	nt
MEAN	111.5	234.4	54.9	7.16	45.10	128.7	13999
S.D.	127.8	142.6	26.7	3.15	10.51	122.8	2630

[T] non stimulated.
[t] stimulated
[†] not tested.
* Stimulated culture samples were diluted 1:20.
[b] > 20,000 pg/ml.

Volunteer 30.04.65
Cycle 31 Days(O=Day 18)

DAY	IFNγ pg/ml	IL-2 pg/ml	IL-4 pg/ml	IL-1ra ng/ml		IL-1β* pg/ml	
				ns[F]	s[t]	ns	s
6	378.8	579.0	42.9	5.33	50.57	40.3	6226
8	233.3	468.6	26.6	27.10	60.57	605.3	7132
12	187.8	747.6	73.9	7.47	35.06	501.2	6970
13	210.7	675.4	56.0	13.49	29.13	397.4	8262
20	148.5	340.8	35.2	4.69	50.26	8.4	6350
22	241.9	634.3	126.5	7.11	34.98	63.5	7894
25	496.6	545.9	96.2	10.63	57.61	>[d]	>[b]
27	39.7	244.3	156.5	nt[†]	nt	nt	nt
29	151.5	723.3	119.2	16.43	56.50	146.4	4866
8	153.9	578.0	85.8	nt	nt	nt	nt
MEAN	224.3	553.7	81.9	11.53	46.84	251.8	6814
S.D.	129.1	162.5	43.0	7.49	12.05	244.6	1136

[F] non stimulated.
[t] stimulated
[†] not tested.
* Stimulated culture samples were diluted 1:10.
[b] > 10,000 pg/ml.
[d] > 1000 pg/ml.

Volunteer 24.02.71
Male Control

DATE	IFNγ pg/ml	IL-2 pg/ml	IL-4 pg/ml	IL-1ra ng/ml		IL-1β* pg/ml	
				ns[F]	s[t]	ns	s
M 15	453.0	303.6	36.4	nt	nt	nt	nt
17	1221.0	262.1	45.5	1.67	nt	<[a]	nt
19	nt[†]	nt	nt	nt	nt	nt	nt
23	729.5	483.0	32.6	1.91	41.25	0.2	<[a]
24	1669.0	668.7	44.4	2.27	32.55	nt	5277
26	599.5	380.2	67.7	1.05	21.70	<	3724
29	1150.0	559.9	31.0	2.73	31.73	<	6728
31	724.2	435.8	60.4	2.01	71.86	6.9	7294
J 2	941.6	531.4	30.8	2.90	49.78	23.9	4596
5	498.6	512.5	54.2	5.33	61.18	174	6178
7	59.4	174.8	31.4	2.63	47.80	5.1	7048
9	1602.0	252.1	33.2	3.03	58.92	24.5	5017
MEAN	877.1	414.9	42.5	2.55	46.31	39.1	5733
SD	496.3	153.2	13.1	1.15	16.13	66.8	1276

[F] non stimulated.
[t] stimulated
[†] not tested.
* Stimulated culture samples were diluted 1:10.
[a] < 1 pg/ml.

Volunteer 06.11.71
Male Control

DATE	IFNγ pg/ml	IL-2 pg/ml	IL-4 pg/ml	IL-1ra ng/ml		IL-1β* pg/ml	
				ns[ŧ]	s[ŧ]	ns	s
M15	154.1	344.9	48.7	nt[†]	nt	nt	nt
17	104.0	212.8	30.3	nt	nt	nt	nt
19	199.8	490.3	56.2	3.13	41.96	2.7	11770
23	107.0	321.3	21.1	5.32	57.82	3.0	16510
24	92.5	445.1	35.4	3.65	57.50	1.9	11220
26	207.9	423.2	21.3	4.99	64.76	3.6	14170
29	202.3	715.6	138.9	6.00	63.09	3.0	17720
31	311.3	616.8	146.3	23.53	62.42	384.5	17020
J 2	113.3	471.3	54.8	2.31	45.66	1.1	15730
5	245.7	213.8	139.8	4.44	>[c]	97.4	>[b]
7	296.7	509.2	105.2	4.21	31.99	110.7	6608
9	471.7	847.8	102.9	4.32	32.37	156.5	8032
MEAN	208.9	467.7	75.1	6.19	50.84	76.4	13198
S.D.	111.1	190.4	48.5	6.18	13.10	122.8	4023

[ŧ] non stimulated.
[ŧ] stimulated
[†] not tested.
* Stimulated culture samples were diluted 1:20.
[b] > 20,000 pg/ml.
[c] > 3.0 O.D.

Volunteer 17.02.75
Male Control

DATE	IFNγ pg/ml	IL-2 pg/ml	IL-4 pg/ml	IL-1ra ng/ml		IL-1β* pg/ml	
				ns[T]	s[t]	ns	s
M24	68.0	633.1	7.8	nt[†]	nt	nt	nt
26	16.9	171.2	<[e]	1.76	>[c]	4.4	5173
J 2	45.5	411.9	17.3	1.12	62.71	2.5	5848
5	88.1	588.4	18.0	2.04	54.32	4.4	10250
7	63.0	536.5	23.2	1.60	43.07	3.4	10890
9	209.1	576.5	15.1	7.59	>	442.2	14500
14	83.7	658.9	38.1	1.23	34.54	5.4	11440
16	94.4	518.9	20.4	2.18	59.03	4.3	13980
19	138.6	807.4	26.9	1.87	61.02	12.2	9363
MEAN	89.7	544.8	20.9	2.42	52.45	59.9	10181
S.D.	56.1	177.0	9.0	2.12	11.26	154.5	3374

[T] non stimulated.
[t] stimulated
[†] not tested.
* Stimulated culture samples were diluted 1:20.
[c] > 20,000 pg/ml.
[e] < 2 pg/ml

Volunteer 16.05.74
Male Control

DATE	IFNγ pg/ml	IL-2 pg/ml	IL-4 pg/ml	IL-1ra ng/ml		IL-1β* pg/ml	
				ns[T]	s[t]	ns	s
J14	nt[†]	nt	nt	nt	nt	nt	nt
19	211.3	397.2	36.3	nt	nt	nt	nt
21	273.8	376.2	74.8	9.12	46.11	165	>[b]
24	482.2	413.9	95.5	9.05	60.37	145.2	nt
26	380.0	824.5	195.2	nt	nt	nt	nt
28	706.7	533.6	146.7	21.57	38.58	264.7	6167
31	nt	201.1	58.6	nt	32.35	nt	nt
A 1	1016	677.4	132.3	nt	nt	nt	nt
3	nt	nt	nt	nt	nt	nt	nt
MEAN	511.7	489.1	105.6	13.24	44.35	191.6	-
S.D.	302.5	208.1	55.5	7.21	12.06	64.1	-

[T] non stimulated.
[t] stimulated
[†] not tested.
* Stimulated culture samples were diluted 1:10.
[b] > 10,000 pg/ml.

Volunteer 19.02.36
PM Control

DATE	IL-2 pg/ml	IL-4 pg/ml	IL-1ra ng/ml		IL-1β* pg/ml	
			ns[T]	s[t]	ns	s
M15	275.6	66.4	nt[†]	nt	nt	nt
19	90.8	5.4	0.17	32.48	2.6	7189
23	151.9	20.6	0.41	59.49	3.4	>[b]
24	308.0	33.9	0.70	59.30	3.5	8587
26	212.9	24.6	0.88	>[c]	9.4	7778
29	263.3	30.6	nt	nt	nt	nt
J12	349.1	82.6	0.71	58.87	16.9	9281
14	210.8	18.4	2.75	>	>[d]	>
16	235.9	36.0	1.33	>	22.3	>
23	393.6	59.2	nt	nt	nt	nt
27	346.6	194.9	nt	nt	nt	nt
28	144.2	77.2	nt	nt	nt	nt
30	147.0	73.9	nt	nt	nt	nt
MEAN	240.7	55.7	0.99	52.54	9.7	8208
S.D.	92.5	48.8	0.86	13.37	8.2	916

[T] non stimulated.
[t] stimulated
[†] not tested.
* Stimulated culture samples were diluted 1:20.
[b] > 20,000 pg/ml.
[c] > 3.0 O.D.l.
[d] > 500 pg/ml.

Volunteer 17.10.38
PM Control

DATE	IL-2 pg/ml	IL-4 pg/ml	IL-1ra ng/ml		IL-1β* pg/ml	
			ns[F]	s[t]	ns	s
M15	415.1	1249	2.32	47.25	0.7	9879
17	485.1	560.4	3.21	nt	1.8	nt
19	484.6	593.3	2.74	52.41	>[d]	10960
23	617.4	1617.5	5.75	>[c]	>	13230
24	523.8	600.2	1.37	51.24	0.8	10530
26	499.5	632.9	1.38	47.48	1.5	7955
29	1306.0	633.4	3.56	66.11	2.1	12510
31	762.5	1340.5	2.19	>	1.8	12440
J 2	551.7	988.0	3.29	>	2.6	12010
5	nt[†]	129.5	12.74	>	251	9665
7	855.6	1344.0	9.34	>	174	12620
9	566.3	414.9	22.52	>	356	>[b]
MEAN	642.5	842.0	5.87	52.90	79.2	11180
S.D.	255.1	453.9	6.27	7.73	132.2	1673

[F] non stimulated.
[t] stimulated
[†] not tested.
* Stimulated culture samples were diluted 1:20.
[b] > 20,000 pg/ml.
[c] > 3.0 O.D.
[d] > 1000 pg/ml.

Table B.31. Means and standard deviations for the phase of the cycle profiles for all the cytokines measured in culture supernatants.

	EF	MF	LF	O	EL	ML	LL	M	EF
IL-2[c] pg/ml									
n	7	8	9	8	8	9	8	8	5
mean	382	445	434	401	521	528	638	532	476
S.D.	249	213	317	317	338	333	337	367	274
IL-4[a,c] pg/ml									
n	6	8	8	7	8	8	7	7	5
mean	38	35	38	28	37	50	55	64	42
S.D.	23	21	33	11	24	34	38	83	23
IFNγ[c] pg/ml									
n	7	8	9	8	8	9	8	8	5
mean	372.6	376.2	364.2	212.2	230.1	270.8	309.8	278.1	263.4
S.D.	272.4	373.9	343.2	154.6	146.8	137.1	143.7	119.4	198.3
IL-1ra[b] ng/ml									
n	5	8	9	8	8	9	7	7	3
mean	4.14	8.36	7.44	6.17	5.65	9.60	9.38	12.05	7.94
S.D.	1.27	8.24	6.58	4.76	4.69	8.37	6.37	10.63	4.57
IL-1ra[c] ng/ml									
n	5	8	9	8	8	9	7	7	3
mean	36.87	38.13	43.00	48.63	43.03	50.62	49.57	44.78	53.84
S.D.	12.65	11.65	11.09	10.53	9.99	14.68	13.83	9.38	11.31

[a] volunteer 09.10.64 excluded.
[b] non stimulated culture supernatants.
[c] stimulated culture supernatants.

Table B.31 continued.....

	EF	MF	LF	O	EL	ML	LL	M	EF
IL-1β[b] pg/ml									
n	3	6	9	8	7	7	7	7	3
mean	26.9	136.8	161.1	52.6	54.4	107.7	100.5	212.2	156.3
S.D.	11.6	232.2	159.1	55.5	89.2	121.2	81.6	142.9	213.7
IL-1β[cd] pg/ml									
n	5	8	8	6	5	6	5	5	1
mean	8571	8752	9850	11289	10205	9664	10044	10485	8153
S.D.	4734	3346	4196	5059	3140	5023	4570	4486	-
IL-1 Ratio[b]									
n	3	6	9	8	7	7	7	7	3
mean	0.541	0.874	3.12	1.10	1.04	1.32	1.82	3.74	1.45
S.D.	0.202	0.755	4.88	1.25	1.56	1.16	3.02	5.37	1.41
IL-1Ratio[cd]									
n	5	8	8	6	5	6	5	5	1
mean	26.94	26.56	22.43	26.11	23.01	18.29	19.73	24.23	19.98
S.D.	18.62	13.77	8.89	11.59	8.24	5.89	6.76	4.42	-

[b] non stimulated culture supernatants.
[c] stimulated culture supernatants.
[d] Volunteer 22.09.75 excluded

Table B.32. Means and standard deviations for the phase of the cycle profiles for all the immune parameters measured in serum.

	EF	MF	LF	O	EL	ML	LL	M	EF
IL-1ra pg/ml									
n	6	8	9	8	8	9	8	8	4
mean	142.7	127.9	255.6	325.9	329.9	303.1	328.9	362.8	507.5
S.D.	83.1	73.5	272.0	297.5	283.2	284.1	285.8	230.8	319.6
IL-1β pg/ml									
n	4	5	8	6	5	5	5	7	2
mean	5.52	3.37	2.86	2.89	4.07	4.86	3.50	2.66	1.83
S.D.	5.65	5.97	4.98	4.98	5.57	5.03	4.10	4.31	2.39
IL-1 Ratio[a]									
n	3	5	7	6	5	5	5	7	2
mean	5.88	2.63	2.50	0.909	1.66	1.58	2.56	0.81	0.88
S.D.	6.15	4.17	4.03	1.20	1.69	0.98	2.46	1.01	1.23
C3 mg/ml									
n	6	8	8	7	8	9	8	8	5
mean	1.08	1.05	1.01	0.95	1.02	1.09	1.07	1.04	1.09
S.D.	0.17	0.14	0.15	0.06	0.18	0.19	0.019	0.13	0.22
CH50 U/L[b]									
n	3	6	4	5	3	6	5	8	4
mean	195	200	192	172	193	208	175	193	204
S.D.	9.9	33.6	9.9	27.3	67.5	46.5	31.6	32.4	61.0

[a] Volunteers 01.04.75 and 30.04.65 excluded.
[b] Volunteer 30.04.65 excluded.

APPENDIX C

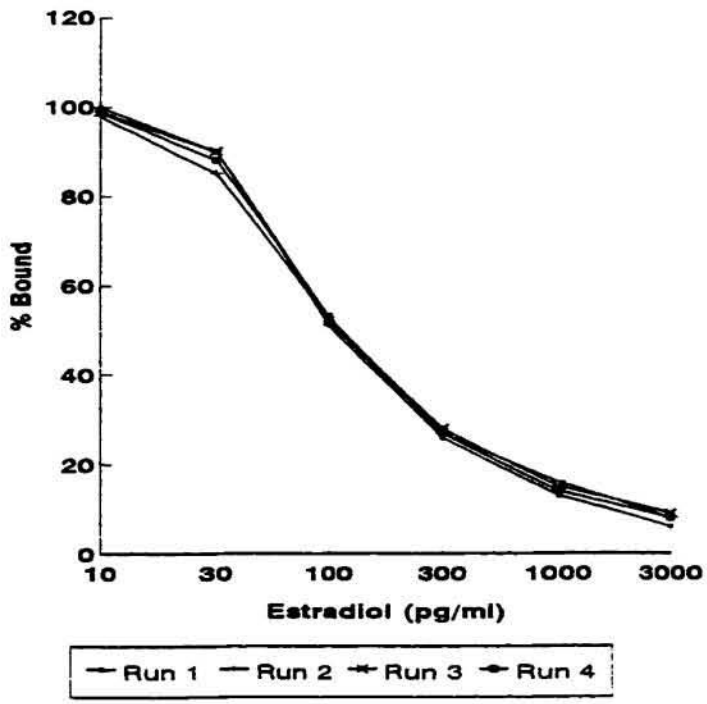

Figure C.1. Standard curves from the Estradiol RIA run at four different times.

Table C.1. Values for the same serum sample run or different RIA estrogen assays with the calculated coefficient of variation.

Assay Number	Value pg/ml
1	24.7
2	26.7
3	21.4
4	21.5
Mean $\pm$ S.D.	23.6 $\pm$ 2.6
C.V.	11.1%

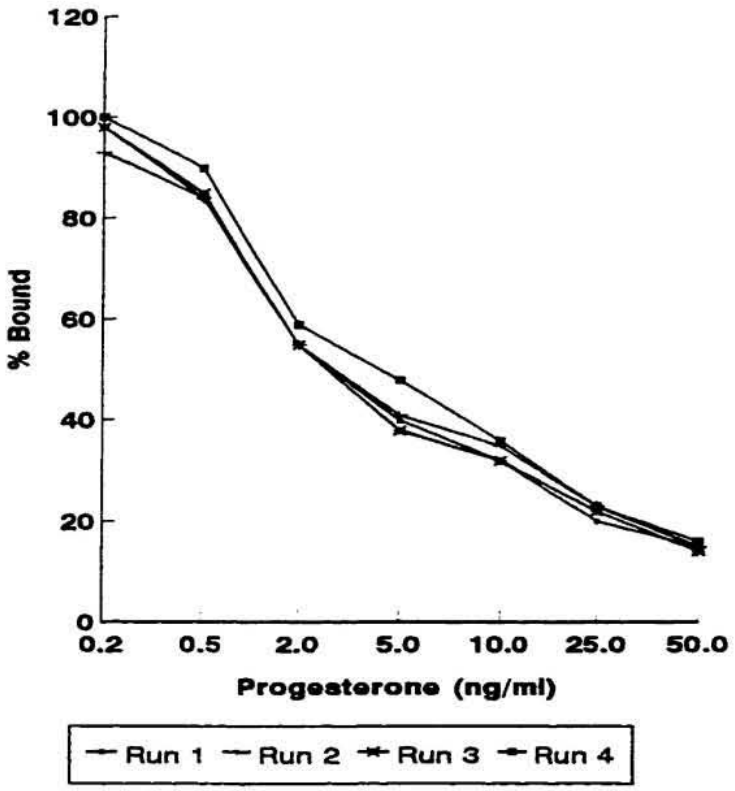

Figure C.2. Standard curves from the Progesterone RIA run at four different times.

Table C.2 Values for the same serum sample run on different RIA progesterone assays with the calculated coefficient of variation.

Assay Number	Value ng/ml
1	0.3
2	0.3
3	0.3
4	0.6
Mean $\pm$ S.D.	0.375 $\pm$ 0.15
C.V.	40.0%

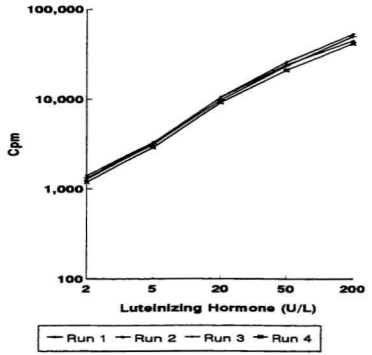

Figure C.3. Standard curves from the Luteinizing hormone RIA run at four different times.

Table C.3. Values for the same serum sample run on different RIA Luteinizing hormone assays with the calculated coefficient of variation.

Assay Number	Value U/L
1	0.08
2	0
3	0
Mean $\pm$ S.D.	-
C.V.	-

Note: The values seen in the above table are not different from 0 thus the luteinizing hormone concentration in the sample run was below the sensitivity of the assay and a coefficient of variation is not available.

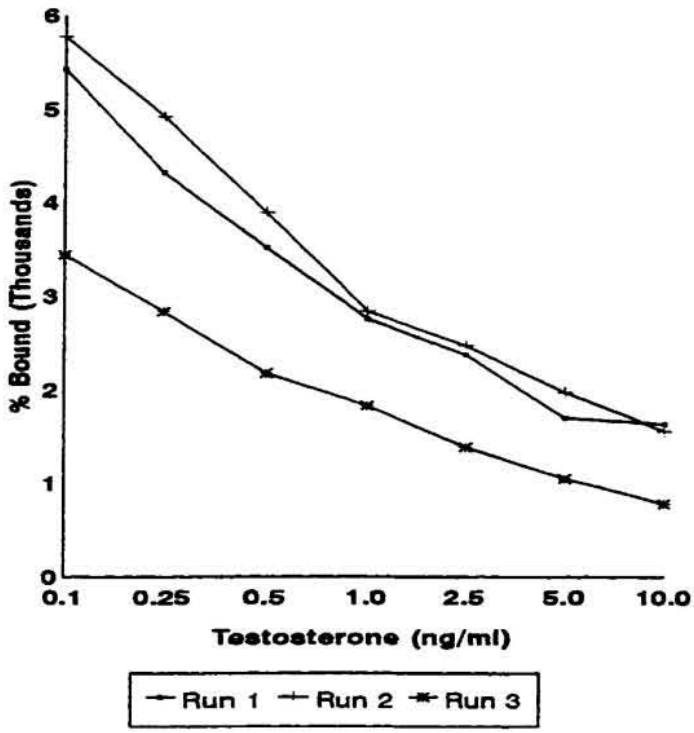

Figure C.4. Standard curves from the Testosterone RIA run at three different times.

Table C.4. Values for the same serum sample run on different testosterone RIA assays with the calculated coefficient of variation.

Assay Number	Value ng/ml
1	0.19
2	0.20
3	0.17
Mean $\pm$ S.D.	0.187 ± 0.015
C.V.	8.0%

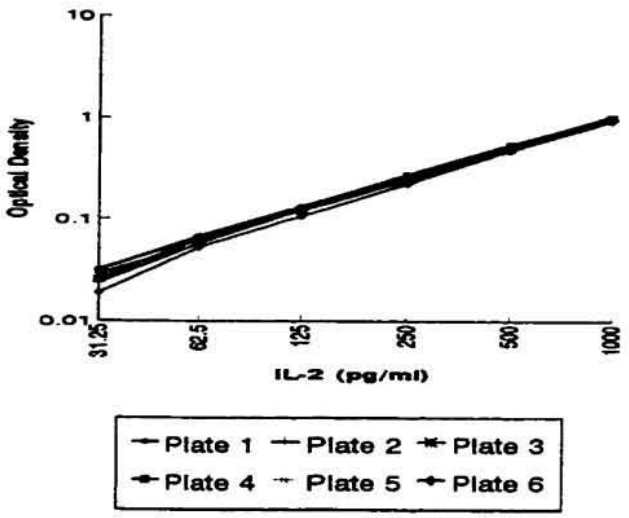

Figure C.5. Standard curves from the IL-2 ELISA run at six different times.

Table C.5. Values for the same culture supernatant sample run on different Interleukin 2 ELISA assays with the calculated coefficient of variation.

Assay Number	Value pg/ml
1	1326
2	1322
3	1378
4	1245
5	1393
6	1359
7	1377
Mean $\pm$ S.D.	1342 $\pm$ 51
C.V.	3.8%

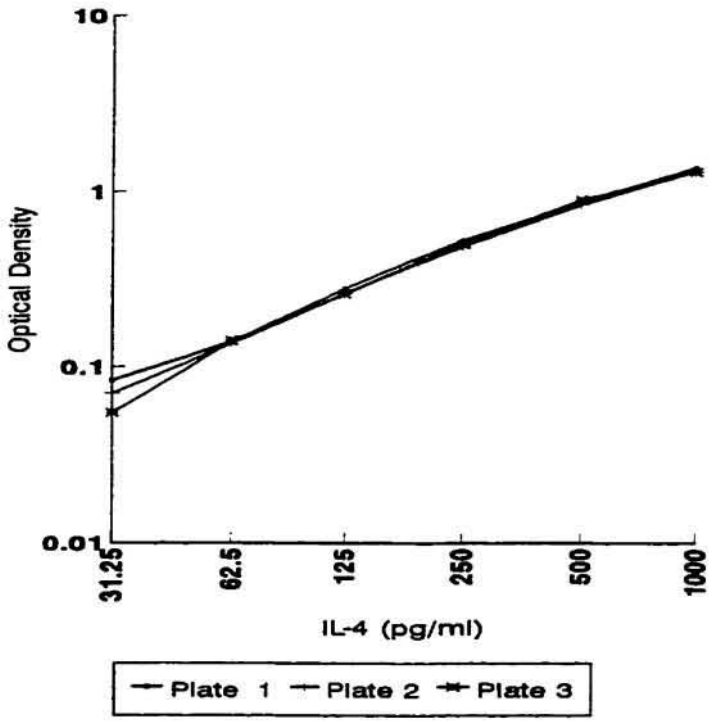

Figure C.6. Standard curves from the R&D Systems IL-4 ELISA run at three different times.

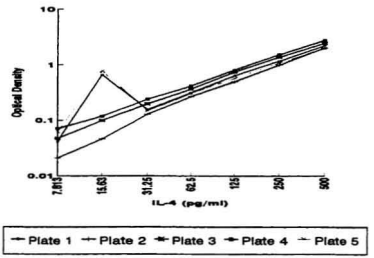

Figure C.7. Standard curves from the Biosource IL-4 ELISA run at five different times.

Table C.6. Values for the same culture supernatant sample run on different Interleukin 4 ELISA assays with the calculated coefficient of variation.

Assay Number	Value pg/ml
1	1200
2	1187
3	1043
4	2998*
Mean ± S.D.	1143 ± 87
C.V.	7.6%

*This value was obtained from the Biosource kits and was not used in the calculation of the coefficient of variation. The remaining four values were all above O.D. 3.0 and not available.

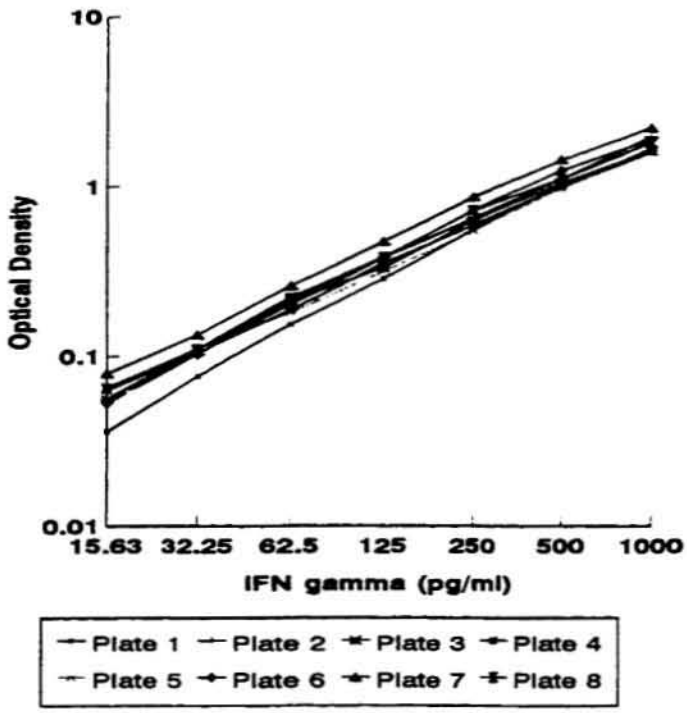

Figure C.8. Standard curves from the IFNγ ELISA run at eight different times.

Table C.7. Values for the same culture supernatant sample run on different Interferon gamma ELISA assays with the calculated coefficient of variation.

Assay Number	Value pg/ml
1	556.7
2	551.4
3	551.6
4	498.6
5	538.0
6	546.4
7	508.0
8	503.4
Mean $\pm$ S. D.	531.8 $\pm$ 24.3
C.V.	4.6%

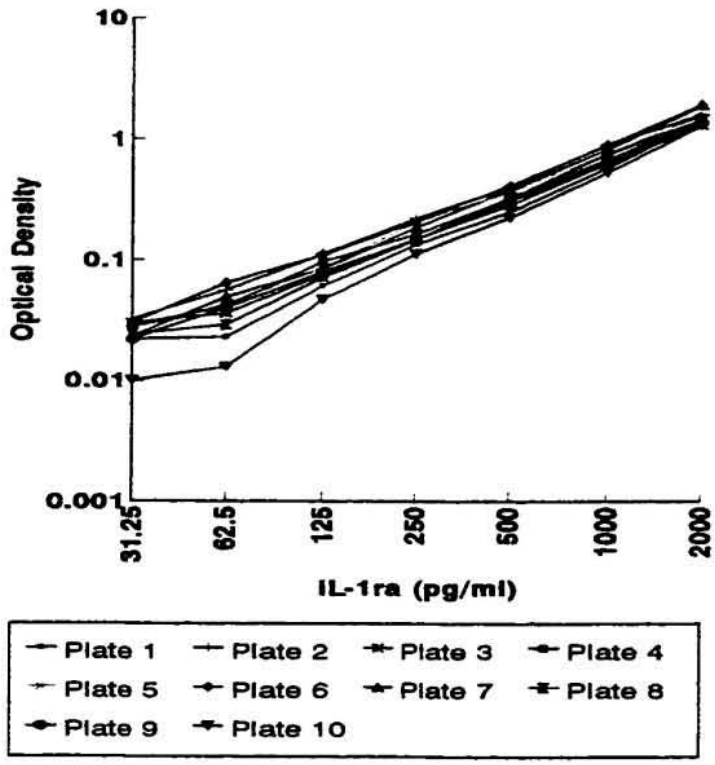

Figure C.9. Standard curves from the IL-1ra ELISA run at ten different times to measure IL-1ra in culture supernatants.

Table C.8. Values for the same culture supernatant sample run on different Interleukin 1ra ELISA assays with the calculated coefficient of variation.

Assay Number	Value ng/ml
1	36.70
2	67.77
3	33.22
4	38.59
5	35.83
6	55.89
7	42.06
Mean $\pm$ S.D.	44.29 $\pm$ 12.75
C.V.	28.79%

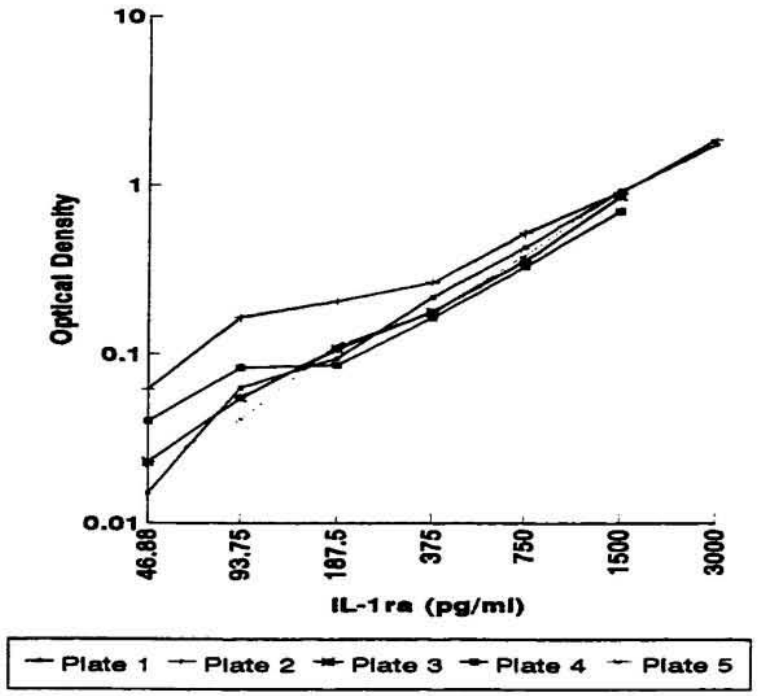

Figure C.10. Standard curves from the IL-1ra ELISA run at five different times to measure IL-1ra in serum.

Table C.9. Values for the same serum sample run on different Interleukin 1ra ELISA assays with the calculated coefficient of variation.

Assay Number	Value pg/ml
1	256.7
2	121.4
3	228.3
4	216.0
5	159.9
Mean $\pm$ S.D.	196.5 $\pm$ 54.8
C.V.	27.9%

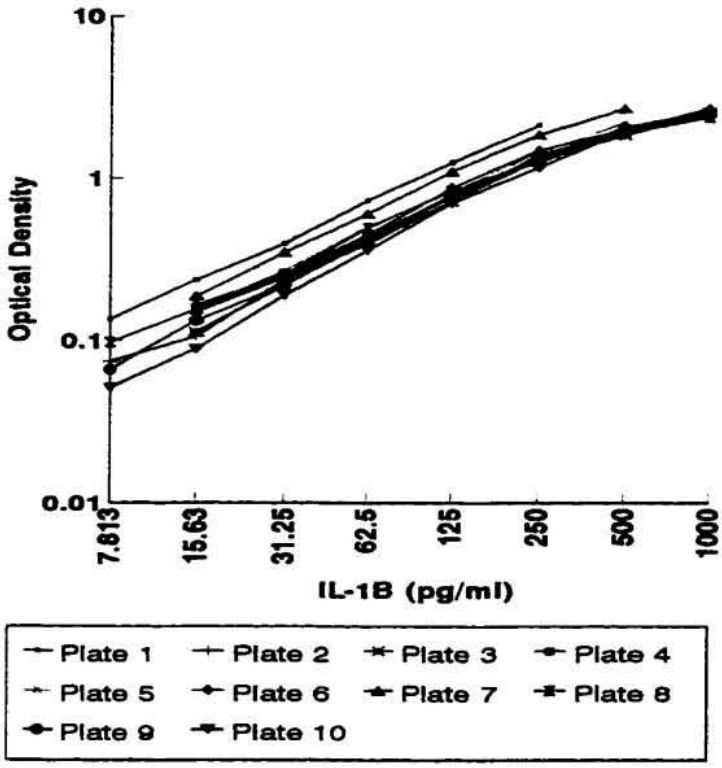

Figure C.11. Standard curves from the IL-1β ELISA run at ten different times to measure IL-1β in culture supernatants.

Table C.10. Values for the same culture supernatant sample run on different Interleukin 1β ELISA assays with the calculated coefficient of variation.

Assay Number	Value pg/ml
1	6596
2	8180
3	8184
4	14570
5	13850
Mean ± S.D.	10276 ± 3658.0
C.V.	35.6%

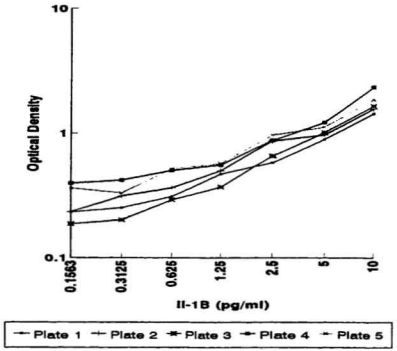

Figure C.12. Standard curves from the IL-1β ELISA run at five different times to measure IL-1β in serum.

Table C.11. Values for the same serum sample run on different Interleukin 1β ELISA assays with the calculated coefficient of variation.

Assay Number	Value pg/ml
1	0.1559
2	< 0.083
3	< 0.083
4	0.1721
5	< 0.083
6	< 0.083
Mean ± S.D.	-
C.V.	-

Note: Many of the values for IL-1β were not measurable in serum as indicated in the above table. Coefficient of variation was not calculated due to this.