Normal Pregnancy and Spontaneous Abortion; Some Immunological Aspects

Thesis presented for the degree of ***M Sc*** *(Med Sci)*

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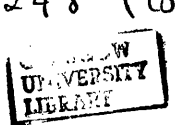

Declaration

The work presented in this thesis was performed in the University Department of Medicine, Royal Infirmary, Glasgow. The detailed planning of the work and its execution were my individual responsibility and, except where indicated, the work was personally performed.

The work in chapter six was published as:

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Abstract

Around one in five pregnancies end in spontaneous abortion. There are many causes for spontaneous abortion and these are discussed in this thesis. The body of a pregnant women has to tolerate a foetus which receives its genetic material from both parents, and as such is essentially an allograft. Normal pregnancy is therefor a complex challenge to the immune system. The cause of some abortions is unclear and it has been suggested that some abortions may be due to immunological factors.

For this study blood samples from normal non pregnant women, normal pregnant women and women undergoing a spontaneous abortion were collected. Isolated peripheral blood lymphocyte cells from the various patient groups were set up in tissue culture. The cells were then stimulated with mitogens and the turn-over of cells and production of immunoglobulins was assessed.

Measurement of some cytokine and thyroid antibody levels in the peripheral blood was carried out using enzyme linked immunoabsorbant assays (ELISA).

Damage caused by the formation of reactive oxygen species, or free radicals, has been implicated in a number of pathologic conditions. In this study samples from the above groups were assayed for free radical scavenger levels.

Compared to normal non-pregnant women, pregnant women were found to have lower IgG levels and raised free radical scavenger levels. Spontaneous aborters also had raised free radical scavenger levels, but no significant difference in IgG levels was detected.

Cytokine levels were significantly different in non-pregnant or pregnant women compared to spontaneous aborters.

Thyroid antibodies were not detected in the serum of either non pregnant or pregnant women. However, 4 out of 11 recurrent aborters (ie women who had suffered more than three spontaneous abortions) were found to be positive for thyroid antibodies.

The significance of these findings is discussed.

Table of Contents

Chapter Two: Methods

Chapter Four: Reactive Oxygen Species

Chapter Seven: Discussion

List of Tables

List of Figures

Chapter One

General Introduction

1.1 Incidence of spontaneous abortion

Spontaneous abortion, or the spontaneous loss of a pregnancy, is emotionally traumatic for the patient and her family and a vexing problem for the physician, especially where the condition recurs. The word abortion comes from the Latin *aboriri*- to go away, to perish.

Reports in the literature of the percentage of pregnancies that end in spontaneous abortion vary to a large degree. This is partly due to study design. Where pregnancy testing is carried out on a daily basis, from ovulation onwards, more positive results will occur than where testing is carried out after missed menses. A number of these "early positives" will abort before pregnancy is clinically diagnosed or by the time of maternal awareness of pregnancy (usually after a missed period). Some studies consider only pregnancies detected at time of maternal awareness. Writing an hypothesis in the Lancet, Roberts and Lowe (1975) suggested "that in England and Wales married women aged between 20-29 may on a conservative estimate abort 78% of their conceptions." They wrote of "Nature resorting to abortion to maintain genetic stability."

Unsuspected pregnancy loss occurs where there is only biochemical proof of pregnancy, and this has led to researchers doubting the validity of other workers research. Whittaker et al (1983) found a spontaneous abortion rate of 12% for clinically obvious abortion and a further 8% of abortions based on circulating levels of human chorionic gonadotropin (HCG).

Figures of between 15 and 20% are usually given in the literature for the rate of spontaneous abortion in clinically recognised pregnancy. If miscarriage always

recurred randomly and we take the figure of 15% of all pregnancies that end in miscarriage, we would expect 0.4% of women to miscarry three consecutive pregnancies. The observed frequency however is about 0.8-1.0% (Regan, 1991), indicating that recurrent miscarriage occurs much more often than would be predicted by chance. Regan says that even after three consecutive miscarriages the chance of a successful pregnancy is as high as 60%, a fact that can confuse statistics, as each success after treatment is more likely to have been due to spontaneous recovery than to the treatment given.

1.2 Classification of abortion (from de Gruyter 1988)

Induced abortion is the deliberate termination of pregnancy.

Spontaneous abortion is the expulsion of a foetus from the womb before 24 weeks of pregnancy. This can be complete, where all the products of conception (foetus, amniotic sac and placenta) have come away, or incomplete, where some products of conception remain in the uterus, the patient presenting with bleeding that continues and symptoms of miscarriage that do not go away.

After 24 weeks the spontaneous ending of pregnancy is termed a still birth if the baby is born dead or premature delivery if the baby is alive.

Primary aborters are women who have never had a successful pregnancy, while women who abort and have had a previous live birth are termed secondary aborters.

A missed abortion occurs where the foetus has failed to develop or has died but is not immediately expelled.

Recurrent abortion is the term used where three or more spontaneous abortions have occurred.

1.3 Causes of spontaneous abortion

The cause of spontaneous abortion may be explained in about half of all cases, the causal factors being;

1.3.1 The foetus

Chromosomal/ non-chromosomal abnormalities. Half of all abortuses of eight to fifteen weeks gestation are found to be chromosomally abnormal (Simpson 1980) The number of spontaneously aborted foetuses with structural (non-chromosomal) abnormalities is extremely difficult to define. Tissue degeneration due to maceration hinders detection of such abnormalities. Neural tube defects were found at a rate of 36 per 1000 spontaneous abortions in one study (MacHenry 1979). The incidence of this defect among spontaneously aborted foetuses is many times greater than is found at birth.

1.3.2 The mother

Fibroids. (benign tumours in the uterus),

Anatomical abnormalities. i.e. malformed uterus, incompetent cervix. One study (Ayers et al 1982) of 66 patients with recurrent abortion found that 42 had a widened uterine isthmus as their only abnormality. First trimester cervical effacement occurred in 90% of these patients. Using a combination of progesterone administration and surgical intervention (McDonald cerclage),

begun in early pregnancy, successful term pregnancies were achieved for 97% of those patients treated. The Medical Research Council and Royal College of Obstetricians and Gynaecologists published results of a multicentre randomised trial of cervical cerclage (McNaughton et al 1993). This study concluded that cervical cerclage should be offered to 'high risk' women i.e. those with a history of three or more pregnancies ending before 37 weeks gestation.

Systemic Disease/Endocrine problems. e.g. kidney disease, hypertension, thyroid disorder, diabetes (Ritchie 1995).

Age. Women over 35 years of age run a higher risk of foetal death than younger women (Fretts 1995). A possible explanation for this is the failure of the uterine vasculature, in older women, to adapt to the haemodynamic demands of pregnancy (Naeye1983).

1.3.3 Both or other

Time of fertilisation. The risk of spontaneous abortion increases with post ovulatory ageing of the ova and also with the age of sperm (Guerrero V, 1975).

Semen abnormalities, especially severe oligospermia (low sperm count) (Daya 1994).

Contraception. Unplanned pregnancies which occur where the mother has an inter-uterine contraceptive device (coil) fitted are at increased risk of spontaneous abortion. 111 out of 177 such pregnancies (62%) spontaneously aborted in one study (Vessey 1979). Spermicidal creams slightly increase the risk of spontaneous abortion.

Diet. Dietary deprivation is known to induce foetal loss in rabbits (Matsuzawa 1981). Diets deficient of certain elements are also known to cause abortion. Poor vitamin A status is associated with prematurity and uterine growth retardation found in poorly nourished populations (Shah 1984). Zinc deficiency has been incriminated in infertility, abortions, malformations, foetal intrauterine growth retardation, premature and postmature births and perinatal death. Risk groups for developing zinc deficiency are found among those with insufficient food intake, especially in protein malnutrition; abnormal mucosal uptake, as in celiac disease; abnormal intestinal losses, as in inflammatory bowel disease; abnormal renal excretion, as in diabetes with insufficient metabolic control; alcoholism; and treatment with diuretic drugs (Jameson 1993).

Smoking. It has been noted that babies born to mothers who smoke while pregnant have a lower birth weight than babies whose mothers do not (Zabriskie 1963). Two possible explanations have been given for this; a) reduced food intake by smokers and b) anoxia. These may be the same factors involved in the increased risk for smokers having spontaneous abortion (Kline 1977). Smoking, certainly in pregnancy, tends to be correlated with alcohol intake. Writing in the Lancet, Harlap (1980) suggested that some studies of smoking and spontaneous pregnancy loss have not considered the effects of drinking alcohol, but taking this into account they still found a higher incidence of abortion among smokers.

Alcohol. More than one quarter of pregnant women drinking twice a week or more are likely to abort, compared with about 14% among women who drink less often (Kline 1980).

Drugs. Abuse of illegal drugs is known to increase the incidence of spontaneous abortion. It is reported that one in four Americans has used cocaine for its

euphorigenic properties. Women in this group have an increased incidence of spontaneous abortion, placental abruption and neonatal neurobehavioural abnormalities. There is also evidence of increased incidence of congenital malformations, intrauterine growth retardation, premature labour and sudden infant death syndrome for cocaine abusers (Das 1994).

Stress. Working on mice subjected to ultrasonic sound stress Arck (1994) suggests that stress may inhibit protective suppressor mechanisms and promote secretion of abortogenic cytokines such as tumour necrosis factor alpha (tnf-α).

Toxic chemicals. Exposure of the mother (or the father prior to pregnancy) to certain harmful chemicals can increase the likelihood of spontaneous abortion. Hospital staff involved in sterilising procedures during their pregnancy had a spontaneous abortion rate of 16.7% compared to 5.6% for non exposed pregnancies. The chemicals implicated were ethylene oxide and, to a lesser degree, gluteraldeyde. (Hemminki 1982). Anaesthetic gases are thought to be responsible for the increased incidence of spontaneous abortion among hospital staff working in operating theatres (Vessey 1980). Other chemicals used in industry and in laboratories are known abortogens e.g. trypan blue stain. Ethylene oxide, mentioned above, is used in the production of antifreeze, floor varnishes and surfactants, as well as for sterilising instruments (Croner 1992).

Exposure to radiation. Women who are, or may be, pregnant are not recommended to undergo X-ray examination because of the risk of abortion or birth defects. The human embryo-foetus is highly radiosensitive and must be protected from excessive exposure to ionizing radiation. The maximum permissible dose equivalent for the developing embryo-foetus is set at 0.5 rem per year (Caprio

1980). Recently there has been the suggestion that exposure to the low frequency magnetic fields of VDTs (visual display terminals) may be a causal factor in abortion (Goldhaber 1988). One study found that women working in clerical positions using VDTs for more than twenty hours per week had double the risk of spontaneous abortion of other women. However, professional women using VDTs for twenty hours or more per week were not found to have a higher risk than women who are nonusers, suggesting that VDT use was not the causal factor in clerical women. In 1991 the US government's National Institute for Occupational Safety and Health published details of the largest study to date and concluded that women working with VDTs were at no increased risk of spontaneous abortion.

Medical techniques. The removal of a sample of amniotic fluid to test for foetal abnormalities (amniocentesis) carries a 1% risk of abortion (Medical Research Council 1978, Tabor 1986). Chorion villus sampling (CVS), a technique performed between 8 and 10 weeks gestation, which involves the sampling of trophoblast (the cellular layer which gives rise to the placenta) via a needle through the mother's abdomen, carries a 2.9% risk of spontaneous abortion (Medical Research Council European Trial 1991). Although the use of ultrasound technology has greatly reduced the risk of abortion caused by these procedures, they are restricted to women for whom there may be a high risk of foetal abnormalities such as those over 40 years old. Ultrasound itself has been implicated in one study as causing spontaneous abortions in physiotherapists working with ultrasound as well as short-wave radiation (Taskinen 1990). While it is recommended that pregnant medical workers avoid unnecessary exposure to ultrasound the risk of diagnostic ultrasound causing harm to the foetus has not

been proven, though a link with repeated ultrasound diagnosis and low birth weight has been noted (Salvesen 1995)

Accident. Pregnancy loss following an accident, such as a fall, is extremely rare as the foetus is well protected in the uterus. Spontaneous abortion caused by trauma is unlikely unless there is a direct penetrating injury (Howie 1995).

Infection. Infection is implicated as an occasional cause of spontaneous abortion (Summers 1994). Listeria monocytogenes, a food borne organism found in soft cheeses, can cause listeriosis, an infectious disease with influenza-like symptoms. Listeriosis can result in spontaneous abortion and stillbirth (Shaw 1995). Pregnant women are advised to avoid soft cheeses and paté. Chlamydial infection is a sexually transmitted bacterial infection that has been linked to spontaneous abortion (Tuormaa 1994).

Fever. A fever of more than 100°F was found to be "antecedent, rather than a symptom of spontaneous abortion" (Kline 1985).

1.4 Immunological factors

1.4.1 The Immune System

The body has a system of differentiating self from nonself and, where a foreign molecule is detected, set up a response to deal with it (Male 1996). The foreign molecule, or antigen, may be a parasite, fungus, bacterium or virus. Reaction to an antigen is highly specific. Burnet's clonal selection theory (1958) states that there is a specific lymphocyte for every possible antigen. Exposure to an antigen leads to clonal expansion of a specific lymphocyte and hence an

immune response.

An important feature of the immune system is immune memory which is the basis of vaccination. When an antigen to which the body has previously been exposed is encountered, T cells which were activated in the initial exposure are still viable and a larger and quicker response can be mounted to the antigen.

1.4.2 Cells of the immune system (Lydyard 1996)

The immune system is made up of a number of organs connected by a network of vessels (figures 1.1 and 1.2). The cells of the immune system are the white blood cells (figure 1.3).

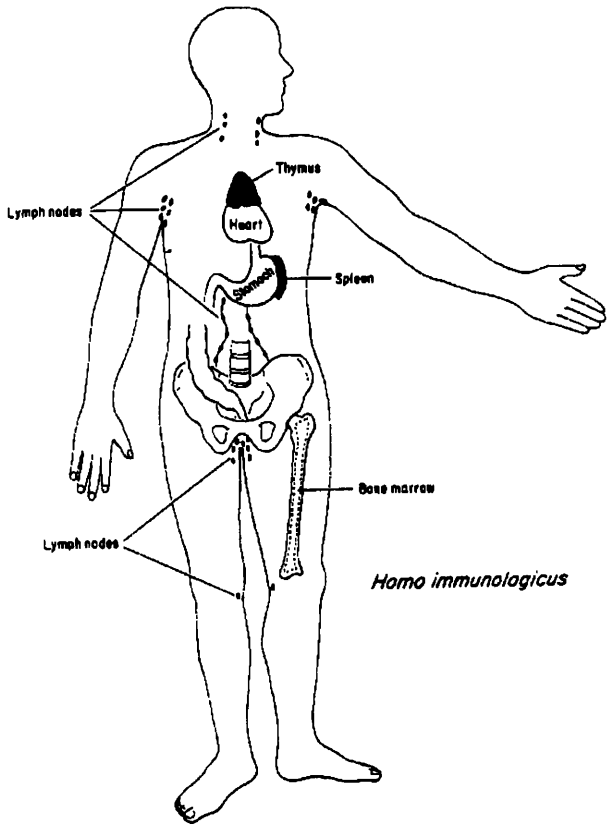


Figure 1.1 **The immune system**

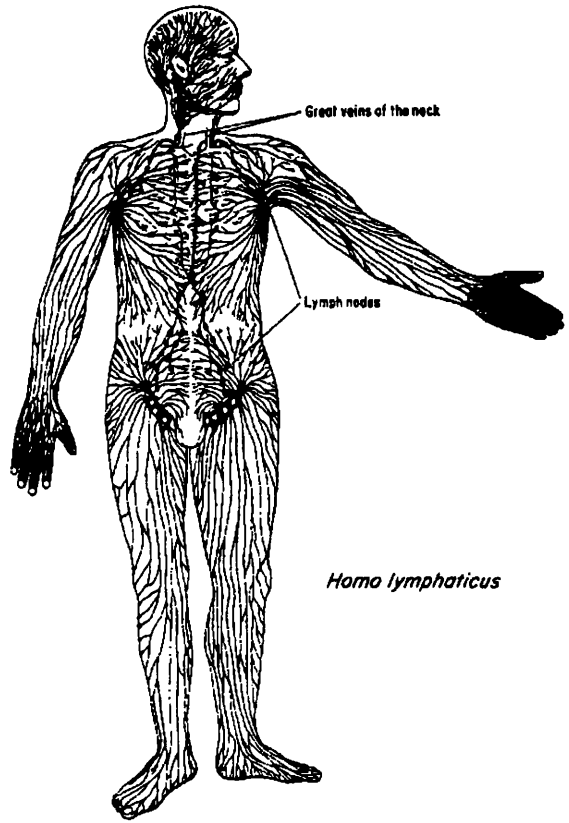


Figure 1.2 **The immune system: network of vessels**

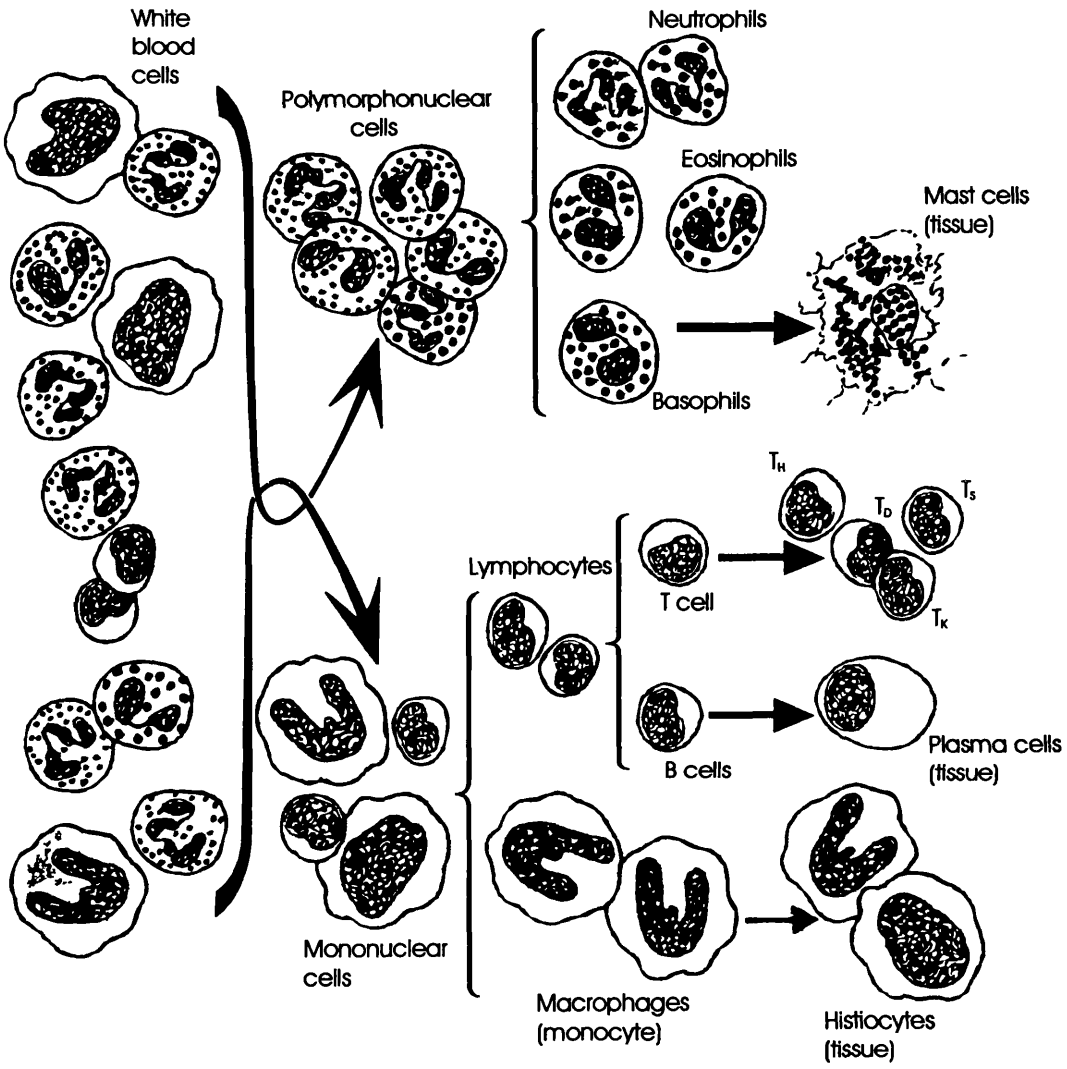


Figure 1.3 **Cells of the immune system**

White blood cells may be divided into two major populations;

1. Mononuclear, or "round" cells with a single nucleus and

2. Polymorphonuclear with segmented nuclei.

Mononuclear cells are further divided into large (macrophage or monocyte) and small (lymphocyte).

Monocytes are formed in the bone marrow and circulate in the blood to tissues where they mature into macrophages. Macrophages are involved in immune responses, they function as scavengers but their prime function is presentation of antigen. Macrophages, along with polymorphonuclear leukocytes, have an active role in the inflammatory response associated with immune reactions. Macrophages may accumulate in large numbers in sites of inflammation, The migration to these sites is believed to be non-antigen specific.

Lymphocytes form 30% of the total of white blood cells and are the main constituents of the immune system. The lymphocyte is responsible for the primary recognition of antigen and is an immunologically specific effector cell. Lymphocytes can be subdivided into two major populations, T cells and B cells, deriving their names from the sites where the immature cells differentiate to their mature forms. Although morphologically very similar, T and B lymphocytes have different cell surface phenotypes (each cell type of the immune system has a diverse array of surface adhesion molecules that allow recognition via discrete corresponding surface receptors).

T Cells. Prothymocytes (T cell precursors) are produced in the bone marrow and circulate to the thymus. Thymus derived cells (T cells) originate in the thymus

from these precursor cells and are then re-released into the circulation. T cells participate in cell mediated reactions and, although they do not themselves produce antibodies, they help regulate antibody production by B lymphocytes (B cells). T cells are divided into subpopulations according to their functions.

When activated by antigen, T helper cells (also known as CD4+ve because they display a molecule called CD4 on their surface) will produce effector molecules that will activate, or deactivate, other lymphocytes. These effector molecules are called interleukins. When cytotoxic, or killer T cells (marked by the surface protein CD8 and therefore called CD8+ve) are activated by antigen they make direct contact with target cells and kill them by secreting toxic molecules. Other T cells induce inflammation (T delayed hypersensitivity cells) and inhibit immune response (T suppressor cells).

T cells are the master regulators of the immune system, they turn other cells in the immune system off or on.

B Cells. These arise from precursors in the bone marrow. B cells are the precursors of plasma cells, the cells that synthesise immunoglobulins (Figure 1.4). Surface immunoglobulin is readily detected on B cells, but not on T cells. When activated by antigen B cells differentiate into antibody (immunoglobulin) secreting plasma cells. Plasma cells are found in large numbers in lymph nodes, spleen and sites of chronic inflammation.

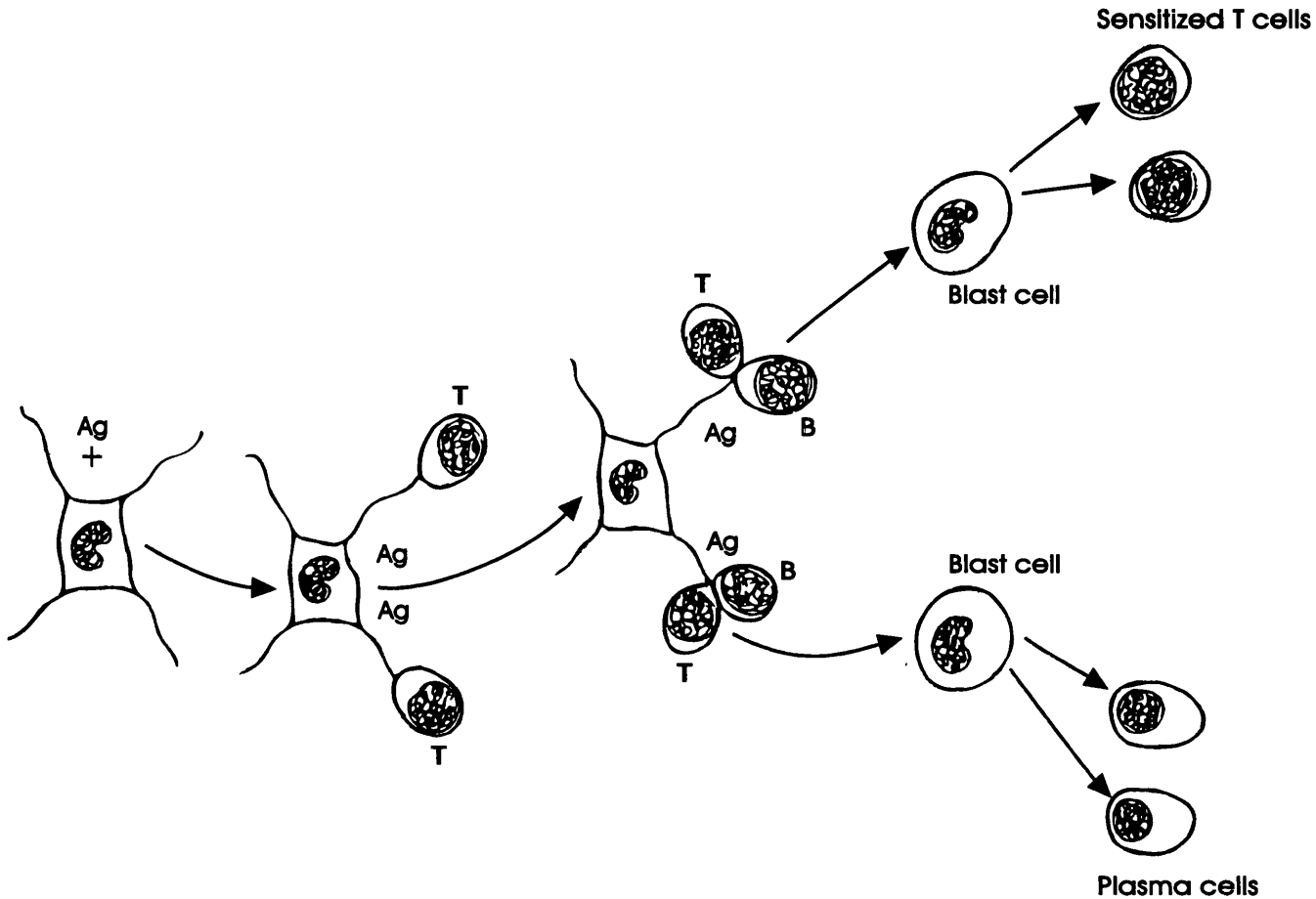


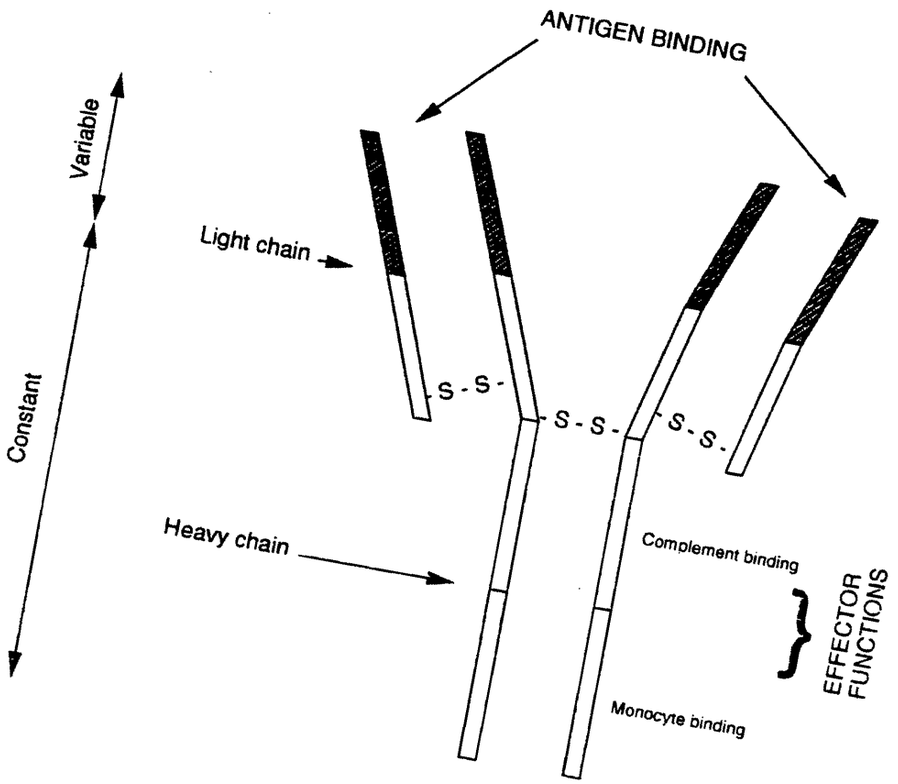
Figure 1.4 **Antibody production**

Immunoglobulins consist of two 'heavy' chains and two 'light' chains held together by inter-chain disulphide bonds (Figure 1.5). The chains have an N-terminal portion that forms the antigen binding site. This N-terminal varies according to the antigen while the rest of the structure remains constant. There are five major classes of immunoglobulins (Ig). These are IgA, IgD, IgE, IgG and IgM. In the bloodstream IgG is the main immunoglobulin found. The immunoglobulins found on the surface of unstimulated B cells are IgM and IgD. When B cells are stimulated they first secrete IgM then switch to secretion of IgG, IgA or IgE.

Blast Cells. These are activated cells in the process of dividing. They are found in lymphoid organs draining sites of antigen injection. They are also found in active inflammatory lesions. Blast cells may be induced *in vitro* in cultures of lymphocytes by certain mitogenic agents. Lymphocytes that recognise antigen are stimulated by that antigen to transform into blast cells. These blast cells proliferate and then differentiate into plasma cells or sensitised T cells.

1.4.3 Interleukins

Interleukins are a series of lymphocytotrophic hormones. They are a group of proteins that act on the immune system in much the same way as hormones and their receptors act on the endocrine system. Eighteen different interleukins have been identified to date, though the list is sure to expand. Interleukins are discussed in more detail in chapter 5.



(S-S denotes disulphide bonds)

Figure 1.5 **Structure of immunoglobulins**

1.5 Immunology of normal pregnancy

Although much work has been carried out in the field of immunology of a normal pregnancy there is still much that is not understood. The foetus receives its genetic material from both parents, and as such is essentially an allograft. An allograft (from Greek *allos* meaning other) is a graft of tissue from a donor to a recipient of the same species, where the two individuals are not genetically identical. A normal pregnancy would thus appear to be against the basic principles of transplant immunology. The body has the ability to differentiate self from non-self, and where foreign material is detected, initiate a response i.e. graft rejection (Gurka 1987). The usual mechanism of immunologic rejection of an allograft involves the presentation of the foreign antigen to regional lymph nodes and processing of the antigen by macrophages. As a result effector T-cells proliferate and produce antibodies and cytotoxic lymphocytes, then return via the bloodstream to eventually destroy the foreign graft. Rejection of the foetus does not normally occur (Medawar 1953). Several theories have been advanced to explain the success of the foetal allograft.

1.5.1 Protection of the foetus

There are a number of possible mechanisms that can protect the foetus from rejection;

1.5.1.1 The Uterus

The uterus is not an immunologically privileged site (immuno-suppressed micro-environment), it is well vascularised, with good lymphatic drainage and

can reject foreign tissue. Some lymphoid cells found in the uterus contain IgA, IgM and IgG. The uterus is therefore well supplied with immune components and is unlikely to protect the foetus.

1.5.1.2 The Placenta

At the placenta the maternal blood is in direct contact with the trophoblast. The trophoblast is the layer of tissue formed by cells that form the outer covering of the blastocyst (the spherical structure produced by cleavage of a fertilised egg). The placenta forms a physical barrier to corpuscular and large molecular particles between the maternal and foetal blood. As well as this physical barrier the placenta is thought to act as an immunologic filter. The immunologic profile of placental tissue as described by Goldsobel et al 1986 indicates considerable cytotoxic activity and several unique phenotypic characteristics that differentiate it from either the maternal or the foetal immune system.

When small numbers of maternal lymphocytes enter the foetal circulation, and vice versa, certain inhibitory substances could be associated with the placenta, causing the opposing cells to be hyporesponsive (Cadet 1995) . This hyporesponsiveness may be manifested by a reduced capacity for the production of cytokines such as interferon gamma, interleukin 1β (IL-1β) and tumour necrosis factor α (TNFα). Cadet proposes interleukin 10 (IL-10) as such an inhibitory substance and has found that the placenta does indeed express IL-10 messenger RNA. Human placental cell lines are also found to produce IL-10. IL-10 has been shown to inhibit the synthesis of interferon gamma, granulocyte-monocyte stimulating factor, IL-1, IL-2, IL-3 and TNFα It also plays a role in the neuroendocrine system as an inducer of ACTH, which upregulates the

production of adrenal glucocortosteroids which are immunosuppresive. All foetal trophoblast populations throughout gestation express high levels of cell surface complement regulatory proteins, providing protection from complement-mediated damage at the materno-foetal interface (Vince 1995). Restricted antigen presentation has also been suggested as an explanation of foetal survival (Wood 1994).

Around the time of implantation and during early pregnancy the human uterine mucosa is infiltrated by large numbers of CD56+ve natural killer (NK) cells. Approximately 80% of maternal leukocytes present in human decidua at this time are CD56+ve NK cells (King 1996). These leukocytes are in close proximity to the foetally-derived invading extravillous trophoblast which expresses at least two HLA class I molecules, HLA-G and HLA-C (Verma 1997). The specific expression of HLA-G in placental trophoblast suggests an important role for this molecule in the immunological interaction between mother and child (Münz 1997). It is thought that maternal recognition of the foetus may be mediated by an NK allorecognition system (Hiby 1997) and that NK inhibition by HLA-G may contribute to the survival of the foetal semiallograft in the mother during pregnancy.

Wegmann (1988) came to the conclusion that maternal T cells, rather than being potentially detrimental to the foetal allograft, promote its growth and viability during normal pregnancy. T cells could be activated by placental trophoblast and produce cytokines that make trophoblast grow better. T-Helper cells are sub divided into T_H1 and T_H2 cells (Wegmann, 1993). T_H1 cells secrete Il-2, IFN-γ and TNF-β. These are cytokines that are deleterious to pregnancy, possibly by inducing NK cell activity. T_H2 cells produce cytokines that are beneficial to

pregnancy, such as Il-3, Il-4, Il-5 and Il-10. These cytokines are involved in B-cell production. Il-10 inhibits T_H1 cells and so a bias away from cell mediated immunity towards antibody-associated immunity is proposed. Possible evidence of this lies in the fact that a majority of patients with rheumatoid arthritis, a cell mediated autoimmune condition, enjoy a remission of disease symptoms during pregnancy, while patients with systemic lupus erythematosis (SLE), a condition of excess auto-antibodies may experience a flare up of disease symptoms whilst pregnant. *In vitro* experiments show that peripheral blood mononuclear cells from women respond to trophoblast antigens with a dichotomous T-helper response (Hill, 1995). These experiments show that "trophoblast cells induce a T_H2-type cytokine response that may benefit pregnancy, whereas many women with unexplained recurrent pregnancy loss manifest an abnormal T_H1-type response following exposure to trophoblast extracts".

1.5.2 Autoimmunity

Autoimmunity is the process by which a humoral or cellular response is directed against a specific component of the host. Examples of autoimmune diseases are systemic lupus erythematosus (lupus), progressive systemic scleroderma, polymyositis, dermatomyotis, mixed connective tissue disease, autoimmune thyroid disease, multiple sclerosis and 'sub clinical autoimmune disease.' Autoantibodies are often found in the blood of older people with no symptom of disease, thus a great deal of the degeneration of our bodies as we age may be due to sub clinical autoimmune disease. Some autoimmune diseases affect women much more than men. Rheumatoid arthritis is 2-3 times more frequent in females, and lupus 6-10 times more, than found in males. In these

diseases which predominantly affect women, there is a tendency for the disease to appear in the childbearing years, leading to speculation that women are more prone than men because they have developed more powerful immune systems in connection with their childbearing function (Clark 1995). That such autoimmune diseases are influenced by sex hormones is shown by experiments on lupus prone F1 hybrid mice (Jacobson et al 1994). The antibodies most prominently found in lupus are to DNA, and are in fact diagnostic for the disease. Castrated male and female mice were injected with gonadotropin releasing hormone (GnRH) agonists and antagonists. GnRH antagonist administration significantly decreased total serum immunoglobulin G and anti-DNA antibodies in castrated male and female mice and significantly improved survival. In contrast, GnRH agonist administration exerted reciprocal effects in castrated mice, leading to early increases in serum anti-DNA and total immunoglobulin G levels.

In spontaneous abortion the most striking autoimmune association is with the presence of antiphospholipid antibodies, lupus anticoagulant and anticardiolipin (Silver 1994). The mechanism of pregnancy loss where these antibodies are present is decidual or placental insufficiency, related to the thrombotic tendency among these patients rather than an immunologic attack on the foetus. Some success in avoiding vascular abnormalities in these patients is achieved by treatment with corticosteroids and low dose aspirin (100 mg/day).

1.5.3 Thyroid Antibodies

The thyroid autoantibodies anti thyroglobulin (anti Tg) and anti thyroid peroxidase (anti TPO) have been implicated as important factors in miscarriage

by Stagnaro-Green et al (1990). In that study thyroid autoantibody positive women miscarried at a rate of 17% compared with 8.4% for thyroid autoantibody negative women. The presence of these autoantibodies may be useful clinical markers for increased risk of spontaneous abortion. The relationship between thyroid autoantibodies and abortion is thought to reflect a generalised activation of the immune system. Anti thyroid antibodies are organ specific, but are increasingly reported in similar distribution patterns to some of the most typical non-organ specific autoantibodies. A substantial number of patients with systemic lupus erythematosus (45%) have thyroid antibody abnormalities (Pratt 1992). Thyroid antibodies are also implicated in reproductive failure. They are found in at least one third of patients with autoimmune induced premature ovarian failure and it is therefore not surprising that they have now been associated with pregnancy loss.

1.5.4 HLA Antigens

Histocompatibility (HLA) antigens are proteins found on the cell surface. Every cell in the body of an individual will have the same HLA antigens on the surface, marking the cell as belonging to that individual. Two different individuals will have different HLA antigens. The immune system is sensitive to differences in histocompatibility antigens and will mount a rejection response against HLA antigens that are not self.

Sharing 2 or more HLA (A,B,DR,DQ) antigens was found to be significantly more frequent in recurrent spontaneous abortion couples than in controls (McIntyre et al 1983, MuellerEckhardt et al 1994). Other studies have failed to confirm the benefit of genetic heterogeneity to maintain human pregnancy

(Balasch 1989, Bellingard 1995) and testing of couples for HLA sharing is not recommended routinely in the evaluation of recurrent abortion (Coulam 1992).The foetus inherits half of its genetic material from the father and this has led to the hypothesis that increases in HLA compatibility between mother and foetus are in some way linked to a deficiency in the development of anti-foetal antibody during pregnancy. As a consequence, the foetus may be deprived of the protection by maternal blocking antibody, which may allow maternal cytotoxic reactions to cause abortion (Unander et al 1983).

1.6 Treatment Regimes for Abortion

Table 1.1 is reproduced from Scott (1994) and summarises his recommendations for an efficient and cost effective diagnostic workup in patients with recurrent miscarriage.

*Suggested Routine Evaluation for Recurrent Pregnancy Loss**	
History	
	Pattern and trimester of pregnancy losses and whether a live embryo or foetus was present
	Exposure to environmental toxins or drugs
	Known gynaecologic or obstetric infections
	Features associated with antiphospholipid syndrome
	Genetic relationship between partners
	Family history of recurrent miscarriage or syndrome associated with embryonic or foetal loss
	Gynaecologic or menstrual disorders
	Previous diagnostic tests and treatments
Physical	
	General physical examination—habitus, galactorrhea, hirsutism, stigmata of endocrine disorders
	Examination of vagina, cervix and uterus for normal discharge, findings suggestive of diethystilbestrol exposure or uterine abnormalities
Tests	
	Hypersalpingogram
	Luteal phase endometrial biopsy; repeat in next cycle if abnormal
	Parental karyotypes
	Lupus anticoagulant and anticardiolipin antibodies
	Other laboratory tests only if suggested by history and physical examination

*Ultrasound examination at 6 weeks' gestation in next pregnancy and chromosome analysis of products of conception from any subsequent miscarriage

Table 1.1. **Diagnostic workup in patients with recurrent miscarriage**

James R. Scott (1994)

Most embryos from first trimester spontaneous abortions are chromosomally or morphologically abnormal (Scott 1994). Treatable maternal or paternal factors are therefor not involved in most cases of spontaneous abortion. Despite the expense, Scott recommends parental chromosome analysis to complete a thorough evaluation for recurrent abortion. Treatment of parental chromosome abnormalities is limited to explanation, donor egg, donor sperm or adoption but advances in reproductive technologies may hold out hope for this problem in the future.

Recurrent miscarriage is a distressing condition, the causes of which are still not fully understood. As a result of this, only limited help is available for women with this condition. Current therapies on offer include the following:

1.6.1 hCG

Human Chorionic Gonadotrophin (hCG) Therapy

In many cases where tests have not given a conclusive reason for recurrent miscarriage a course of hCG is given from first confirmation of pregnancy until about fourteen weeks. Some centres (Quenby 1994) claim to have success rates of 86% where the patients suffered from oligomenorrhea (from Greek *oligo* meaning little), a reduction in the frequency of menstruation with cycles usually exceeding 40 days, compared to a 40% success for such patients taking placebo. HCG therapy for patients with normal periods had a success rate of around 85% but the placebo group also had 85% success. Others remain sceptical about the effectiveness of this treatment.

1.6.2 Immunotherapy

As indicated in chapter one, the concept of an allo-immune cause of recurrent abortion remains a contentious matter. Despite the lack of specific data to support the hypothesis of allo-immune abortion, many centres have attempted to immunise mothers with either paternal or third party leukocytes in the hope that this will induce the appropriate immune response and protect the foetus from rejection (Mowbray 1985, Ho 1991). A marginal benefit from immunotherapy has been reported but this must be weighed against the potential complications of the treatment. Hepatitis (infective and autoimmune), anaphylactic shock, virus transmission and transfusion reactions have all been reported (Mowbray 1994).

1.6.3 Corticosteroids, anti-coagulants and aspirin

Systemic lupus erythematosis is an autoimmune disease which causes joint pains and can affect internal organs. Ten percent of the 30,000 women in UK with lupus also have antiphospholipid syndrome (APS), a disorder which makes blood sticky (Lima 1996). Blood clots can form in the placenta of these women and destroy areas of the placenta, thus causing a miscarriage. It is now thought that antiphospholipid antibodies have an adverse effect on embryonic implantation (Rai 1996). Success with anticoagulant (heparin or warfarin), antiplatelet (dipyridamole) and low dose aspirin has caused some doctors to call for APS screening after one miscarriage.

Other centres have suggested a treatment regime of glucocorticosteroids, anticoagulants and platelet inhibitor therapy for patients with APS associated

with lupus anticoagulant and a poor obstetric history (Menashe 1993). The use of steroids in pregnancy, however, has been reported as being detrimental and associated with a significant excess of pre-term deliveries and pre-eclampsia (Cowchock 1992, Silver 1993).

1.6.4 Surgical Intervention

Cervical incompetence is cited as a cause for, mainly second trimester, abortion. Congenital malformations of the uterus are associated with recurrent pregnancy loss. After surgical correction there is a pregnancy success rate of about 70 to 90% (Scott 1989). Other workers believe that cervical incompetence is over-diagnosed and recommend careful evaluation of this syndrome before cervical cerclage is carried out (Rai 1996).

1.6.5 Psychological Support

As early as 1940 psychotherapy was deemed necessary as part of therapy to prevent repeated pregnancy loss (Javert 1940). This psychotherapy consisted of 'reassurance, the creation of a strong bond of confidence in and dependency on the physician and repeated suggestions that the pregnancy would succeed'. Post psychotherapy a miscarriage rate of 7.5% was reported, compared to a rate of 79% before therapy (Javert 1954). Other workers reported similar success using psychotherapy (Mann 1959). One study (Stray-Pederson 1988) evaluated the effect of 'tender loving care' in 116 women. Tender loving care was defined as psychologic support along with weekly medical and ultrasound examinations. Successful pregnancy outcome was reported in 85% of the women who received tender loving care compared to only 36% of women who did not participate in

the study.

Although none of these studies were properly designed to evaluate the role of supportive psychotherapy (Keye 1994), reports of patients treated in this way suggest that supportive therapy is important to reduce the anxiety and distress felt by these women. Special clinics, usually found in large teaching hospitals, dedicated to patients with recurrent pregnancy loss offer supportive therapy, as well as investigate the cause of miscarriage. Unfortunately there are not many clinics of this type in the UK.

Finally it is important for clinicians, when assessing the efficacy of treatment given to patients with recurrent abortion, to remember that even without treatment about 60% of these women will be successful in their next pregnancy (Regan). Control groups in trials of various treatments for recurrent pregnancy loss have varied from 29% to 76% (Coulam 1992).This spontaneous cure rate, while holding out hope for patients, can be an obstacle for workers looking for funding for research into recurrent abortion.

1.7 Normal Menstruation and Pregnancy

In order to study and understand what goes wrong in a pregnancy to result in miscarriage it is first necessary to look at normal pregnancy. What follows is a brief description of normal menstruation and pregnancy along with definitions used in the thesis (de Gruyter 1988).

1.7.1 The Menstrual Cycle

The menstrual cycle is the time from day one of menstruation till the day before the next period. The duration of the menstrual cycle is, on average 29.5 days, with large variations between women. However, individuals show some constancy in cycle duration. The cycle, which is under hormonal control, can be divided into four phases:

day 1-4	menstruation
day 5-12	follicular development
day 12-17	intermenstruum, ovulation occurs
day 17-28	luteal phase

Menstruation (from the Latin *menstruum* monthly flow) is the monthly shedding of the endometrium that occurs throughout the reproductive life from menarche (Greek menos month; arche beginning) to menopause. True menstruation occurs only when a corpus luteum has formed in an ovary in the previous cycle following ovulation. Where there has been no fertilisation and nidation (embedding of a fertilised ovum into the endometrium) the production of ovarian hormones from the degenerating corpus luteum decreases. The endometrium need no longer be maintained and is shed. This is the desquamation phase or menstruation *per se* (Figure 1.6).

Follicular development (Figure 1.7) . The follicle develops from a primary follicle 50μm in diameter to a mature Graaf's follicle which is 20mm in diameter. Follicle stimulating hormone (FSH) stimulates oestrogen production in the mature follicle of the ovary which itself stimulates the proliferation of the endometrium. There is a sharp rise in FSH and Luteinizing hormone (LH) on

approximately day 12 of the cycle. This can occur earlier or later and it is the length of time of this proliferation phase that determines the length of the cycle, the secretory phase which follows ovulation being fixed at 14 days (Ojeda 1992).

Ovulation. The mature Graaf's follicle ruptures with the expulsion of the mature ovum into the fallopian tube.

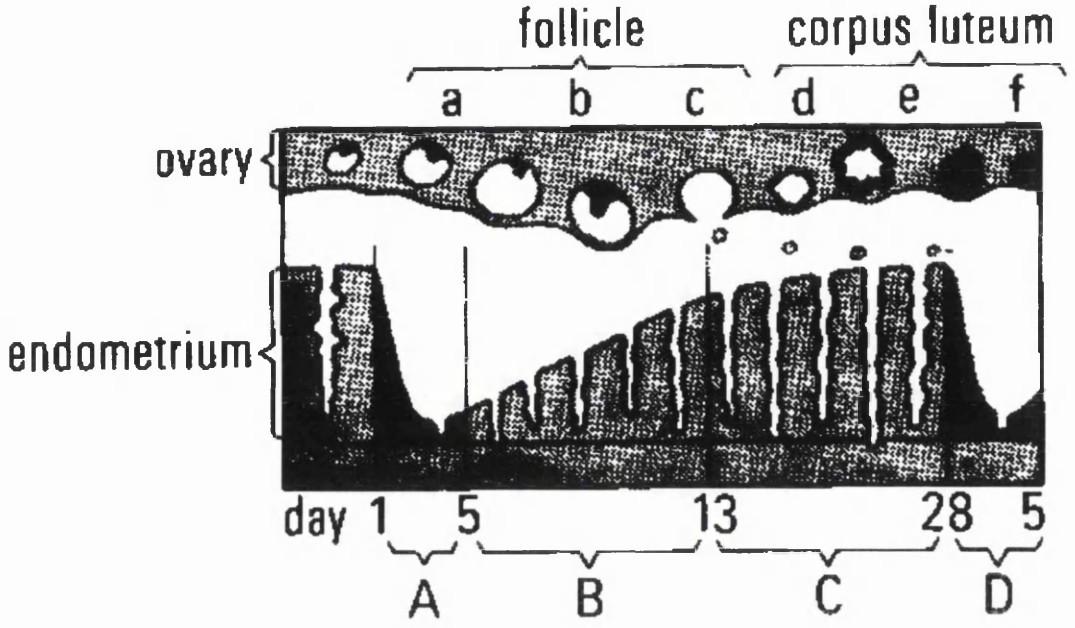


Figure 1.6 **Menstrual cycle**

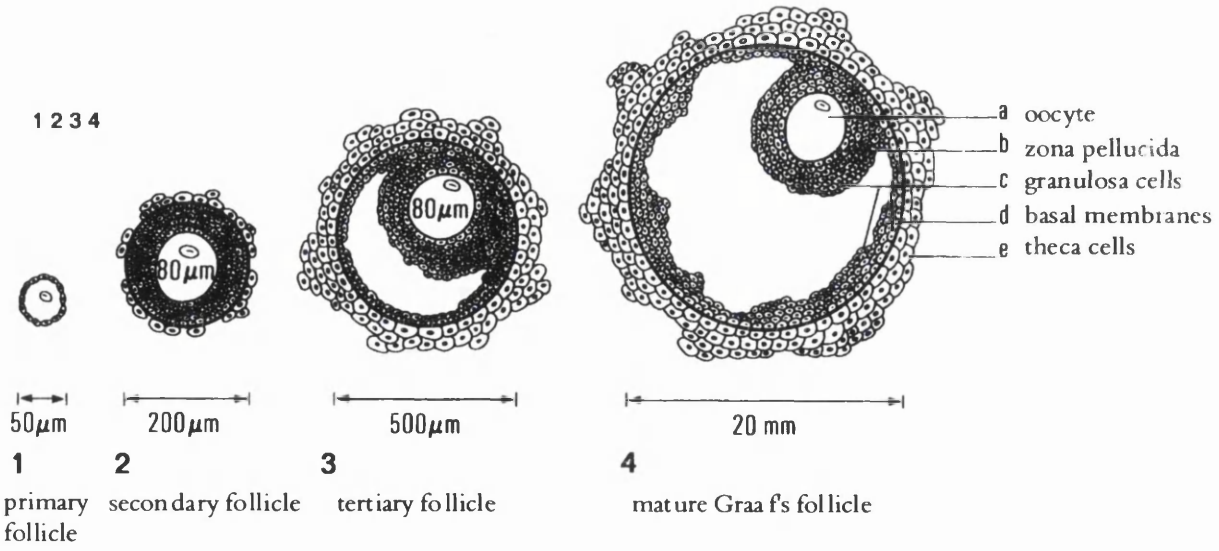


Figure 1.7 **Follicular development**

Luteal phase. Under the influence of LH, and later luteotropic hormone (LTH) (prolactin), the corpus luteum produces progesterone. Progesterone transforms the endometrium into the secretory phase. Levels of progesterone in blood rise from around 1,500pg/ml in the follicular phase to 3,000-20,000pg/ml in the luteal phase (Figure 1.8).

Follicle stimulating hormone (FSH). A gonadotropin produced by the anterior pituitary lobe (APL). Release from the APL is controlled by FSH-releasing hormone (this is probably identical to luteinizing hormone releasing hormone LH-RH). FSH promotes granulosa cell growth in Graaf's follicle, glycolysis and amino acid uptake in the ovary and plays a central role in the regulation of the menstrual cycle.

Luteinizing hormone (LH). A gonadotropin of molecular weight 28,000 which consists of two amino acid chains (a and b) the a-chain, being identical to the a-chains of HCG, FSH and TSH, contains 89 amino acids. The b-chain, which consists of 115 amino acids, is responsible for the biological action and also has an immunogenic action. LH induces follicular maturation and ovulation and brings about the development and function of the corpus luteum (and hence the synthesis of oestrogens and progesterone) Secretion of LH is controlled by luteinizing hormone releasing hormone (LH-RH), a decapeptide produced by the hypothalamus. LH-RH also stimulates the release of FSH.

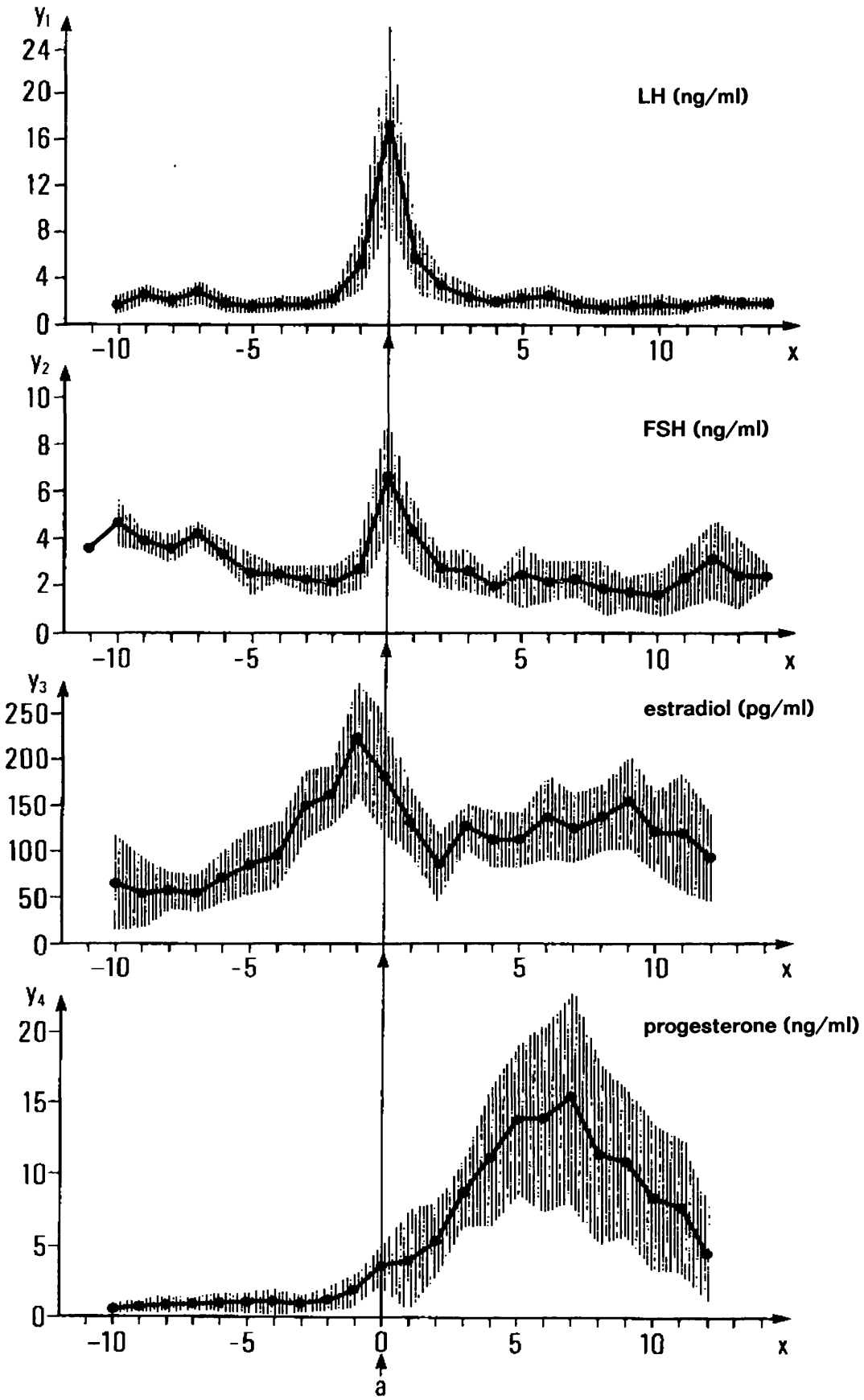


Figure 1.8 **Hormone levels in menstrual cycle**

1.7.2 Pregnancy

Pregnancy occurs as a result of the fertilisation of an ovum by sperm. With fertilisation cell division begins, and the fertilised egg develops into a mass of cells called the morula that move through the fallopian tube into the uterus. The morula continues to divide until it becomes a hollow clump of about a hundred cells called the blastocyst. About seven or eight days after fertilisation, the blastocyst settles on the wall of the uterus (nidation). Some of the cells covering the blastocyst, known as the trophoblast, begin to eat into the lining of the uterus and anchor the blastocyst to the wall of the uterus (Figure 1.9). The trophoblast eventually develops into the placenta.

The blastocyst is composed of two layers, the upper, ectoderm layer, and the lower, endoderm layer. An amniotic cavity and yolk sac soon appear in the blastocyst. The amnion lines the chorion, the outermost envelope that furnishes a protective and nutritive covering for the zygote, as the fertilised egg is now called. The embryonic disk, a flat area in the cleaved ovum in which the first traces of the embryo are seen, is suspended from the chorion and is composed of three germ layers, the ectoderm, mesoderm, and endoderm. All organs of the embryo are developed from these three germ layers.

From the ectoderm the nervous system, sense organs, and epidermis, among others, are developed. The circulatory, excretory, skeletal, muscular, and reproductive systems are developed from the mesoderm. The endoderm produces the respiratory and digestive systems, along with their linings.

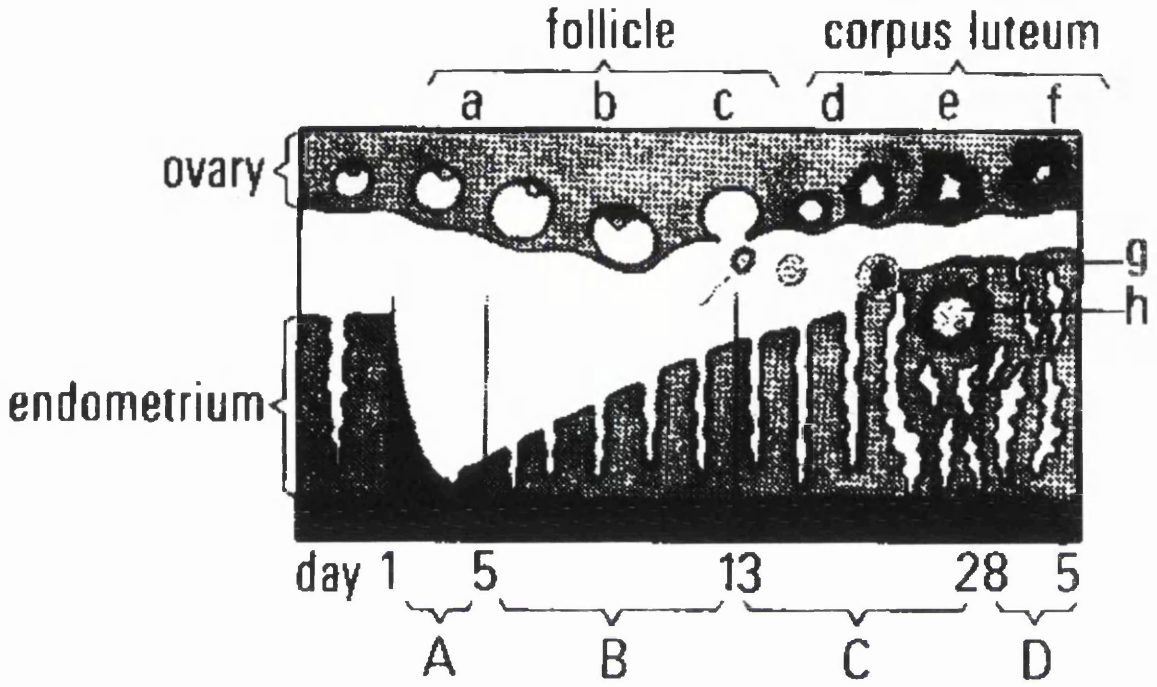


Figure 1.9 **Pregnancy**

On average, a normal pregnancy lasts for 38 weeks from conception till birth. As conception occurs midway through the menstrual cycle, just after ovulation, and on a date that is not known exactly, doctors calculate pregnancy from the first day of the last menstrual period. Therefore the average pregnancy is said to last 40 weeks. Pregnancy is divided into three, approximately equal, trimesters. It is in the first trimester, when the various organs of the foetus are formed, that miscarriage is most likely to occur. During the second and third trimesters the foetus develops and matures until full term i.e. between 38 and 42 weeks.

Parity. This is defined in the Concise Oxford Dictionary as the fact or condition of having borne children, or the number of children previously borne. A primigravida is a woman who is pregnant for the first time. Doctors represent this as $0+^{0}$ i.e. zero (0) live births and zero (^{0}) pregnancies that did not continue beyond 28 weeks. $3+^{1}$ represents a multigravid woman who has borne three children and has also had one miscarriage.

1.8 Free Radicals, Antioxidants and Reactive Oxygen Species

Free radicals have recently been implicated as having a role in spontaneous abortion (Sane 1991).

A free radical is any chemical species (atom or molecule) capable of independent existence that contains one or more unpaired electrons in their outermost orbitals. Electrons are more stable when paired together in orbitals and therefore radicals are, on the whole, more reactive than non-radical species (Halliwell 1989). When two radicals meet they can combine their unpaired electrons and

join to form a covalent bond. An unpaired electron is represented as (*).

$$A^{\star} + A^{\star} \rightarrow A\text{-}A$$

A radical might donate its unpaired electron to another molecule or steal from another in order to pair. However in doing this the other molecule itself becomes a radical, and so a feature of the reactions of free radicals is that they proceed as chain reactions.

Free radical mechanisms can have both beneficial and detrimental actions *in vivo*. Such mechanisms play a fundamental role in many physiological reactions such as mitochondrial oxidation, oxygen transportation by haemoglobin and cytochrome P450 activity.

At a cellular level the innate high reactivity of free radicals can cause cellular oxidative damage. When tissue is exposed to high level radiation, such as gamma radiation, most of the energy taken up is absorbed by the cell water. The radiation causes one of the oxygen-hydrogen covalent bonds in water to split, leaving a single electron on hydrogen and one on oxygen, thus creating two radicals.

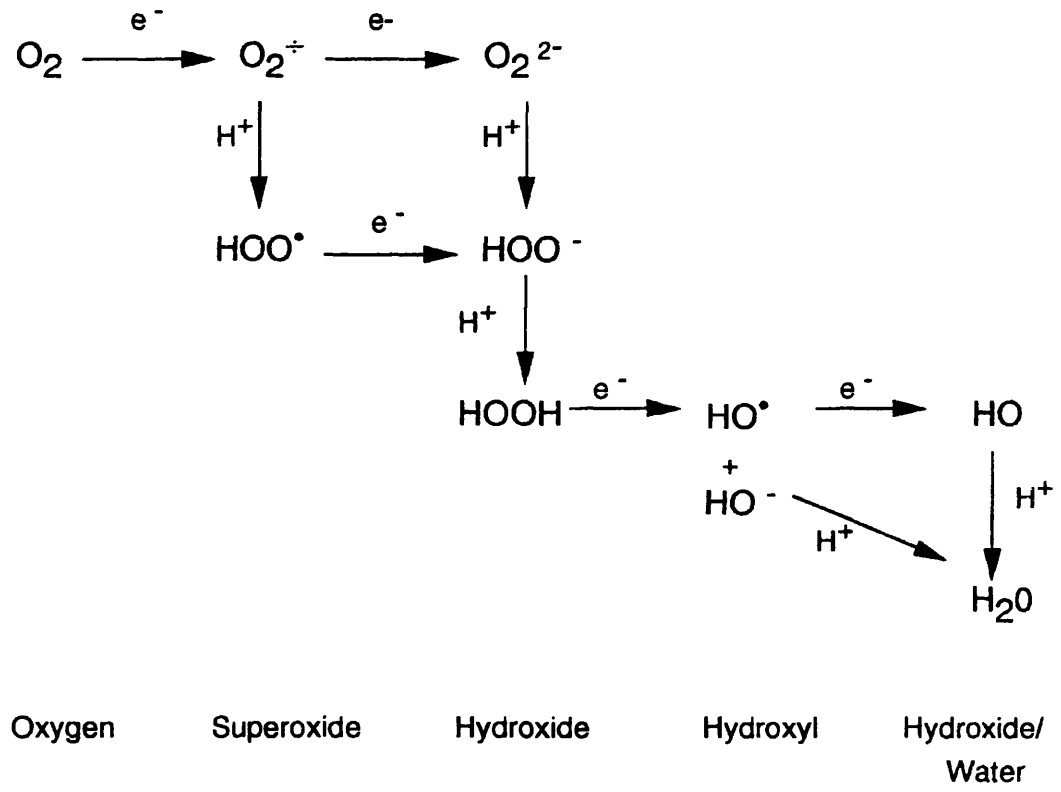
$$H\text{-}O\text{-}H + \text{radiation} \rightarrow H^{\star} + {}^{\star}OH$$

$H^{\star}$ is the hydrogen radical, $^{\star}OH$ is the hydroxyl radical.

Oxygen, which has two unpaired electrons in separate orbitals acts as a free radical. Oxygen is required to transform various substances for the release of energy, to oxidise endogenous compounds and to detoxify xenobiotics (compounds not occurring in nature, especially synthetic organic compounds

that are recalcitrant to biodegeneration) (Sinclair 1990). Oxygen acts as a terminal 4-electron acceptor and is eventually converted to water. The reduction of oxygen is frequently incomplete and a series of reactive chemical intermediates are produced. These intermediates are hydrogen peroxide, the superoxide radical ($O_2^{\star}$) and the hydroxyl radical ($OH^{\star}$) (figure 1.10).

The hydroxyl radical is the most reactive radical species known to chemistry and can attack and damage almost every molecule found in living cells. Polyunsaturated fatty acids, proteins, DNA and carbohydrates are all susceptible to free radical damage particularly from the hydroxyl radical.



From Sinclair et al, 1990.

Figure 1.10 **Electron reduction of oxygen**

Lipid peroxidation is a free radical chain reaction stimulated by the hydroxyl radical (*OH). This occurs when the *OH is generated close to cell membranes and attacks the fatty acid side chains of the membrane phospholipids. The *OH pulls off an atom of hydrogen from one of the carbon atoms in the side chain and combines to form water.

Lipid peroxidation causes a reduction in membrane fluidity and permeability. The reactions can be halted by the action of free radical-scavenging molecules such as vitamin E or vitamin C. These molecules produce more stable radical derivatives (Porter 1984). The chain reactions which result from lipid peroxidation can cause injuries at sites distant from the original site of free radical generation.

When the hydroxyl radical attacks DNA, free radical chain reactions propagate through the DNA and cause chemical alterations of the bases or deoxyribose sugars. This can lead to mutations and strand breakage. Imperfect repair of such damage can lead to oncogene activation and carcinogenesis. Paradoxically, hydroxyl radical generation is the major mechanism by which malignant cells are destroyed during radiotherapy.

Mammalian DNA has evolved in such a way as to offer protection from free radical damage. Nuclear DNA is compartmentalised away from mitochondria and peroxisomes where most radicals are probably generated and most non-replicating nuclear DNA is surrounded by histones which may have a protective role against free radical attack.

Free radical induced damage to proteins may result in fragmentation, cross-linking, aggregation of protein and the induction of autofluorescence. This

damage can be manifested by an inactivation of certain enzymes or indeed activation of enzymes by inactivation of the relevant inhibitor. Extracellular proteins with a large proportion of disulphide bridges such as IgG or albumin appear to be particularly susceptible to hydroxyl and peroxy radical attack.

Free radical production is required for host protection from bacterial invasion, but once formed free radicals can cause tissue damage (McCord 1985). Production of free radicals may be increased during various disease states such as inflammation and during metabolic conditions such as hypoxia and tissue ischaemia (Dormandy 1983).

1.8.1 Sources of free radicals in biological systems

Table 1.2 shows the two main sources of free radicals: cellular and environmental.

Source		**Examples**
Cellular metabolism	Prostaglandin synthesis	
	Mitochondrial electron transport	
	Endoplasmic reticulum oxidation	
	Enzymic activity	NADPH oxidase
		Xanthine oxidase
	Oxyhaemoglobin	
	Auto-oxidation	Adrenalin
		Melanin thiols
		Reduced riboflavin
		Flavin
		$FMNH_2$
		Flavin adenine dinucleotide
		$FADH_2$
Environmental	Drugs	Halothane
		Paracetamol
	Cytotoxics	Bleomycin
		Doxorubicin
	Pesticides	Paraquat
	Photochemical air pollutants	
	Tobacco smoke	
	Radiation	X-ray water radiolysis
		Light Photoionisation
		Photoexcitation of organic molecules

Table 1.2. **Sources of Free Radicals in Biological Systems**

1.8.2 Anti-oxidant systems

To protect themselves from the effects of free radicals aerobic organisms have developed antioxidant systems. These take the form of enzymes such as superoxide dismutase (SOD) and low molecular weight free radical scavengers such as vitamin E. These limit the cellular concentrations of free radicals and prevent excessive oxidative damage (oxidative stress).

1.8.3 Detection of free radicals

Free radicals are highly reactive and consequently have a life span of microseconds or less and as such can only be detected *in flagrante* by the use of electron-spin resonance (ESR) spectroscopy (Dormandy 1989). An electron occupying an orbital by itself can choose its direction of spin. ESR measures the energy changes that occur when they change their direction of spin.

Detection of free radicals by an indirect approach is more common in the biological laboratory. While free radicals themselves are undetectable, some products of free radical mediated reactions are relatively stable. These products serve as markers of free radical activity.

1.8.4 Superoxide Dismutase

The enzyme superoxide dismutase was discovered in 1938 (Mann and Klein), though its biological activity as a catalyst of dismutation was not described until 1968 (McCord & Fridovich). They demonstrated that erythrocyprein (superoxide dismutase) catalysed the dismutation of superoxide

radicals generated during the reduction of cytochrome C.

$$2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$$

Misra and Fridovich developed an assay based on the photo-oxidation of dianisidine sensitized by riboflavin. This involved a complex series of chain reactions with the SOD ion as a chain propagating species. They proposed that the mechanism of photo-oxidation of O-dianisidine involves the following reactions;

$$Rb + h\nu \rightarrow Rb^{\star} \qquad (a)$$

$$Rb^{\star} + DH_2 \rightarrow RbH^{\bullet} + DH^{\bullet} \qquad (b)$$

$$RbH^{\bullet} + O_2 \rightarrow Rb + O_2^- + H^+ \qquad (c)$$

$$DH^{\bullet} + O_2^- + H^+ \rightarrow DH_2 + O_2 \qquad (d)$$

$$DH^{\bullet} + DH^{\bullet} \rightarrow \underline{D} + DH_2 \qquad (e)$$

$$O_2^- + O_2^- + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2 \qquad (f)$$

DH_2 is O-dianisidine Rb is Riboflavin

D is the product formed by photo-oxidation and is measured at 460nm.

In reaction (a) riboflavin absorbs a photon and becomes electronically excited. In reaction (b) the excited riboflavin oxidises dianisidine, yielding a flavin semiquinone and a dianisidine radical which, in the absence of competing reactions, would dismute as in reaction (e) to yield oxidised dianisidine which absorbs at 460nm. However, the flavin semiquinone produced (in reaction b) can reduce O_2 to O_2^- (reaction c) and the O_2^- can in turn reduce the dianisidine radical (reaction d). SOD which scavenges the O_2^- (reaction f) would thus

prevent the reaction (d) relative to reaction (e). Thus in the presence of SOD an increase in absorbance will be observed.

1.8.5 Caeruloplasmin

Caeruloplasmin (Cp) is a blue copper protein that accounts for 90% of total plasma copper. Copper is incorporated into caeruloplasmin at the time of synthesis in the liver. There are 6 or 7 copper atoms bound to each molecule of Cp. Cp has a small thiol (SH) titre of less than 0.5 SH / mole but extra thiol (1 to 4 groups per mole, depending on what technique is used) appears on removing the copper from the reduced protein using chelating agents, suggesting that the metal is bound to thiol groups. Cp oxidises Fe^{2+} to Fe^{3+} which can bind to transferrin (Dormandy 1978). This has the effect of inhibiting iron-dependant and copper-dependant lipid peroxidation.

1.9 Aims of study

The aims of this study were:

1. To evaluate free radical scavenger levels in different phases of the normal menstrual cycle and compared to levels in males.

Interest in the role of free radicals in inflammation and tissue destruction has increased in recent years. One aim of this study was to see if free radical scavenger levels, and therefore free radical levels, altered at different phases of the normal menstrual cycle or if indeed free radical levels in males differed to those of non pregnant women measured at various phases of their menstrual cycle. Measured in the study were two extracellular (plasma thiols and

caeruloplasmin) and two intracellular (lysate thiol and superoxide dismutase) components of the antioxidant system.

2. To investigate some immunological aspects of miscarriage:

Free radical scavengers, thyroid antibodies, mitogen stimulation and antibody production by lymphocytes and cytokine levels.

The study examined whether free radical scavenger levels in women admitted to hospital undergoing, or following, a spontaneous abortion differed from those in healthy pregnant women.

As discussed earlier, the anti thyroid antibodies, anti-thyroglobulin and anti-thyroid peroxidase, have been identified as useful clinical markers for increased risk of spontaneous abortion by Stagnaro-Green et al (1990). For this study samples were assayed to detect thyroid antibody levels in normal pregnant and aborting women to see if a relationship could be found between antibody levels and pregnancy loss.

Other immunologic characteristics of miscarriage were looked at for this study. Mitogen stimulation and antibody production of lymphocytes in tissue culture and estimation of cytokine levels in sera from pregnant, non pregnant, spontaneous aborters and recurrent aborters were assayed. The aim was to form an overall impression of the immunological changes occurring in pregnancy and abortion and see if particular immunological abnormalities are associated with abortion.

Chapter Two

Methods

2.1 Stimulation of Lymphocytes

2.1.1 Tissue culture reagents

Foetal calf serum was supplied by Northumbria Biologicals Ltd., Cramlington, UK. The same batch of serum was used throughout the study.

Phosphate buffered saline (PBS) tablets from Oxoid Ltd., UK were reconstituted according to manufacturers instructions.

Lymphoprep was obtained from Nyegaard, Oslo Norway.

Thymidine was supplied by Gibco BRL Ltd., UK.

Stock thymidine solution. A 5 mM solution was prepeared by dissolving 121mg of thymidine in 100ml PBS This was filter sterilised and stored at 4°C.

^{3}H thymidine (TRK 418, specific activity 40-60 Ci/mM) was supplied by Amersham International plc, UK.

Working ^{3}H thymidine solution. This was prepared by mixing 500 μCi ^{3}H thymidine (0.5 ml of 1mCi/ml) with 0.1 ml of stock thymidine solution and made up to 10 ml in RPMI.

RPMI 1640 from Gibco BRL, UK was supplied at 10x concentration and was diluted prior to use as follows:

Sterile distilled water (431.5 ml).

RPMI 1640 10X (50ml).

Sodium bicarbonate (13.5ml).

Penicillin (10,000 U/ml) and Streptomycin (10mg/ml) (5ml).

L-glutamine 200mM from Gibco BRL Cat No 25030 (5ml).

NaOH solution (6M) was added dropwise to bring to neutral pH.

White cell diluting fluid. A few crystals of crystal violet were added to 1% acetic acid in PBS.

Phytohaemagglutinin (PHA). Sigma Cat. no. L9132. A whole vial (5mg) was resuspended in 5 ml of sterile distilled water (1000 μg/ml). This was stored at -20°C in 50 x 100 μl aliquots. Before use one aliquot was thawed and made up to 10 ml in RPMI to give a working dilution of 10 μg/ml.

Concanavalin A (Con A). Sigma Cat no. C2010. A whole vial (25mg) was resuspended in 5 ml of sterile distilled water (5 mg/ml). This was stored at -20°C in 50 x 100 μl aliquots. Before use one aliquot was thawed and made up to 5 ml in RPMI to give a working dilution of 100 μg/ml.

Pokeweed mitogen (PWM) for the mitogen stimulation assay. Sigma Cat. no. L9379. A whole vial (5mg) was suspended in 100 ml of RPMI (50 μg/ml). This was stored at -20°C in 20 x 5ml aliquots. One 5ml aliquot was divided into 50 small aliquots of 100 μl each. These were also stored at -20°C. Before use one small aliquot was thawed and made up to 25 ml in RPMI to give a working dilution of 0.2 μg/ml.

Pokeweed mitogen for the B-cell stimulation assay. This was obtained from Gibco BRL, Life technologies Ltd., PO Box 35, Trident House, Renfrew Rd., Paisley PA3 4EF Cat. no. 061-05360B. This was resuspended in sterile PBS and stored at -20°C in aliquots of 220μl. Prior to use one aliquot was thawed and 200μl was added to 9.8 ml of RPMI. This gives a 1:50 dilution. An aliquot (3ml) of this was

added to 12 ml of RPMI to give a working dilution of 1:250.

2.1.2 Lymphocyte separation

Details of this method are described in Boyum A. (1968) (Figure 2.1). Heparinised venous blood was aseptically added to an equal volume of RPMI 1640. Diluted blood (5ml) was floated onto Lymphoprep (5ml) in sterile 15ml plastic conical tubes. The tubes were spun at 1200 RPM for 30 minutes. The lymphocytes settle in a dense white band in the centre of the tube. These were removed with a Pasteur pipette and washed three times with PBS. After the last wash the pellet of cells was resuspended in 2 ml RPMI. To count the cells 10µl of the suspension was diluted 1/10 in white cell diluting fluid and counted on a haemocytometer. The number of cells per ml was calculated and then diluted in RPMI 1640 to give final working dilutions of 10^6 per ml for phytohaemagglutinin and concanavalin A and $2x10^6$ per ml for pokeweed mitogen.

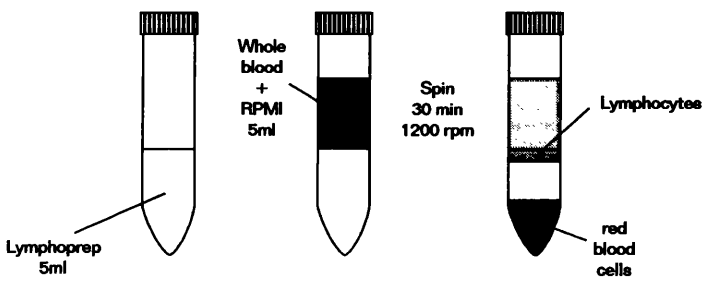


Figure 2.1. **Ficoll-Hypaque separation of lymphocytes**

2.1.3 Mitogen stimulation

To minimise variation, all mitogens used were taken from the same batch throughout the study, and were prepared as described above. Assays were carried out in 96-well sterile plates (Greiner UK Ltd.).

RPMI (100µl) for a control (C) and 100 µl of mitogens diluted as described previously were added to appropriate wells. Cells were made up in RPMI containing 20% human serum to a concentration of 10^6/ml for C, PHA and Con A and at 2 x 10^6/ml for PWM. Cell suspensions (100µl) were added to appropriate wells.

Plates were incubated for 68 hours at 37°C in a humidified atmosphere of 5 % CO_2 / air. After the incubation 20 µl of ^3H thymidine (made up as previously described) was added to each well. The plate was then returned to the incubator for 4 hours. After the incubation the contents of the plates were harvested using a Packard Micromate 196 harvester. The harvester removed cells by a vacuum onto disks on fibre filter mats (Packard). Unbound ^3H passed through into a reservoir. The filters were washed, then dried in a microwave oven for 2 minutes before being counted in a Packard Matrix 96 direct beta counter. Triplicate means were calculated and results were expressed as a stimulation index (SI) ie

$$\text{SI (PHA)} = \frac{\text{total counts PHA - total counts control}}{\text{total counts control}}$$

$$\text{SI (Con A)} = \frac{\text{total counts Con A - total counts control}}{\text{total counts control}}$$

$$\text{SI (PWM)} = \frac{\text{total counts PWM - total counts control}}{\text{total counts control}}$$

2.1.4 Immunoglobulin production

This method is described in Wilson (1988). Assays were carried out in 24-well sterile plates from Greiner Ltd. All cultures were set up in duplicate and there was a total volume of 2ml per well. Control wells contained 1ml cells at a concentration of 10^6 per ml and 1ml of 20% foetal calf serum (FCS) in RPMI (ie a total cell concentration of $5x10^5$ per ml). Pokeweed mitogen at a concentration of 1:250 was added to test wells in duplicate, the concentration of FCS and cell numbers being the same as in the control wells. The controls were set up to account for spontaneous release of immunoglobulins. The plate was incubated for 7 days at 37°C in a humidified atmosphere of 5 % CO_2 / Air. After incubation the media were pipetted off and the duplicates pooled in 15ml conical tubes. The tubes were spun at 700G for ten minutes, after which the supernatants were taken off and stored in a -20°C freezer until they were assayed.

2.1.5 Immunoglobulin ELISA reagents

IgG and /IgM standards. Frozen aliquots of standard were stored at -70°C and thawed immediately prior to use. 8 μl of standard in 20ml of diluting buffer gave a concentration of 400 ng/ml. From this serial dilutions were made to give standards of 200, 150, 100, 75, 50, 25 and 10 ng/ml.

Coating buffer. This was prepared by mixing 2.2mls 0.2M Na_2CO_3 solution (2.12g/100ml) with 2.8mls 0.2M $NaHCO_3$ (1.68g/100ml) and making up to 100mls with deionised water.

Anti-Human IgG (g-chain specific). Sigma Cat. No. I-7883

Anti-Human IgM (m-chain specific). Sigma Cat. No. I-8633.

Immunoglobulin standards. IgG (Cat. No. I-4506) and IgM (Cat. No. I-8260) were obtained from from Sigma Ltd.

Alkaline Phosphatase Conjugate. Anti-human IgG (Cat. No. A-3150) and Anti-human IgM (Cat. No. A-3275) were obtained from Sigma Ltd.

Substrate buffer. This was prepared by mixing 2.2mls 0.2M Na_2CO_3 solution(2.12g/100ml), 2.8mls 0.2M $NaHCO_3$ solution(1.68g/100ml) and 2.0mls 0.01M MgCl2 solution(0.95g/100ml) and making up to 20mls with deionised water.

Diluting (blocking) buffer. This was prepared by dissolving bovine serum albumin (5g) and sodium azide (0.5g) in 1l of PBS.

Washing buffer. This was prepared by dissolving bovine serum albumin (5g),

sodium azide (0.5g) and 0.5ml Triton X100 (or Tween 20) in 1l of PBS.

Quality control material. Using samples from previous studies, a pool of supernatants with high immunoglobulin content was made.

2.1.6 ELISA for estimation of IgG and IgM

This method was described by Wilson et al (1989). The inter assay coefficient of variation was calculated for this study by running QC samples with each assay and was found to be 14.6% for IgG and 22.4% for IgM.

Pre-coating Plates. Flexible plates (96-well) from Greiner were used. Goat anti-human IgG or IgM (200μl) (10μl/10ml coating buffer) was added to each well with two wells left empty to estimate non specific binding. The plate was incubated at 37°C for 1 hour, then washed 4 times with wash buffer. After blotting the plate dry, 200μl blocking buffer was added to each well, and the plate stored at 4°C. The plate was stored at this stage for up to 2 months.

Standards (100μl), quality control samples and samples were added in duplicate to designated wells. An aliquot (100μl) of the highest concentration standard (400ng/ml) was added to the non specific binding well. The plate was incubated for 2 hours at 37°C. After the incubation the plate was washed 4 times and blotted dry on paper towel.

Anti-human IgG/IgM (10 μl) conjugate (0.6 mg conjugate/ml) was diluted in 10ml of diluting buffer. of this Diluted conjugate (100 μl) was added to each well. The plate was incubated for 2 hours at 37°C. After the incubation the plate was washed 4 times and blotted dry on paper towel.

P-nitrophenyl phosphate (PNP) substrate (100 μl) (Sigma) (1mg/ml in substrate buffer) was added to each well. The plate was incubated for 30 minutes at 37°C. After the incubation the reaction was stopped by the addition of 100 μl of 3M NaOH to each well.

Optical density was measured at 410 nm on a Dynatech MR700 microplate reader. The plate was zeroed using the non-specific binding well.

A calibration curve was constructed by plotting optical density of at 410 nm against standard concentration. Test samples were then read off from this curve.

For quality control purposes aliquots from the QC pool were assayed 20 times to estimate intra-assay variation. The pool of samples was then split into aliquots of 300μl and stored at -20°C. These were used as QC samples and were run with each assay. A QC profile was made up and results were only accepted if QC was within 2 standard deviations of the mean.

2.2 Free Radical Estimations

Free radical methods were based on those described by Ellman (1959), Menden (1977) and Misra (1977)

2.2.1 Reagents

PBS was prepared from tablets supplied by Sigma and kept at 4°C.

Chloroform:ethanol 3:5. A mixture of chloroform (90ml) and ethanol (150ml) was prepared and stored at -20°C.

Sodium phosphate buffer (0.1M). This was prepared as follows:

Two solutions were prepared:

Solution A. 8.89g Na_2HPO_4 .$2H_2O$ in 500mls deionised water (pH 9.5).

Solution B. 7.80g NaH_2PO_4 .$2H_2O$ in 500mls deionised water (pH 4.5).

All of solution A was used with solution B added dropwise until the pH was 7.6. The buffer was stored at 4°C for up to 2 weeks.

DTNB (0.01M). A 0.01M solution of 5,5' dithiobis-(2-nitrobenzoic acid)(DTNB) in 0.1M Na_2HPO_4 at pH 7.6 was made up and kept on ice and used within 1 hour of preparation.

Sodium acetate buffer (1.195 M) for Caeruloplasmin assay. Sodium acetate (24.5g) was dissolved in 250 ml H_2O and the pH was adjusted to 5.4 by the addition of glacial acetic acid. This buffer was stored for up to 2 weeks at 4°C.

p-Phenylenediamine (PPD) solution (9.25mM) for Caeruloplasmin assay. p-Phenylenediamine (0.050g) was dissolved in 50mls sodium acetate buffer in a beaker covered in tin foil to protect the solution from light. The solution was kept on ice and used within 1 hour of preparation.

Sodium azide solution (3mM) for Caeruloplasmin assay. Sodium azide (0.0487g) was dissolved in 250 mls of distilled H_2O. This solution was freshly prepared for each assay and was kept on ice.

0.01M potassium phosphate buffer pH 7.5. This was prepared as follows:

Solution A: KH_2PO_4 (molecular weight 136.1) 0.68g/500ml

Solution B: K_2HPO_4 (molecular weight 174.2) 0.87g/500ml

All of solution B (the alkali salt) was used and solution A was added dropwise to bring down the pH to 7.5.

Riboflavin solution $1.3x10^{-5}$. This solution was made up freshly for each assay and as riboflavin is light sensitive, was kept in a beaker covered with aluminium foil. Because of the small amount of chemical to be weighed, this solution was made up at 10x strength then diluted ie riboflavin (0.005g) was made up in 100mls of phosphate buffer then 10 mls of this was made up to 100mls in phosphate buffer.

O-dianisidine solution (0.01M). This solution was made up freshly for each assay. O-dianisidine (0.061g) was dissolved (after a lot of mixing) in ethanol (25mls) and kept in a universal container covered in foil.

Superoxide dismutase standards. Superoxide dismutase from Sigma Product number S2515, activity 3,000 units per mg. This was kept in -20°C freezer. SOD was reconstituted in phosphate buffer and a new set of standards were made up every four weeks. These were kept at 4°C.

Plasma and lysate Quality Control materials. This was prepared from pools of lysate or plasma samples and stored in small aliquots at -70°C. A QC sample was run with each assay.

2.2.2 Specimen preparation

Heparinised blood (10 ml) was stored at 4°C for a maximum of 24 hours before assay. The blood was spun for 10 minutes at 3000 rpm and 4°C. The plasma was removed and stored at 4°C. The packed red cells were washed twice

with phosphate buffered saline. Washed red cells (1 ml) were incubated with an equal volume of distilled H_2O for 2 hours at 4°C. Haemoglobin was precipitated out of these lysed cells by the addition of 800 μl of chloroform : ethanol 3:5 (v/v) per 2 ml lysed cell solution. This produced a brick red paste which was kept on ice for 10 minutes and mixed occasionally by stirring with a glass rod. After the incubation 3 drops of chloroform : ethanol and 300 μl distilled H_2O were added, and again the paste was mixed with the glass rod. The sample was spun for 10 minutes at 3000 rpm and 4°C. The pale yellow/clear supernatant was removed and lysate thiol and superoxide dismutase activity were assessed.

2.2.3 Plasma and lysate thiol levels

Thiol levels in plasma (PSH) and lysate (LSH) were determined by the method of Ellman (1959). The basis for the analysis is the thiol disulphide interchange reaction between 5,5' dithiobis-(2-nitrobenzoic acid) (ESSE) and biological thiols.

$$ESSE + RSH \leftrightarrow ESSR + ESH$$

At pH 7.6 ESH is ionised to ES^-, a colourless species with bond maximum at 412 nm which can be used to determine thiol concentration.

An array of tubes was set up in a rack at room temperature. The assay is summarised in the table 2.1. All samples were assayed in duplicate.

		Na_2HPO_4 buffer (mls)	Sample (plasma/lysate) (μl)	DTNB (μl)
A	blank	3.0		
B	reagent blank	2.5		500
C	plasma blank	2.8	200	
D	plasma test	2.3	200	500
E	lysate blank	2.8	200	
F	lysate test	2.3	200	500

Table 2.1. **Summary of thiol determination in plasma and lysate**

The DTNB buffer was added to tubes at timed intervals (30 seconds) and absorbance was measured (440nm) against respective blanks after 5 minutes. The value for the reagent blank was subtracted from the sample test values.

PSH Intra assay precision (cv = 1.2%) was calculated by running 20 samples of plasma from the same heparinised blood sample in one assay.

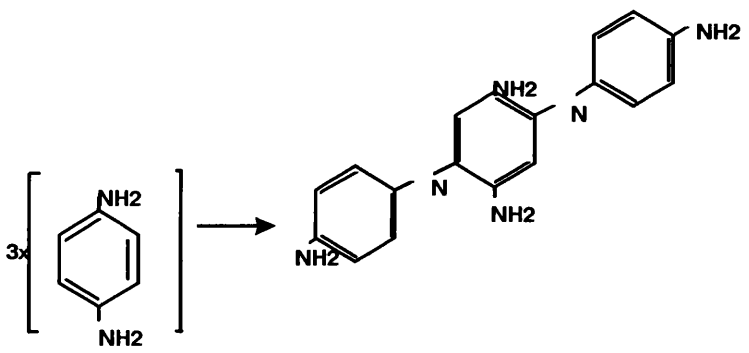
LSH Intra assay precision (cv = 16.7%) was calculated by assaying 20 different lysates from one heparinised blood sample in one batch.

LSH Inter assay precision (cv = 7.9%) was calculated by assaying aliquots of lysate made for QC sample with each assay.

2.2.4 Plasma Caeruloplasmin Assay

Caeruloplasmin oxidase activity was measured by the method of Menden (1977). The method is based on the caeruloplasmin catalysed oxidation

of p-phenylenediamine to Bandrowski's base (figure 2.2).



p-phenylenediamine Bandrowski's Base

Figure 2.2. **Bandrowski's base**

Plasma was separated from whole blood in lithium heparin bottle. This was kept at 4°C for a maximum of 7 days prior to assay.

Plastic conical tubes (15ml) were set up in a rack on ice. Three tubes per sample were labelled, one for a blank and two test samples. Plasma samples were removed from the fridge prior to assay and allowed to reach room temperature. Plasma (50μl) was added to each tube then sodium azide solution (5ml) was added to blank tubes only. P-phenylenediamine solution (1ml) was then added to all tubes and the rack was shaken by hand before being incubated in a shaking water bath at 37°C. The rack was taken out of the bath after exactly 15 minutes and placed on ice. Sodium azide solution (5ml) was added to all test samples (not the blanks) to stop the reaction. The rack was dipped in the water bath to allow the tubes to return to room temperature. The absorbance of each test sample was read against its blank at 525nm on a Pye Unicam SP8-400 spectrophotometer. This measurement of Bandrowski's base at 525nm serves as the basis for defining the molecular activity of caeruloplasmin. Activity was converted to concentration

by referring to a calibration curve using known concentrations of purified human caeruloplasmin. This curve cuts the x and y axes at zero, the gradient of the line is $y = 0.00526\,x$.

Results are expressed as units mg/100ml.

The intra assay %cv was calculated by running 20 plasma samples from the same whole blood in one assay and was found to be 3.8%. The inter assay %cv was calculated by assaying an aliquot of frozen pooled plasma and was found to be 22.5%. Previous studies stated that Cp was only stable for 7 days at 4°C and this high inter assay %cv may be indicative of Cp instability.

2.2.5 Lysate Superoxide Dismutase assay

This method is based on the increase in rate of photo-oxidation of O-dianisidine sensitized by riboflavin and was described by Mizra and Fridovitch (1977). The measurement was carried out on lysate samples prepared as described above.

A specially constructed light box was required for this assay. This consisted of a wooden, foil lined box with a door. Inside the box were two Philips fluorescent bulbs (8w) placed 6" apart. In the centre was an upturned beaker onto which cuvettes were placed. The box was 38cm high and had a base measuring 648cm^2.

On the day of the assay the samples and standards were removed from the fridge and allowed to reach room temperature. The spectrophotometer (Pye Unicam SP8-400) was switched on and the wavelength adjusted to 460nm. Quartz cuvettes (3ml) were covered with foil and the light box was switched on.

Riboflavin solution was added to each of 4 cuvettes (2.94ml for first cuvette (blank) and 2.88ml for the rest). Standard or lysate sample (60μl) was added to all but the blank cuvette. O-dianisidine solution (60μl) was added to all cuvettes each now containing a total volume of 3mls. The cuvettes were inverted to mix the sample, then the foil was removed and the absorbance of each was measured at 460nm. The samples were illuminated for exactly 4 minutes each then removed from the light box and the absorbance was again read at 460nm. This procedure was continued, in groups of 4 cuvettes at a time till all standards and samples had been assayed.

The difference in absorption between illuminated and baseline samples was calculated and a standard curve was plotted. Unknown samples were read off from this plot. A quality control sample was run with each assay.

Results were multiplied by a correction factor (78.125) and expressed as μmol/L.

The intra assay %cv was 9.2% and the inter assay %cv was 39.5% This very high inter assay variation may be due to SOD degrading in freezer during prolonged storage.

2.3 Cytokines

2.3.1 Human Interleukin 1β (hulL1β)

This was measured using an ELISA kit from the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service. The kit is specific for bioactive huIL1β and recognises natural and recombinant IL1β equivalently. There is no detectable cross reactivity for recombinant mouse IL1β, recombinant

human IL1α, recombinant mouse IL1α, recombinant human IL2, recombinant human IL3, recombinant human IL4, recombinant human IL6, IL7, IL8 and recombinant human M-CSF, recombinant human GM-CSF and recombinant human TNFα.

Because of the expense, not enough kits were run to calculate an "in house" %cv. The inter assay coefficient of variation quoted by the manufacturer was 8.8%.

The kit is a "sandwich-type" of enzyme immunoassay in which a monoclonal anti-human IL1β antibody has been pre-coated to polystyrene microtitre wells. Human IL1β, present in a measured volume of standard or sample is bound by the antibody on the microtitre plate. Non-bound material is then removed by washing. Subsequently a rabbit antibody to huIL1β is added. This binds to the huIL1β-antibody complex present in the microtitre well. Excess of the rabbit antibody is removed by washing, followed by addition of horseradish peroxidase (HRP) conjugated goat-anti-rabbit antibody, which binds onto the rabbit side of the huIL1β sandwich. After removal of non-bound HRP conjugate by washing, substrate solution is added to the wells. A blue coloured product is formed in proportion to the amount of IL1β present in the standard or sample. After the reaction has been stopped absorbance is measured in a microtitre plate reader. From the absorbance of samples and those of a standard curve, the concentration of IL1β can be determined by interpolation with the standard curve.

Six standards (500, 250, 100, 50, 10 and 0 pg/ml) were supplied lyophilised and had to be reconstituted prior to use in serum diluent. A standard or serum sample (100 μl) was added to designated wells, leaving one well empty as a blank.

The plate was gently tapped for 15 seconds, then covered and incubated for 2

hours at 37°C.

After the incubation the plate was washed 4 times, then blotted dry on paper towel. Anti- IL1β (100 μl) was added to all but the blank well. The covered plate was incubated for 1 hour at 37°C.

After the incubation the plate was washed 4 times, then blotted dry on paper towel. HRP-conjugated Goat anti-rabbit antibody (100μl) was added to all but the blank well. The covered plate was incubated for 30 minutes at 37°C.

After the incubation the plate was washed 4 times, then blotted dry on paper towel. Substrate solution (100 μl) was added to all wells, including the blank. The plate was incubated for 15 minutes at room temperature, in the dark.

The reaction was stopped by addition of concentrated $H_2 SO_4$ (100 μl). The plate was read at 450 nm on a Dynatech MR 700 microplate reader.

A calibration curve was constructed by plotting optical density at 450 nm against huIL1β concentration in pg/ml on log-linear paper. The best fitting curve was drawn and test sample values were then read off from this curve.

2.3.2 Interleukin 2 Receptor (IL2-R) ELISA

This was measured using an enzyme immunoassay for the quantitative determination of IL2-R supplied by T Cell Diagnostics, Inc. The manufacturer's quoted assay sensitivity was 50U/ml. Because of the expense, not enough kits were run to calculate an "in house" %cv. The inter assay coefficient of variation quoted by the manufacturer was 4.8-5.6%. Intra assay variation was 2.2-3.4%.

A 96 well plate was pre-coated with murine monoclonal antibody to IL2-R.

IL2-R standard and sample serums (50 μl) were added to wells in duplicate.

Horseradish Peroxidase conjugated murine monoclonal antibody to human IL2-R (100μl) was added to all but blank wells. The plate was agitated by tapping for 15 seconds, then sealed and incubated at room temperature on a rotator at 150 RPM.

The plate was then aspirated and washed with a buffer of 0.1% surfactant in PBS This step was repeated another 2 times, then the plate was blotted on paper towel.

Chromogen solution, containing O-Phenylenediamine, (100μl) was added to all wells and the uncovered plate was incubated at room temperature for 30 minutes.

Stopping solution (2N H_2 SO_4) (50μl) was added to all wells.

The optical density was obtained for each well by reading the plate in the Dynatech MR 700 plate reader at 490nm.

A standard curve was generated by plotting the optical densities (at 490nm) for the standards versus their concentrations. The best curve was drawn (log/linear) and unknown patient samples were read off from this.

2.3.3 Interleukin 6 (IL6) ELISA

An enzyme immunoassay for the quantitative determination of IL6 was supplied by Eurogenetics. The manufacturer's quoted assay sensitivity was 5pg/ml. Because of the expense, not enough kits were run to calculate an "in house" %cv. The inter assay coefficient of variation quoted by the

manufacturer was 3.8-8.7%. Intra assay variation was 5.5-9.4%.

2.3.3.1 Supplementary reagents

The following solutions for the assay and were made up in advance:

Coating buffer. Carbonate/Bicarbonate buffer (0.1M) at pH 9.6.

PBS stock solution. Phosphate Buffered Saline (0.2M) at pH 6.8-6.9.

PBS working strength buffer. Phosphate Buffered Saline (0.01M) at pH 7.2-7.4.

Blocking buffer. Supplied blocking reagent (500μl) in 25ml working strength PBS.

Washing buffer. PBS (0.01M) with 0.005% TWEEN 20.

Dilution buffer. This was supplied 5x concentrated with kit and diluted to working strength in distilled water

Substrate buffer. Acetate buffer (0.11M) at pH 5.5.

TMB Stock Solution. 6mg/ml 3,5,3,5'-tetramethylbenzidine in dimethylsulfoxide

Hydrogen peroxide solution. Hydrogen peroxide (3%) in distilled water.

Substrate solution. This was prepared by mixing substrate buffer (2ml) with TMB stock solution (200μl) and hydrogen peroxide solution (12μl).

Stop solution. 1.8M Sulphuric Acid

2.3.3.2 Assay procedure

Monoclonal antibody to IL6 (120μl) was diluted in coating buffer

(12ml). 100μl was added to each well of a 96 well plate and then incubated at room temperature overnight.

Supernatants were aspirated from all wells and the wells were washed 5 times with working strength PBS. The plate was blotted on paper towel.

Blocking buffer (100μl) was added to all wells. The plate was incubated at room temperature for 1 hour.

The plate was aspirated and washed 5 times in washing buffer, then blotted dry on paper towel. 100μl standards prepared in dilution buffer and serum samples diluted 1:2 in dilution buffer were added, in duplicate, to wells, leaving 2 wells empty as blanks.

The plate was incubated at room temperature for 1 hour, then aspirated and washed 5 times in washing buffer and blotted dry on paper towel.

Biotinylated Il-6 antibody (100μl) was added to all but blank wells.

The plate was incubated at room temperature for 1 hour, then aspirated and washed 5 times in washing buffer and blotted dry on paper towel.

Streptavidin-HRP conjugate (100μl) was added to all but the blank wells and the plate was incubated for 30 minutes at room temperature.

The plate was washed 5 times in washing buffer and blotted dry on paper towel.

Substrate solution (100μl) was added to all wells and the plate was incubated at room temperature, in the dark for 30 minutes.

Stop solution (100μl) was added to all wells. The optical density was obtained for

each well by reading the plate in the Dynatech MR 700 plate reader at 450nm.

A standard curve was generated by plotting the optical densities (at 450nm) for the standards versus their concentrations. The best curve was drawn (log/linear) and unknown patient samples were read off from this.

2.3.4 ELISA For Interleukin 8 (IL8)

This assay was performed with IL8 kit from Amersham International plc, Amersham, (UK) (Kit code no. RPN 2147).

This assay employs the quantitative immunometric, "sandwich" enzyme immunoassay technique.

Because of the expense, not enough kits were run to calculate an "in house" %cv. The inter assay coefficient of variation quoted by the manufacturer was 12.2%. The intra assay coefficient of variation was 9.2%.

A 96 well plate was pre-coated with a monoclonal antibody specific for IL8. IL8 standard was supplied lyophilised and was reconstituted prior to use. This was at a concentration of 6000 pg/ml. Serial dilutions were carried out to give standards of 3000, 1500, 750, 375, 187.5 and 93.8 pg/ml.

Substrate solution was supplied as solutions A and B. These were mixed immediately prior to use.

Assay diluent (100 μl) was added to each well. Standard or serum sample (100 μl) was added to each well. After gentle tapping for 1 minute, the plate was covered and incubated for 2 hours at room temperature.

After the incubation the plate was washed 4 times with wash buffer then blotted dry on paper towel.

IL8 conjugate (200 μl) was added to each well. The plate was then re-covered and incubated for 2 hours at room temperature.

After the incubation the plate was washed 4 times with wash buffer then blotted dry on paper towel. Substrate solution (200 μl) was added to each well. The plate was kept at room temperature and after 20 minutes the reaction was stopped by addition of stop solution (50μl).

The optical density was obtained for each well by reading the plate in the Dynatech MR 700 plate reader at 450 nm. A standard curve was generated by plotting the optical densities (at 450nm) for the standards versus their concentrations. The best curve was drawn and unknown patient samples were read off from this.

2.4 Thyroid Antibodies

2.4.1 ELISA for Autoantibodies to Thyroglobulin

This was measured with an ELISA kit from RSR Ltd, Cardiff, UK. A 96 well plate was pre-coated with purified thyroglobulin. Positive control serum and patient samples were diluted 1:20. Standards, controls and diluted samples (50μl) were added in duplicate to appropriate wells and the covered plate was incubated on a plate shaker for 30 minutes at room temperature. After the incubation the plate was washed 4 times with diluted wash solution, then blotted dry on paper towel. Protein A-phosphatase conjugate solution (100μl) was

added to each well. The covered plate was incubated on a plate shaker for 30 minutes at room temperature. After the incubation the plate was washed 4 times (2 times with diluted wash solution and 2 times with distilled water), then blotted dry on paper towel. Substrate solution (100μl) (containing 1mg/ml PNPP in substrate buffer) was added to each well and the plate was incubated for 15 minutes in the dark. After this a yellow-green colour developed and the reaction was stopped by adding 1M NaOH (100μl). Optical density was measured at 405nm on a Dynatech MR 700 microplate reader. A calibration curve was constructed by plotting optical density at 405nm against antibody concentration. Test sample values were then read off from this curve.

2.4.2 ELISA for Autoantibodies to Thyroid Peroxidase (anti TPO)

This was measured with an ELISA kit from RSR Ltd, Cardiff, UK. A 96 well plate was pre-coated with purified thyroglobulin-free TPO Positive control serum and patient samples were diluted 1:20. Standards, controls and diluted samples (50μl) were added in duplicate to appropriate wells and the covered plate was incubated on a plate shaker for 30 minutes at room temperature. After the incubation the plate was washed 4 times with diluted wash solution, then blotted dry on paper towel. Protein A-phosphatase conjugate solution (100μl) was added to each well. The covered plate was incubated on a plate shaker for 30 minutes at room temperature. After incubation, the plate was washed 4 times (2 times with diluted wash solution and 2 times with distilled water), then blotted dry on paper towel. Substrate solution (100μl) (containing 1mg/ml PNPP in substrate buffer) was added to each well and the plate was incubated for 15 minutes in the dark. After this a yellow-green colour developed and the reaction

was stopped by adding 1M NaOH (100μl). Optical density was measured at 405nm on a Dynatech MR 700 microplate reader. A calibration curve was constructed by plotting optical density at 405nm against antibody concentration. Test sample values were then read off from this curve.

2.5 Subject Population

The following groups were included in the study.

Non pregnant females

Samples were obtained from women, not on the oral contraceptive pill, with a mean menstrual cycle of 28 ± 3 day duration. The women were aged 29.4 ± 4.5 years. The following subgroups were looked at;

Follicular phase (day 5-9)

Luteal phase (day 18-22)

Ovulating (day 14)

Menstruating

Normal males

These healthy male volunteers were not taking any medication and were aged 30.5 ± 6.3 years.

Pregnant

Samples from women in the first trimester of pregnancy were assayed. The women were aged 27.2 ± 5.5 years.

Aborters

Samples were taken from women admitted to hospital suffering a spontaneous abortion in the first trimester of pregnancy. The women were aged 27.5 ± 5.8 years. Blood was taken from these women prior to them going to theatre. They were grouped as follows;

First pregnancy aborters (primary aborters)

First time aborters with previous successful pregnancy (secondary aborters)

Women aborting for the second time

Women aborting three or more times (recurrent aborters)

Of the 22 first pregnancy aborters 13 were resampled at a three months follow-up visit and 9 were resampled in a subsequent pregnancy.

Chapter Three

Lymphocyte Proliferation and Function

3.1 Tissue Culture

This chapter concerns the setting up into cell culture of isolated peripheral blood lymphocyte cells from various patient groups. Maintaining cells in culture (*in vitro*), according to the methods described in chapter two, provides a system whereby we can stimulate cells and extrapolate from their behaviour to what may happen *in vivo*, within the body.

3.1.1 Cell Proliferation

The technique used involves the centrifugation of a suspension of whole blood (diluted 1:1 with RPMI) on Ficoll-Hypaque (Lymphoprep) gradients (Boyum 1968). Ficoll-Hypaque is an aqueous solution of density 1.077 containing Ficoll 400, a high molecular weight polymer and diatrizoate sodium, which has high density and low viscosity. In this separation system red blood cells and granulocytes aggregate in the presence of Ficoll, which increases their sedimentation rate, and they therefore settle at the bottom of the centrifuge tube. Lymphocytes and other mono-nuclear cells remain at the interface of the plasma and the Ficoll-Hypaque due to their lower density. The cells from this interface are removed, washed in saline then set up in culture at known concentrations.

Autoradiography is the method used in the detection of cell proliferation (Sigal 1985). A constant amount of labelled DNA precursor, ^{3}H thymidine, is added to cells growing in medium. The ^{3}H thymidine is then incorporated into the replicating DNA of the cells. The uptake of ^{3}H correlates with the number of cells in the S phase of the cell cycle. After incubation with ^{3}H thymidine an

automated cell harvesting machine aspirates the cells onto glass fibre filters. The filters retain DNA with incorporated isotope since the cells are broken up on the filters and unincorporated isotope is washed away. Proliferation of cells grown in a control medium is compared to cells stimulated by mitogens.

3.1.2 Lymphocyte Activation (Mitogenesis)

Lymphocyte transformation assays are used to assess the responsiveness of lymphocytes. Stimulants used in lymphocyte transformation may be specific (antigens) or non specific (mitogens). The discovery, by Nowell (1960), that plant lectins such as phytohaemagglutanin (PHA) are mitogenic for lymphocytes began the understanding of T cell growth. Mitogens (e.g. lectins) activate lymphocytes via receptors apparently distinct from antigen recognition receptors (Fudenberg 1971) and cause non specific transformation of 60 to 90% of the total lymphocyte population, depending on the agent used.

The activation of lymphocytes by lectins *in vitro* provides us with a means of assessing the responsiveness of the lymphocyte population. Lectins are proteins, or glycoproteins, of non immune origin whose precise physiological role in nature is unknown.

Phytohaemagglutinin (PHA) is a T helper/inducer (CD4+ve) cell mitogen. It is a lectin which is an extract of Phaseolus vulgaris, the red kidney bean (Nowell 1976).

Concanavalin A (ConA) is a T cytotoxic/suppresser (CD8+ve) cell mitogen. It is a lectin extracted from Conavalia ensiformis, the jack bean.

Pokeweed mitogen (PWM) is a T cell dependent, B cell mitogen. This means it will activate B cells only in the presence of T cells. PWM is a lectin extracted from the roots of Phytolacca americana, American pokeweed.

Because of the predominance of T cells in blood T cell mitogens are the most utilised in the laboratory.

3.1.3 B Cell Function

The measurement of immunoglobulin (Ig) production *in vitro* allows us to assess B cell function. Pokeweed mitogen (PWM) is used to trigger Ig production. PWM stimulates a small proportion (around 1%) of B cells to secrete Ig (Sigal 1985). The amount of Ig secreted into culture medium after a seven day incubation is quantified using enzyme-linked immunoabsorbant assays (ELISA) for immunoglobulin G (IgG) and immunoglobulin M (IgM). Ig concentration in supernatants from medium containing stimulated cells is compared with the concentration in medium from unstimulated, control cells incubated under twin conditions. A stimulation index (S.I.) is calculated i.e.

$$SI = \frac{\text{concentration IgG in stimulated cells} - \text{concentration IgG in control cells}}{\text{concentration IgG in control cells}}$$

An absence of response with PWM stimulation would suggest a lack of B cell function.

3.2. Mitogenesis

3.2.1 Subject Population

Non pregnant females (Group 1). Samples were obtained from women, not on the oral contraceptive pill, with a mean menstrual cycle of 28 plus or minus 3 days duration. The samples were obtained from 19 women at the following stages of their menstrual cycle;

5 follicular phase (day 5-9)

3 luteal phase (day 18-22)

5 ovulating (day 14)

6 menstruating

Pregnant (Group 2). A total of 7 samples from women in the first trimester of pregnancy were assayed. Of these 3 were from primigravada women and 4 were from women who had been seen at the start of this study when admitted to hospital suffering a spontaneous abortion, but these samples taken in a subsequent pregnancy.

Aborters (Group 3). A total of 18 samples were taken from women admitted to hospital suffering a spontaneous abortion. They were grouped as follows;

10 first pregnancy aborters (primary aborters)

5 first time aborters with previous successful pregnancy (secondary aborters)

3 women aborting for the second time

For the purpose of statistics, analysis was carried out on the following groups;

18 all aborters

15 first aborters (with or without previous pregnancy)

Of the 10 first pregnancy aborters 6 were sampled at a 3 months follow up visit and 4 were sampled in a following pregnancy.

3.2.2 Results

Patient lymphocytes were stimulated by mitogens and incubated according to the method in chapter two. Incorporated ^{3}H thymidine in stimulated cells is compared to control cells. Results are expressed as a stimulation index (S.I.), calculated as follows:

$$\text{S.I.} = \frac{\text{c.p.m stimulated cells - c.p.m control cells}}{\text{c.p.m control cells}}$$

where c.p.m. = counts per minute

A stimulation index was chosen as the best way to express results where there is a wide variation in incorporation of ^{3}H thymidine into control cells within and across all groups. Comparison of radioactive counts only fails to recognise this range of starting point.

3.2.2.1 Inter-cycle variation S.I.

Because of the small group numbers (n=3) it was impossible to predict

the distribution of the data and therefore a non parametric statistical analysis (Mann Whitney) was carried out. A p value of <0.05 was considered significant.

	ConA		PHA		PWM	
S.I.	mean	sd	mean	sd	mean	sd
follicular n = 5	105.3	66.0	227.9	208.1	84.1	50.8
luteal n = 3	110.0	79.3	147.1	149.7	55.8	44.6
day 14 n = 6	59.1	85.2	96.9	92.6	43.5	58.1
menstruating n = 5	41.7	30.6	170	249.0	46.4	47.5

Table 3.1. **Inter cycle Variation Mitogen Stimulation Indexes**

There were no statistical differences found between any phase of the menstrual cycle for any of the parameters measured. There was, however, a wide range of response to the various stimulants, as seen in the following box and whiskers plots which show minimum, maximum, inter-quartile and median values for each group.

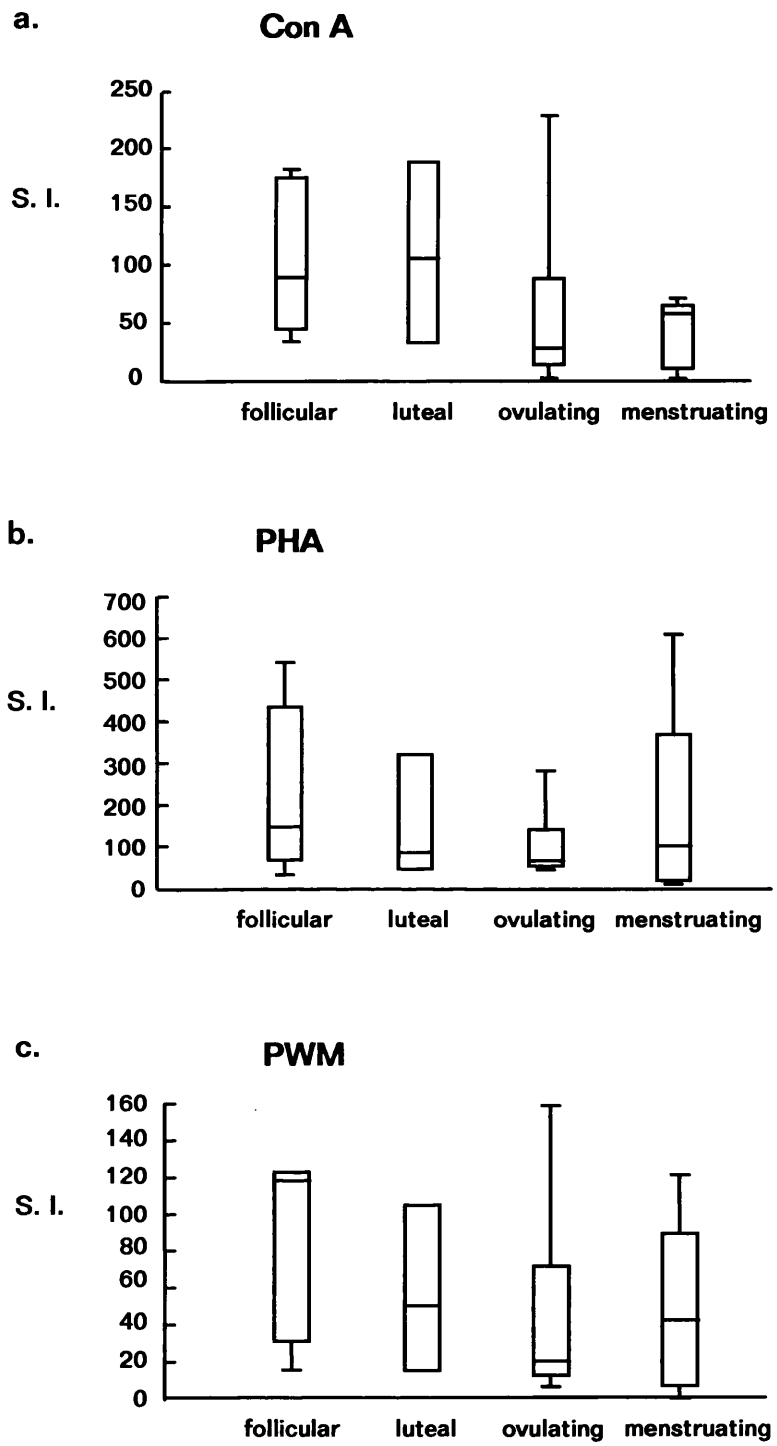


Figure 3.1. **Box and Whisker plot; Mitogen Stimulation Indexes Menstrual Cycle; a, ConA; b, PHA; c, PWM.**

3.2.2.2 Non Pregnant v Pregnant S.I.

There was no statistical difference between stimulation indexes from pregnant and non pregnant samples for any of the mitogens added.

	ConA		PHA		PWM	
S.I.	mean	sd	mean	sd	mean	sd
Non pregnant n=19	74.7	68.9	158.7	175.8	56.9	50.3
Pregnant n=7	52.5	43.2	82.5	55.7	26.17	16.77

Table 3.2. **Non pregnant v Pregnant Mitogen Stimulation Indexes**

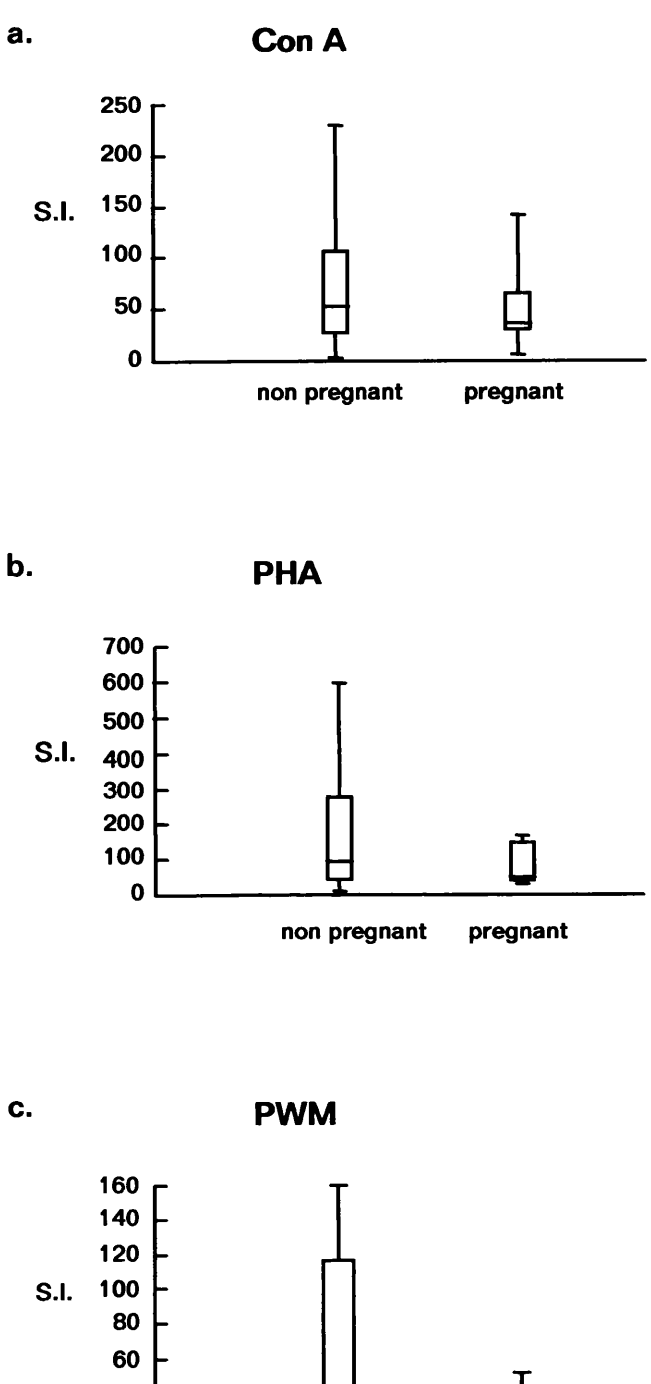


Figure 3.2. **Box and Whisker plot; Mitogen Stimulation Indexes, Non Pregnant v Pregnant; a, ConA; b, PHA; c, PWM.**

3.2.2.3 Non pregnant v Abortion S.I.

There was no statistical difference between stimulation indexes of any of the mitogens added between the non pregnant group and any abortion subgroup.

	Con A		PHA		PWM	
S.I.	mean	sd	mean	sd	mean	sd
non pregnant n=19	74.7	68.9	158.7	175.8	56.9	50.3
1st abortion n=10 primary aborter	41.5	33.8	131.6	137.9	31.1	34.8
1st abortion n=5 secondary aborter	26.6	23.5	80.5	139.9	44.94	20.48
1st abortion n=15	36.53	30.72	114.6	135.8	35.71	30.70
2nd abortion n=3	49.5	27.3	156.1	44.0	33.67	15.92
abortion n=18	38.69	29.83	121.5	125.2	35.37	28.40

Table 3.3. **Non pregnant v Abortion Mitogen Stimulation Indexes**

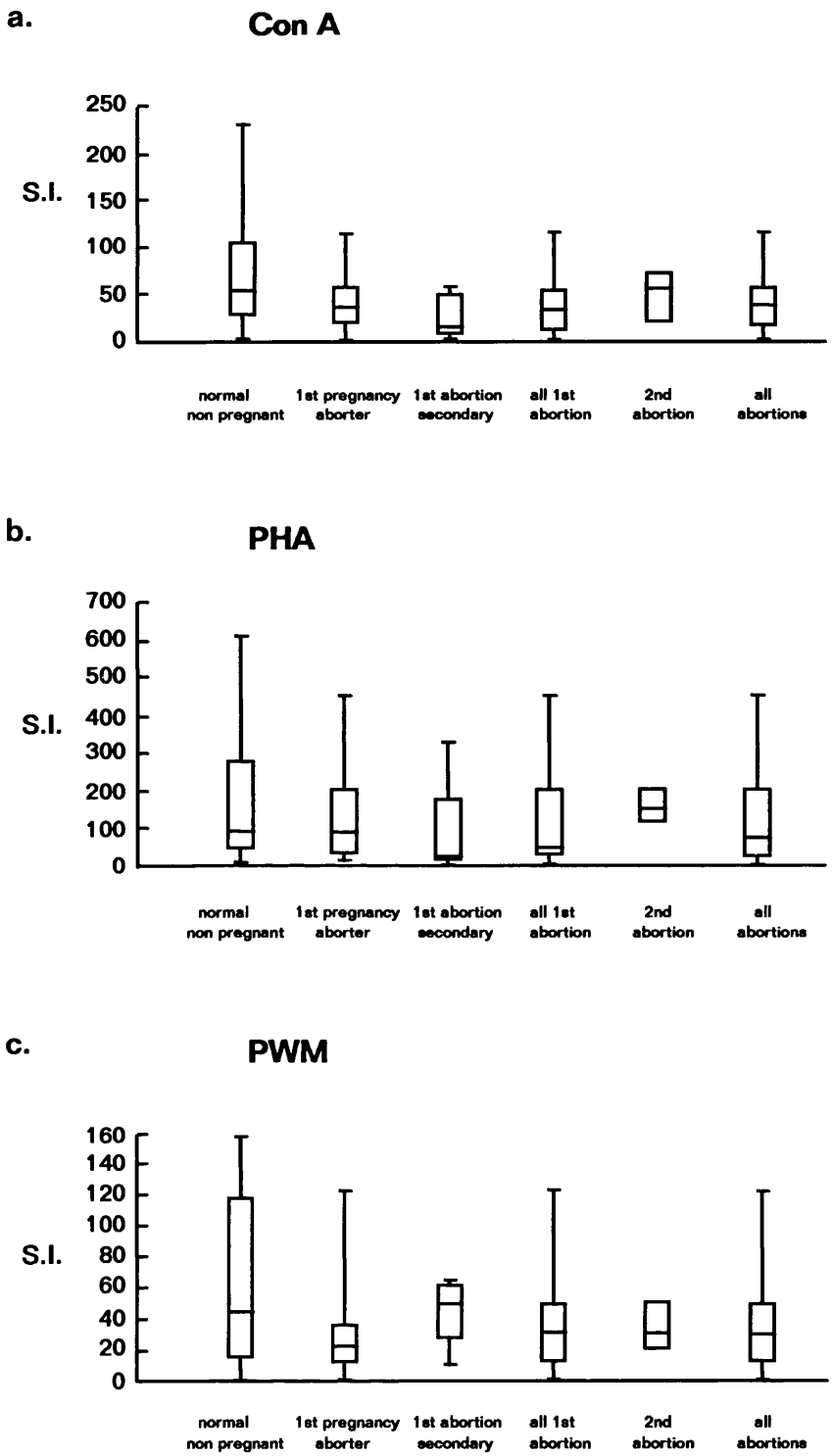


Figure 3.3. **Box and Whisker plot; Mitogen Stimulation Indexes, Non Pregnant v Abortion; a, ConA; b, PHA; c, PWM.**

3.2.2.4 Pregnancy v Abortion S.I.

There was no statistical difference between stimulation indexes of any of the mitogens added between the pregnant group and any abortion subgroup.

	ConA		PHA		PWM	
S.I.	mean	sd	mean	sd	mean	sd
Pregnant n=7	52.5	43.2	82.5	55.7	26.17	16.77
1st abortion n=10 primary aborter	41.5	33.8	131.6	137.9	31.1	34.8
1st abortion n=5 secondary aborter	26.6	23.5	80.5	139.9	44.94	20.48
1st abortion n=15	36.53	30.72	114.6	135.8	35.71	30.70
2nd abortion n=3	49.5	27.3	156.1	44.0	33.67	15.92
abortion n=18	38.69	29.83	121.5	125.2	35.37	28.40

Table 3.4. **Pregnant v Abortion Mitogen Stimulation Indexes**

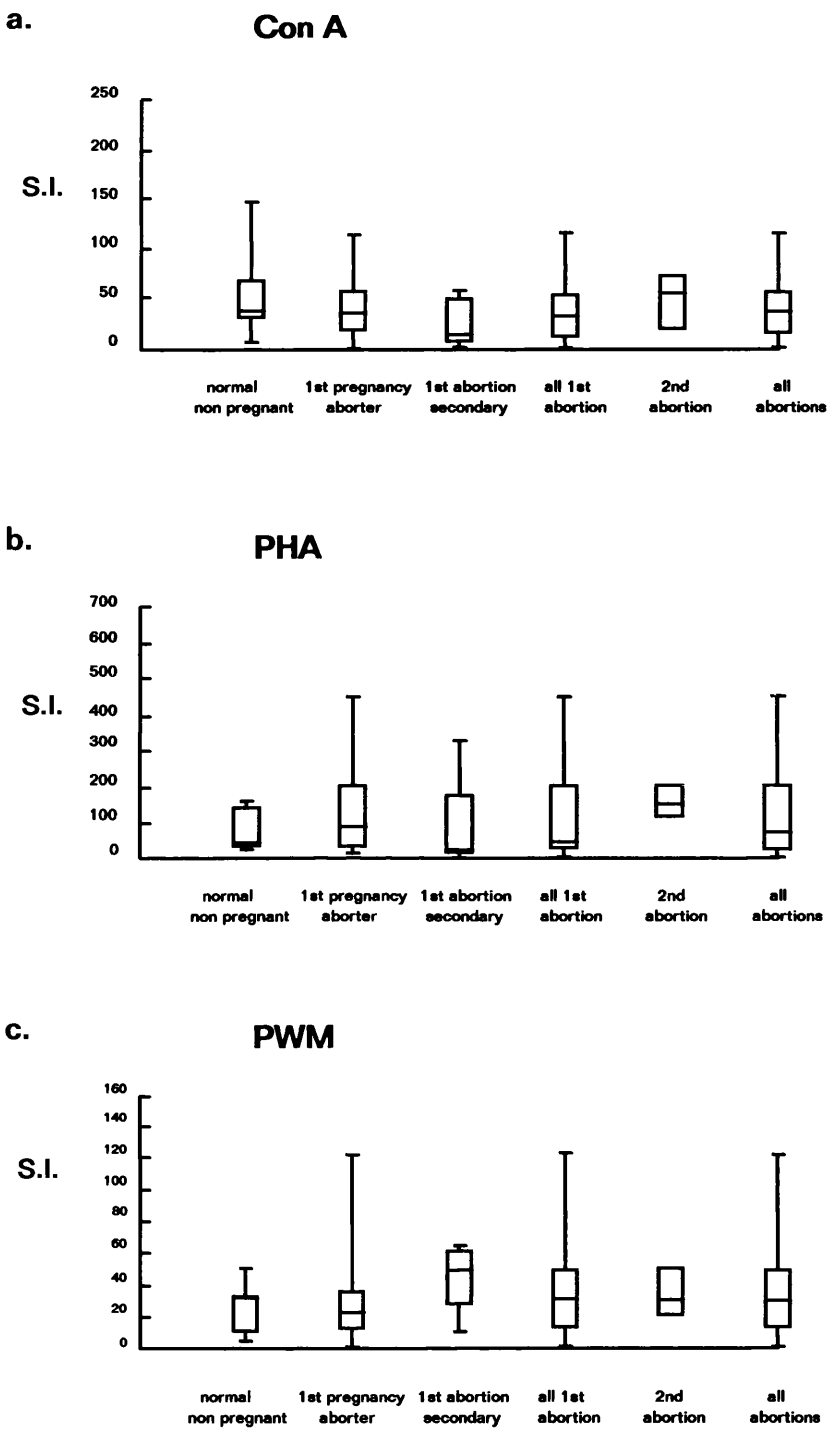


Figure 3.4. **Box and Whisker plot; Mitogen Stimulation Indexes, Pregnant v Abortion; a, ConA; b, PHA; c, PWM.**

3.2.2.5 Abortion

There was no significant difference detected in mitogen stimulation index between any of the abortion sub groups for any of the mitogens.

3.2.3 Discussion

Previous work on mitogen stimulation has shown conflicting results. Sumiyoshi et al (1981) found that a reduced response to PHA occurred in pregnancy. MacLean et al (1992) found that the ability of lymphocytes to respond to stimulation with PWM, PHA or ConA was significantly higher throughout pregnancy. This present study has found there to be no significant difference in lymphocytes response to mitogen stimulation between non pregnant and pregnant subjects. The reasons for this difference are unclear. Numbers of patients involved may be a factor, as the numbers were relatively small in this study compared to those of MacLean et al.

Lymphocytes response to mitogen stimulation was not found to fluctuate throughout the course of the menstrual cycle in this study.

The various sub groups of abortion could not be separated by their lymphocyte response to stimulation. This may be in partly attributable to small numbers in this study. Although no statistical differences were found, wide variations were obtained in how patients' lymphocytes responded to mitogenic stimulation. It may be that the mechanism responsible for miscarriage differs between patients in the one group.

3.3 B Cell Function: Immunoglobulin production

3.3.1 Subject Population

Normal non-pregnant females (Group 1). Samples were obtained from women, not on the oral contraceptive pill, with a mean menstrual cycle of 28 plus or minus 3 days duration. The samples were obtained from 48 women at the following stages of their menstrual cycle;

10 follicular phase (day 5-9)

16 luteal phase (day 18-22)

14 ovulating (day 14)

8 menstruating

Normal Pregnancy (Group 2). A total of 29 samples from women in the first trimester of pregnancy were assayed. Of these 10 were from primigravida women, 2 were from women with a history of infertility, 6 were from women with a history of successful pregnancy outcome and 2 were from women who had suffered a miscarriage previous to this study (one of these patients also had a previous successful pregnancy). The remaining 9 samples were from women who had been seen at the start of this study when admitted to hospital suffering a spontaneous abortion, but these samples taken in a following pregnancy. For statistical analysis this group of 9 women were also treated as a sub group.

Aborters (Group 3). A total of 49 samples were taken from women admitted to hospital suffering a spontaneous abortion, with blood samples taken prior to surgery. They were grouped as follows;

26 first pregnancy aborters (primary aborters)

11 first time aborters with previous successful pregnancy (secondary aborters)

10 women aborting for the second time

1 woman aborting for the third time

1 woman aborting for the fourth time.

For the purpose of statistics, analysis was carried out on the following groups;

49 all aborters

37 first aborters (with or without previous pregnancy)

12 two or more abortions

Of the 26 first pregnancy aborters 10 were sampled at a 3 months follow up visit and 9 were sampled in a following pregnancy.

3.3.2 Results

Tables below show means and standard deviations (sd). Where there is insufficient data to calculate the sd an asterisk * has been inserted.

3.3.2.1 Inter-cycle variation Stimulation Index

Because the data were not normally distributed, a non parametric statistical analysis (Mann Whitney) was carried out.

	Ig G		**Ig M**	
	mean	sd	mean	sd
follicular n = 10	3.56	9.66	7.55	11.31
luteal n = 16	1.212	0.667	11.54	24.64
day 14 n = 14	2.229	0.812	4.42	4.99
menstruating n = 8	0.259	0.096	1.64	9.14

Table 3.5. **Inter cycle Variation Immunoglobulin Stimulation Indexes**

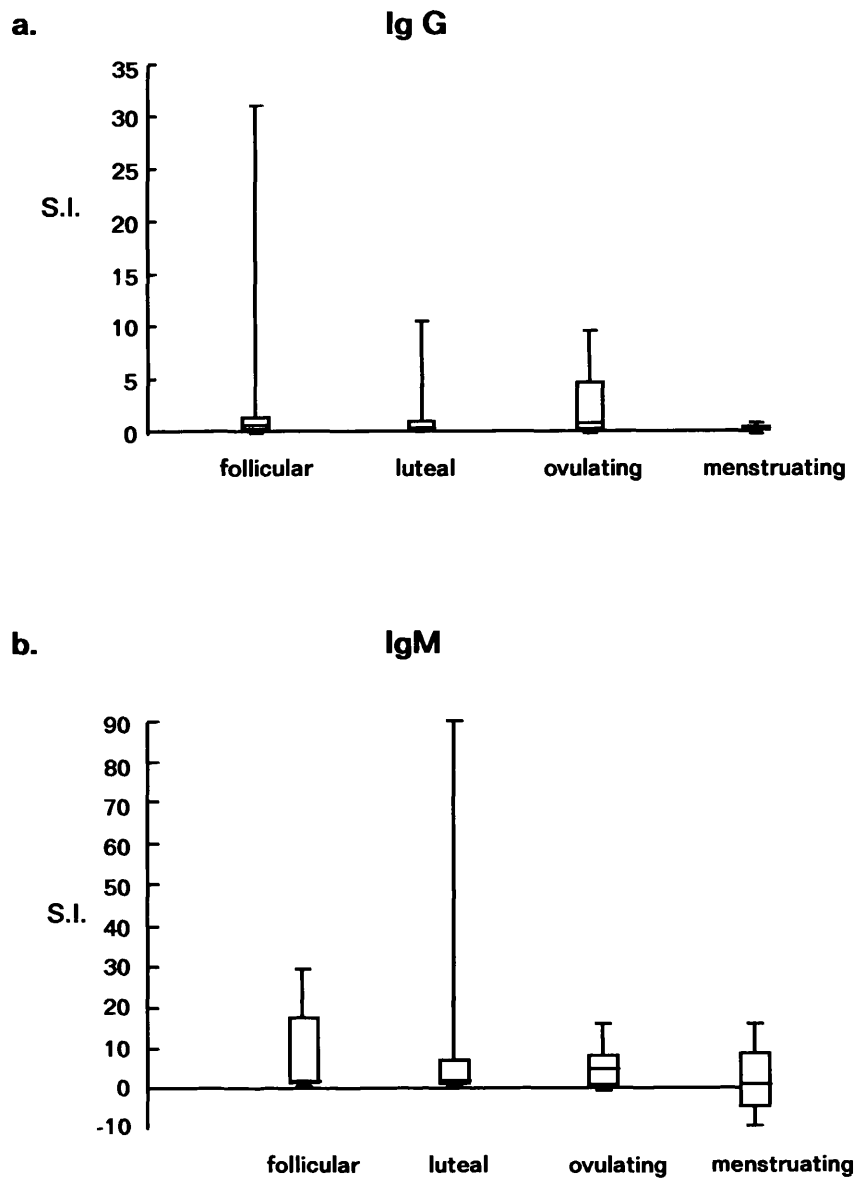


Figure 3.5. **Box and Whisker plot; Immunoglobulin Stimulation Indexes Menstrual Cycle; a. IgG; b. IgM.**

The following table summarises the statistics carried out on the above data. Nsd = no significant difference.

versus	**follicular**	**luteal**	**day 14**
luteal	nsd		
day 14	nsd	nsd	
menstruating	nsd	nsd	nsd

Table 3.6. **Inter cycle Variation Statistics Immunoglobulin**

Comparison of the results obtained at the various stages of the menstrual cycle, as outlined above, showed there to be no significant difference either IgG or IgM.

3.3.2.2 Non pregnant v Pregnant Stimulation Index

	Ig G		Ig M	
S.I.	mean	sd	mean	sd
Normal non-pregnant n = 48	1.839	4.894	7.29	16.25
Normal pregnant n = 20	0.807	2.111	5.53	7.38
$0+^{0}$ n = 9 (pregnancy sub group)	1.045	2.691	6.76	9.59
$1+^{0}$ n = 2 (pregnancy sub group)	-0.20	*	-0.13	*
$2+^{0}$ n = 3 (pregnancy sub group)	1.74	3.14	4.05	5.95
$3+^{0}$ n = 1 (pregnancy sub group)	7.29	*	7.29	*
$1+^{1}$ n = 1 (pregnancy sub group)	0.59	*	10.21	*
Pregnant no previous abortion n = 15	1.434	2.915	5.34	7.99
Multi-parous no previous abortion. n = 6	2.02	3.4	8.69	9.64
all pregnant n = 29	0.664	1.805	4.19	6.55

* denotes insufficient data to obtain these figures

Table 3.7. **Non Pregnant v Pregnant Immunoglobulin Stimulation Indexes**

The following table summarises the statistics carried out on the above data.

versus	**normal non pregnant**	**$0+^0$**	**pregnant no previous abortion**	**multi-parous no previous abortion**
$0+^0$	nsd			
pregnant no previous abortion	nsd	nsd		
multi-parous no previous abortion	nsd	nsd	nsd	
all pregnant	IgG p=0.0355 (pregnant lower)	nsd	nsd	nsd

Table 3.8. **Non Pregnant v Pregnant Statistics Immunoglobulin**

The results suggest a reduced capacity of B cells to produce IgG in pregnant women when compared to non pregnant women.

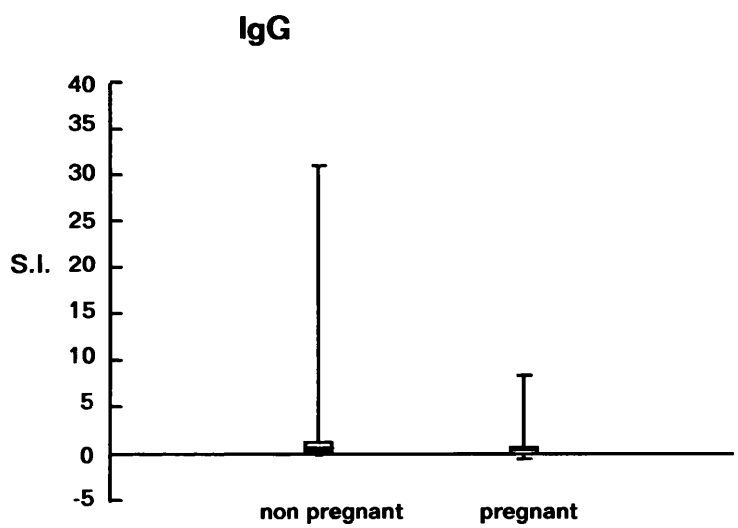


Figure 3.6. **Box and Whisker plot; IgG Immunoglobulin Stimulation Indexes, Non Pregnant v Pregnant**

3.3.2.3 Non pregnant v Abortion Stimulation Index

	Ig G		Ig M	
S.I.	mean	sd	mean	sd
non pregnant n = 48	1.839	4.894	7.29	16.25
1st pregnancy aborter n = 26	3.15	8.36	7.14	14.32
1st aborter (regardless parity) n = 11	2.69	3.92	20.03	42.6
all 1st aborters n = 37	3.02	7.27	10.98	25.8
all aborters n = 49	5.11	19.31	8.78	22.49

Table 3.9. **Non Pregnant v Abortion Immunoglobulin Stimulation Indexes**

The following table summarises the statistics carried out on the above data.

versus	**non pregnant**	**1st pregnancy aborter**	**1st aborter regardless parity**	**all 1st aborters**
1st pregnancy aborter	nsd			
1st aborter regardless parity	nsd	nsd		
all 1st aborters	nsd	nsd	nsd	
all aborters	nsd	nsd	nsd	nsd

Table 3.10. **Non Pregnant v Abortion Statistics Immunoglobulin**

The results indicate no alteration of B cell function between non pregnant women and those undergoing abortion. There is also no significant difference in

B cell function between various first abortion sub groups.

3.3.2.4 Normal pregnancy v Abortion Stimulation Index

	Ig G		Ig M	
S.I.	mean	sd	Mean	sd
pregnant $0+^0$ n = 9	1.045	2.691	6.76	9.59
all pregnant n = 29	0.664	1.805	4.19	6.55
1st pregnancy aborter n = 26	3.15	8.36	7.14	14.32
all 1st aborters n = 37	3.02	7.27	10.98	25.8
>1 abortion n = 12	11.6	37.3	2.57	4.37
all aborters n = 49	5.11	19.31	8.78	22.49

Table 3.11. **Pregnant v Abortion Immunoglobulin Stimulation Indexes**

The following table summarises the statistics carried out on the above data.

versus	**0+0**	**all pregnant**	**1st pregnancy**	**all 1st aborter**	**>1 abortion Aborters**
all pregnant	nsd				
1st pregnancy aborter	nsd	nsd			
all 1st aborters	nsd	nsd	nsd		
>1 abortion	nsd	nsd	nsd	nsd	
all aborters	nsd	nsd	nsd	nsd	nsd

Table 3.12. **Pregnant v Abortion Statistics Immunoglobulin**

The results indicate no alteration of B cell function between normal pregnant women and those undergoing abortion. There is also no significant difference in

B cell function between various abortion sub groups. Recurrent aborters ie >1 abortion do not differ significantly from spontaneous aborters for B cell function.

3.3.2.5 Abortion Stimulation Index

	Ig G		Ig M	
S.I.	mean	sd	mean	sd
1st pregnancy aborter n = 26	3.15	8.36	7.14	14.32
1st aborter regardless parity n = 11	2.69	3.92	20.3	42.6
all 1st aborters n = 37	3.02	7.27	10.98	25.8
2nd aborters n = 10	13.9	40.8	2.83	4.72
3rd aborters n = 1	-0.19	*	-0.72	*
4th aborters n = 1	-1.0	*	3.59	*
> 1 aborter n = 12	11.6	37.3	2.57	4.37
all aborters n = 49	5.11	19.31	8.78	22.49

Table 3.13. **Abortion Immunoglobulin Stimulation Indexes**

The following table summarises the statistics carried out on the above data.

versus	**1st pregnancy aborter**	**1st abortion regardless parity**	**all 1st aborters**	**2nd aborters**	**>1 abortion**
1st abortion regardless parity	nsd				
all 1st aborters	nsd	nsd			
2nd aborters	nsd	nsd	nsd		
>1 abortion	nsd	nsd	nsd	nsd	
all aborters	nsd	nsd	nsd	nsd	nsd

Table 3.14. **Abortion Statistics Immunoglobulin**

Again no significant difference in B cell function is detected between these abortion sub groups.

3.3.2.6 Results at follow up of aborters Stimulation Index

	Ig G		Ig M	
S.I.	mean	sd	mean	sd
1st visit n = 26 (aborting)	3.15	8.36	7.14	14.32
3/12 follow-up n = 10	3.02	9.76	2.02	5.58
2nd pregnancy n = 9	0.344	0.811	1.082	1.928

Table 3.15. **Follow Up Patients Stimulation Indexes Immunoglobulin**

The following table summarises the statistics carried out on the above data.

versus	**1st pregnancy aborter**	**3/12 follow up**
3/12 follow up	nsd	
2nd pregnancy	nsd	nsd

Table 3.16. **Follow Up Patients Statistics Immunoglobulin**

Comparison was also carried out between the group of 2nd pregnancy samples with the normal pregnant group. While there was no significant difference between these groups for IgG, IgM was found to be significantly higher ($p=0.0464$) in normal pregnant than in the "follow up" pregnant samples.

3.3.3 Discussion

No significant differences in production of immunoglobulin was detected throughout the menstrual cycle. Levels of IgG in vaginal fluids have

been reported to fluctuate with the menstrual cycle (Usala 1989), being relatively high after menstruation, declining in the ovulatory phase and remaining low in the luteal phase. De Punzio et al (1992) also found this fluctuation in IgA levels in vaginal fluids. The variations were small and not statistically significant. Vaginal fluid consistency and volume alters throughout the menstrual cycle and this, along with fluctuating pH levels in the vagina, may affect the levels of immunoglobulins present. From this present study it would appear that this local fluctuation is not reflected in the peripheral blood.

No significant differences in immunoglobulin levels were detected between the various sub groups of pregnancy.

Pregnant women were found to have lower S.I. for IgG than non pregnant women. Yasuhara et al (1992) found serum levels of IgG, IgA and IgM decreased throughout pregnancy. Levels of agalactosyl IgG were found to decrease during pregnancy by one group (Thompson et al 1992). The level of agalactosyl IgG correlates with the severity of arthritis. The symptoms of arthritis are known to be less severe during pregnancy. This may be a case of a temporary immunosupression having a beneficial effect on a disease of immune origin. If the T_H2 bias of pregnancy postulated by Wegmann (1993) and discussed in the introduction of this thesis is correct then B-cells taken from pregnant woman will be up-regulated and producing large amounts of antibody. Stimulation of these, already up regulated cells, might not result in the production of large amounts of antibody and the stimulation index would therefore be smaller as was found when comparing pregnant with non pregnant women.

Immunosuppression in pregnancy has been much considered over the years. The maternal immune system must be directed to tolerate the foreign foeto-

placental unit, immunosupression would aid such tolerance. It has been noted that pregnant women suffer more severe symptoms from some viral infections such as influenza and pneumonia than non pregnant women but are not at risk from more frequent or more severe infections from most viral or bacterial pathogens (Adelsberg 1985). Adelsberg proposed that immunosuppression does play a role in the maintenance of a normal human pregnancy but that it is likely to be quite specific, utilising specific antibodies, immune complexes or lymphocytes, and/or quite localised, i.e., that the development of reactive maternal cells is prevented in and around the placenta but not necessarily elsewhere in the mother. Hegde (1991) found very little change in the immune competence of the mother during pregnancy. This makes sense, as generalised immunosupression would be a risky way to ensure the survival of the foetus, the mother being prone to infection. Declining titres of anti-viral antibodies have been noted in the blood of pregnant women (Baboonian 1983), but this is thought to be predominantly a manifestation of haemodilution (ie the increased volume of blood in pregnant women).

Some thyroid diseases, particularly autoimmune thyroid disease, tend to be less severe during pregnancy and other autoimmune diseases are also known to go into remission. During pregnancy levels of circulating lymphocyte subsets such as CD4+ve are reduced and natural killer cell activity is depressed (Lazarus 1994). This modification of the maternal immune response appears to have a significant influence on disease activity in autoimmune disorders.

No statistical difference in B cell production of immunoglobulins was detected between women undergoing abortion and non pregnant women. This was also the case between women undergoing abortion and pregnant women. There was

also no difference found in the various sub groups of abortion. MacLean et al (1991) reported higher levels of IgG and IgM in pregnant women as well as spontaneous aborters when compared to non pregnant women. Their data however, was calculated on total immunoglobulin produced by stimulated B cells whereas this study looks at a stimulation index. The stimulation index is a measure of B cells capacity to produce immunoglobulin under stimulation and is an attempt to compensate for the large variation of total immunoglobulin found in individuals' controls. For instance a sample with 10ng/ml IgG in the control and 100ng/ml in the stimulated sample would have a SI of 9 and a sample with 90ng/ml IgG in the control and 100ng/ml in the stimulated sample would have a SI of 0.11. Both of these examples would have the same value in total IgG measured. Maclean et al reported large standard deviations in their data and also had a lower number of patients than this study.

Chapter Four

Reactive Oxygen Species

4.1 Reactive Oxygen Species

Reactive Oxygen Species (ROS) are also sometimes known as free radicals, a term that is not strictly correct as not all biologically important oxidants are radicals (e.g. hydrogen peroxidase). Radical reactions are an integral part of homoeostasis in cellular processes. Many cellular enzymes catalyse reactions by utilising one-electron transfers that produce reactive intermediate oxygen species. This is also the case for energy producing electron transport systems. Molecular oxygen, found in all aerobic organisms, readily accepts electrons. This results in the formation of ROS. The production of ROS must be tightly controlled as they can be highly destructive to cells. The body has a complicated anti-oxidant system with enzymatic and non enzymatic components to keep in check levels of ROS. Thus the body receives the benefits of ROS biochemistry without the risk of uncontrolled reactions harming its physiology. ROS can cause damage to tissue when the balance between their production and levels of anti-oxidant protective mechanisms tips in favour of ROS production.

4.1.1 Reactive Oxygen Species and Disease

Damage caused by the formation of ROS has been implicated in a number of pathologic conditions. Mutagenesis through oxidative DNA damage is widely hypothesised to be a frequent event in the normal human cell. ROS-related lesions that do not cause cell death can stimulate the development of cancer (Dreher 1996). It is believed that ROS play a critical role in the development of cardiovascular disease (Barber 1994), the autoimmune disease rheumatoid arthritis (Banford 1982) and in thyrotoxicosis (Wilson 1988). ROS damage has been implicated in the

atherogenic process, whereby there is degeneration of the arteries due to the formation in them of fatty plaques and scar tissue (Tse 1994). Oxidative injury may be increased in diabetes mellitus because of a weakened defence to reduced endogenous antioxidants such as vitamin E and reduced glutathione (Giugliano 1995). Hypertension (high blood pressure) is a pathological state that may be associated with a loss of the balance between pro-oxidation and antioxidation (RomeroAlvira 1996). Pregnancy induced hypertension (PIH) develops in the latter stages (usually after week 20) of pregnancy and resolves at about 6 weeks post partum. Changes in the antioxidant systems in late pregnancy and in PIH have been noted (Wisdom 1991, Pandey 1995). In normal pregnancy lipid peroxides increase, but antioxidants also increase to offset their toxic actions (Walsh 1994). However, this is not the case in pre-eclampsia. In women with pre-eclampsia, circulating levels of lipid peroxides are increased, but net antioxidant activity is decreased as compared to normally pregnant women.

Extracellular and intracellular antioxidant buffering levels were found to be decreased in patients with PIH, especially in those with proteinuria, compared to levels found in normal pregnancy (Chen 1994).

4.2 Subject Population

In this study two intracellular anti-oxidants, lysate thiol (LSH) and superoxide dismutase (SOD), as well as two extracellular anti-oxidants, plasma thiol (PSH) and caeruloplasmin (Cp) were measured. The methods are described in chapter 2.

The assay for measurement of the thiol groups was based on the thiol-disulphide

interchange reaction between 5,5'-dithio-bis'-nitrobenzoic acid and biological thiols. Caeruloplasmin activity in plasma was assessed on the basis of the caeruloplasmin catalysed oxidation of p-phenylenediamine to Bandrowski's base. The increased rate of photo-oxidation of *o*-dianisidine was the basis of superoxide dismutase measurement. When looked at together, these four parameters are a measure of the redox status of the cell and the oxidative stress across the cell membrane , as reflected by two intra and two extra cellular parameters.

In vitro studies of cultured luteal cells (cells from the corpus luteum) have provided evidence which suggests that ROS play an important role in luteolysis (degeneration of the corpus luteum) in the rodent ovary (Rueda 1995). Recent findings have indicated that luteal cells employ ROS at specific sites in controlling the production of progesterone over the course of the menstrual/reproductive cycle, and inhibiting its synthesis during regression (Carlson 1993). Levels of anti oxidants in the peripheral blood may therefor differ according to the stage in the menstrual cycle. To see if this is so, samples were taken from women with a normal menstrual cycle at various stages and anti oxidant levels were measured. Levels of anti oxidants for these women were also compared to those found in normal males.

Free radical toxicity in pregnancy and abortion (induced and spontaneous) has previously been noted (Sane 1991). Blood samples from normal pregnant women and women suffering spontaneous abortion were collected for measurement of antioxidant levels.

The following groups were included in the study:

Non pregnant females (Group 1). Sixty samples were obtained from women, not on the oral contraceptive pill, with a mean menstrual cycle of 28 plus or minus 3 days

duration. The women were aged 29.4 ± 4.5 years. The samples were obtained from 43 women at the following stages of their menstrual cycle;

12 follicular phase (day 5-9)

18 luteal phase (day 18-22)

18 ovulating (day 14)

12 menstruating

Normal Males (Group 2). Anti-oxidant levels were measured in samples from 19 healthy male volunteers. They were not taking any medication and were aged 30.5 ± 6.3 years

Pregnant (Group 3). A total of 28 samples from women in the first trimester of pregnancy were assayed. The women were aged 27.2 ± 5.5 years. Of these 10 were from primigravida women, 7 were from women with a history of successful pregnancy outcome and 2 women had suffered a miscarriage previous to this study (one of these also had a previous successful pregnancy). The remaining 9 samples were from women who had been seen at the start of this study when admitted to hospital suffering a spontaneous abortion, but these samples taken in a following pregnancy. For statistical analysis this group of 9 women were also treated as a sub group.

Aborters (Group 4). A total of 60 samples were taken from women admitted to hospital suffering a spontaneous abortion. Blood was taken from these women prior to them going to theatre. They were grouped as follows;

22 first pregnancy aborters (primary aborters)

19 first time aborters with previous successful pregnancy (secondary

aborters)

16 women aborting for the second time

2 women aborting for the third time

1 woman aborting for the fourth time.

For the purpose of statistics, analysis was carried out on the following groups;

60 all aborters (age 27.5 ± 5.8 years)

41 first aborters (with or without previous pregnancy) (age 25.5 ± 4.8 years)

19 two or more abortions (age 29.1 ± 6.1 years)

Of the 22 first pregnancy aborters 13 were re-sampled at a 3 months follow up visit and 9 were re-sampled in a following pregnancy.

4.3 Results

Tables below show means and standard deviations (sd). Where there is insufficient data to calculate the sd an asterisk (*) has been inserted.

4.3.1 Normals (Male v Female)

	PSH (U/ml)		LSH (U/ml)		Cp (mg/dl)		SOD (µg/ml)	
	mean	sd	mean	sd	mean	sd	mean	sd
female	427.75	62.87	407.80	149.2	18.02	5.55	42.52	23.00
male (n=19)	516.80	32.70	397.4	96.5	15.52	2.05	43.51	13.87

Table 4.1. **Female v Male Free Radicals**

Because the data was not normally distributed, a non parametric statistical analysis (Mann Whitney) was carried out.

The results of the group of all non pregnant females was compared with the group of males. No significant difference occurred between the groups for LSH, Cp or SOD. For PSH, male levels were significantly increased when compared to female (P=0.0001).

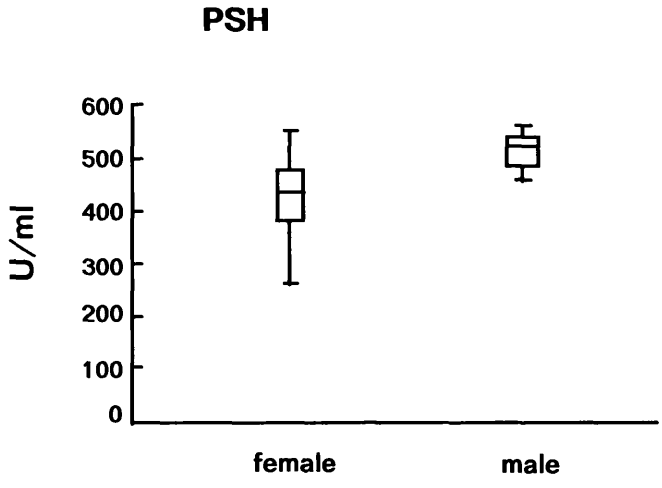


Figure 4.1. **Box and Whisker plot; PSH female v male.**

4.3.2 Inter-cycle variation

	PSH (U/ml)		LSH (U/ml)		Cp (mg/dl)		SOD (µg/ml)	
	mean	sd	mean	sd	mean	sd	mean	sd
follicular	414.8	65.2	492.9	164.8	17.5	7.35	31.08	13.39
luteal	440.3	64.3	364.3	130.0	17.78	6.31	48.59	31.87
day 14	428.5	52.7	379.9	162.0	18.66	3.87	44.07	20.53
menstruating	420.8	75.9	427.5	115.6	17.98	4.82	41.55	14.46

Table 4.2. **Inter cycle Variation Free Radicals**

Comparison of the results obtained at the various stages of the menstrual cycle, as outlined above, showed there to be no significant difference in any of the parameters measured, apart from LSH, where samples measured in the follicular phase were found to be significantly higher than those in the luteal phase (P=0.0466).

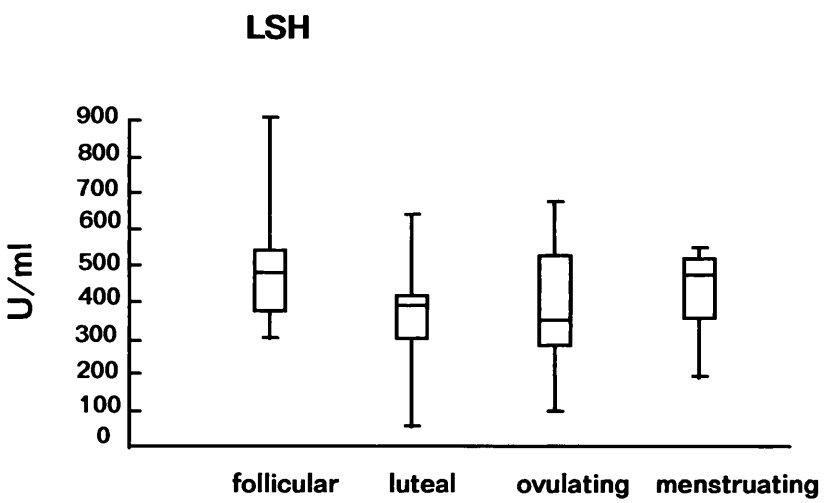


Figure 4.2. **Box and Whisker plot; LSH Menstrual Cycle.**

4.3.3 Non pregnant v Pregnant

	PSH (U/ml)		LSH (U/ml)		Cp (mg/dl)		SOD (µg/ml)	
	mean	sd	mean	sd	mean	sd	mean	sd
Non pregnant	427.75	62.87	407.8	149.2	18.02	5.55	42.52	23.0
0+0	445.1	78.3	385.9	251.2	21.95	3.86	53.93	21.38
1 +0	386.3	88.7	342.0	227.0	25.67	8.58	54.8	42.2
2 +0	406.0	*	373.0	196.0	27.33	6.66	25.93	10.55
3 +0	295.0	211	166.0	*	30.5	*	74.0	*
0 +1	413.0	*	478.0	*	33.0	*	64.0	*
1 +1	415.0	*	234.0	*	33.0	*	66.0	*
Pregnant	412.8	102.1	363.0	222.8	24.06	5.57	50.32	25.53
Multi-parous	359.0	121.0	330.3	188.6	27.07	6.5	45.2	31.6
all pregnant	443.7	93.1	356.5	177.2	23.96	5.71	51.92	21.91

* denotes insufficient data to obtain these figures

Table 4.3. **Non Pregnant v Pregnant Free Radicals**

Results from non pregnant women were compared to those from pregnant women. No significant difference was detected in the measurements of the plasma or lysate thiols between results from non pregnant women and any of the pregnancy sub groups.

For Cp the result of the non pregnant samples was found to be significantly lower

than the pregnant samples (P=0.0001). Non pregnant samples were significantly lower than the primigavidae samples (P=0.0198), the multigravidae samples (P= 0.0017) and the samples from pregnant women with no history of abortion (P= 0.0003).

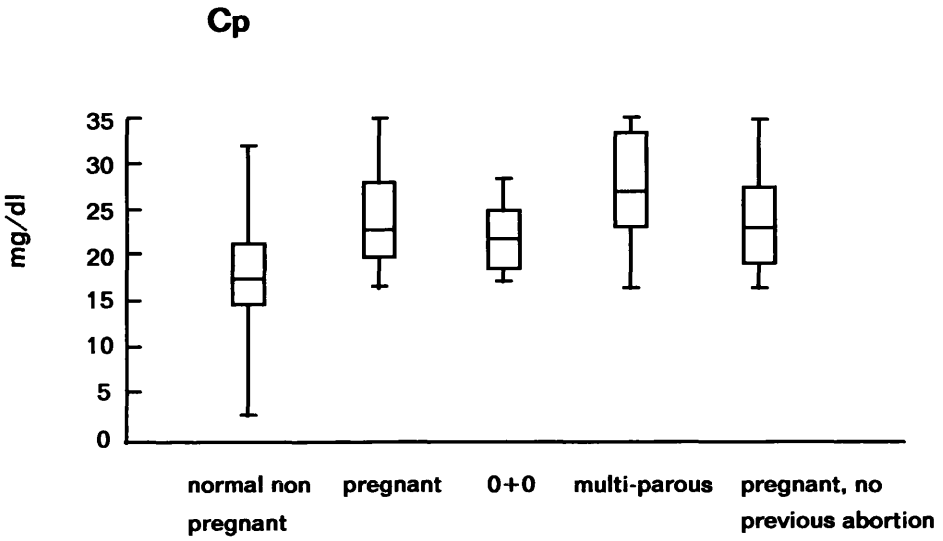


Figure 4.3. **Box and Whisker plot; Cp Non Pregnant v Pregnant.**

Levels of SOD were significantly raised in the group of all pregnant women compared to non pregnant (P=0.0219) and also in the group not including the 9 follow up samples (P =0.0187). There was no significant difference in SOD value between non pregnant and any of the pregnancy sub groups (i.e. prim, multi, no history of abortion).

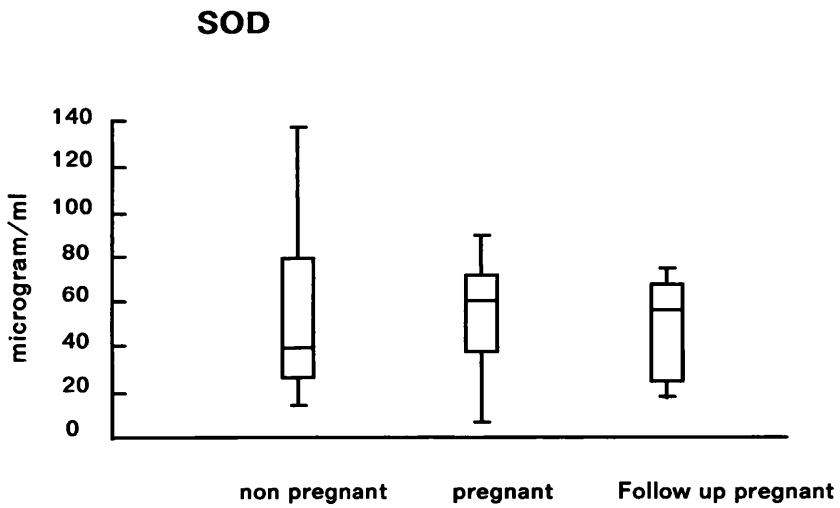


Figure 4.4. **Box and Whisker plot; SOD Non Pregnant v Pregnant.**

4.3.4 Non pregnant v Abortion

	PSH (U/ml)		**LSH** (U/ml)		**Cp** (mg/dl)		**SOD** (µg/ml)	
	mean	sd	mean	sd	mean	sd	mean	sd
non pregnant	427.75	62.87	407.8	149.2	18.02	5.55	42.52	23.0
1st pregnancy aborter	406.5	68.3	422.4	150.4	19.9	6.15	38.75	20.43
1st aborter (regardless parity)	417.1	75.8	391.4	166.7	22.07	9.4	33.28	15.61
all 1st aborters	411.4	71.1	408.0	156.9	20.87	7.75	36.29	18.4
all aborters	406.7	85.8	398.6	170.5	21.13	7.06	38.52	17.85

Table 4.4. **Non Pregnant v Abortion Free Radicals**

Results from normal, non pregnant, women were compared to those from aborting women. No significant difference was detected in the measurements of the plasma

or lysate thiols or for levels of SOD.

For Cp the result of all abortion samples (*group 3*) was found to be significantly higher than the non pregnant (*group 1*) samples (P=0.0048). While no significant difference between non pregnant samples and first pregnancy abortion occurred non pregnant samples were found to be significantly lower than secondary aborters (P= 0.0129). Non pregnants were significantly lower than the group of first time aborters (with or without a previous successful pregnancy) (P=0.021).

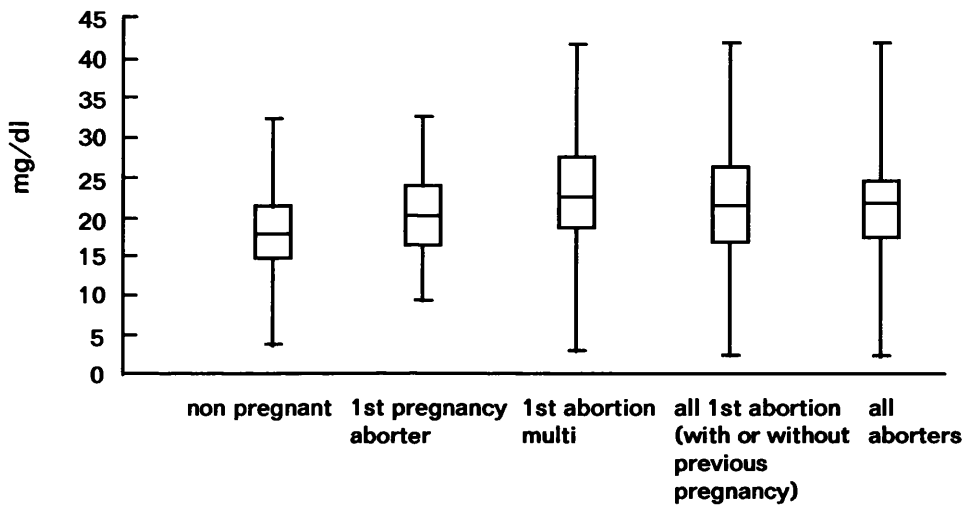


Figure 4.5. **Box and Whisker plot; Cp Non Pregnant v Abortion.**

4.3.5 Pregnancy v Abortion

	PSH (U/ml)		LSH (U/ml)		Cp (mg/dl)		SOD (μg/ml)	
	mean	sd	mean	sd	mean	sd	mean	sd
pregnant $0+^0$	445.1	78.3	385.9	251.2	21.95	3.86	53.93	21.38
all pregnant	443.7	93.1	356.5	177.2	23.96	5.71	51.92	21.91
1st pregnancy aborter	406.5	68.3	422.4	150.4	19.9	6.15	38.75	20.43
all 1st aborters	411.4	71.1	408.0	156.9	20.87	7.75	36.29	18.4
>1 abortion	279.0	112.9	378.1	199.8	21.68	5.48	43.46	15.93
all aborters	406.7	85.8	398.6	170.5	21.13	7.06	38.52	17.85

Table 4.5. **Pregnant v Abortion Free Radicals**

Normal primigravid women were compared with women aborting in their first pregnancy. There were no significant differences for PSH, LSH or Cp, but for SOD results for these pregnant women were significantly higher than this group of aborting women (P=0.0463).

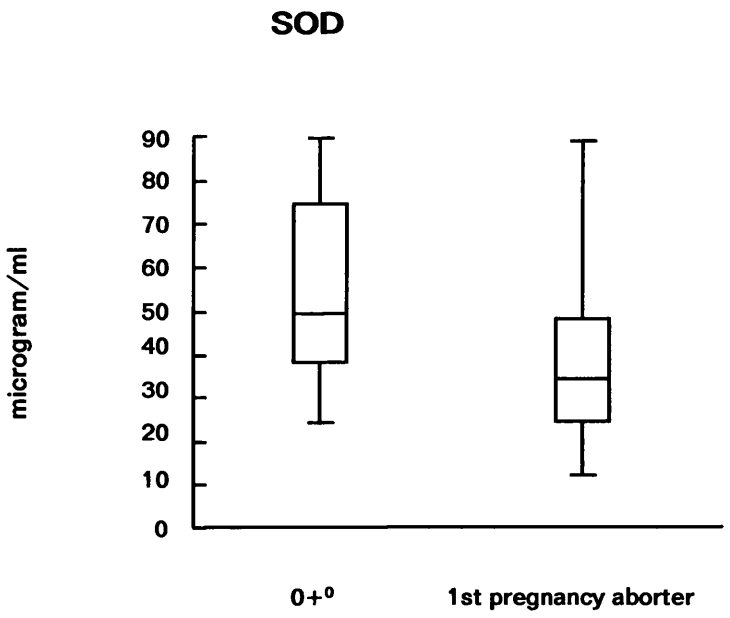


Figure 4.6. **Box and Whisker plot; SOD 0+ ⁰ v 1st Pregnancy Aborters.**

Normal primigravid women were compared with women aborting for the first time, though not necessarily in a first pregnancy. There were no significant differences for PSH, LSH or Cp, but for SOD results for these pregnant women were significantly higher than this group of aborting women (P=0.0129).

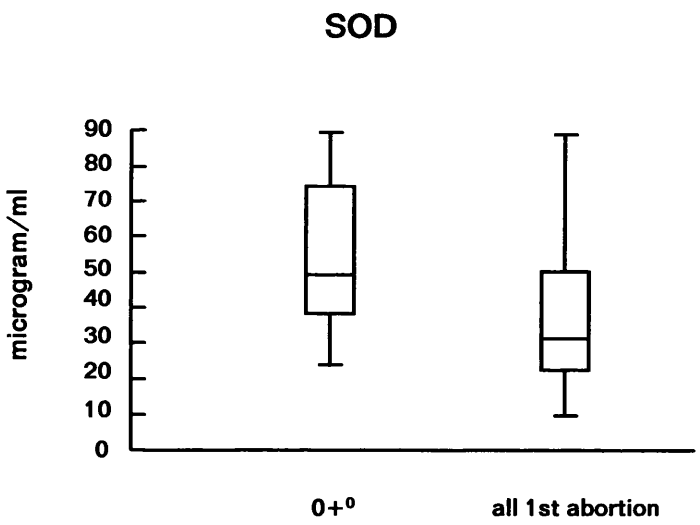


Figure 4.7 **Box and Whisker plot; SOD 0+⁰ v All 1st Aborters**

Normal primigravid women were compared with women who had suffered more than one abortion. There were no significant differences for PSH, LSH, Cp or SOD results.

Normal primigravid women were compared with the group of all aborters. Again there were no significant differences for PSH, LSH or Cp, but SOD results for these pregnant women were significantly higher than this group of aborting women (P=0.0339).

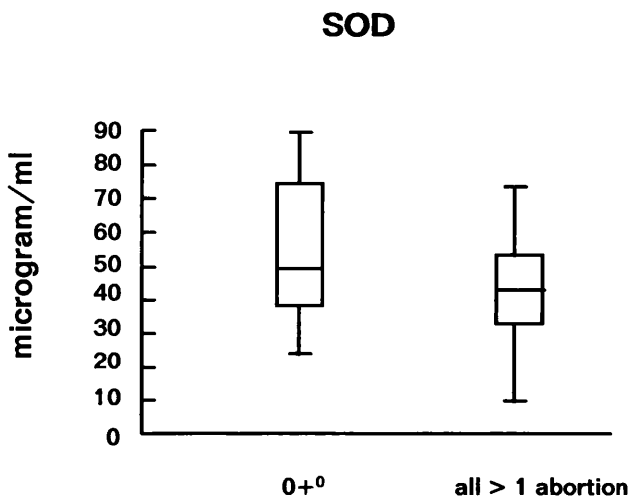


Figure 4.8. **Box and Whisker plot; SOD 0+ ⁰ v All >1st Aborters.**

The group of all pregnant samples was compared to first pregnancy aborters, No significant difference was detected for PSH or LSH.

Cp levels were significantly higher in the group of all pregnant women than in the group of first pregnancy aborters ($p=0.0323$).

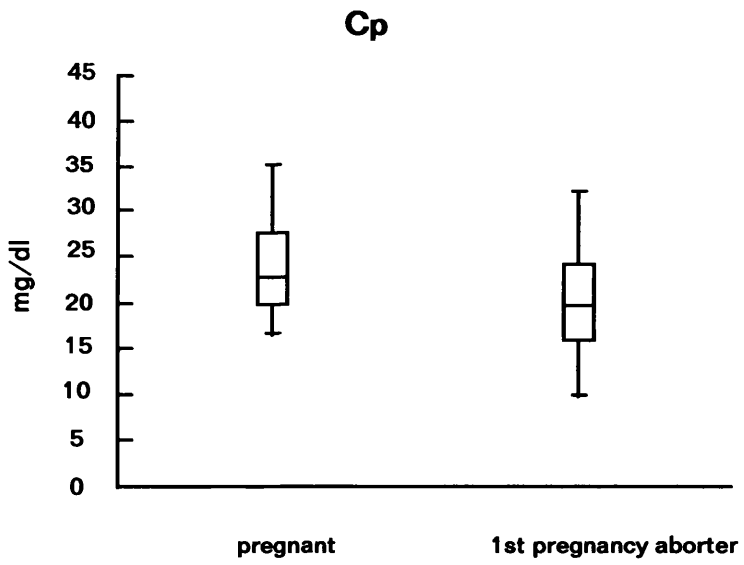


Figure 4.9. **Box and Whisker plot; Cp Pregnant v 1st Pregnancy Aborters**

SOD levels were significantly higher in the group of all pregnant women than in the group of first pregnancy aborters (p=0.0218).

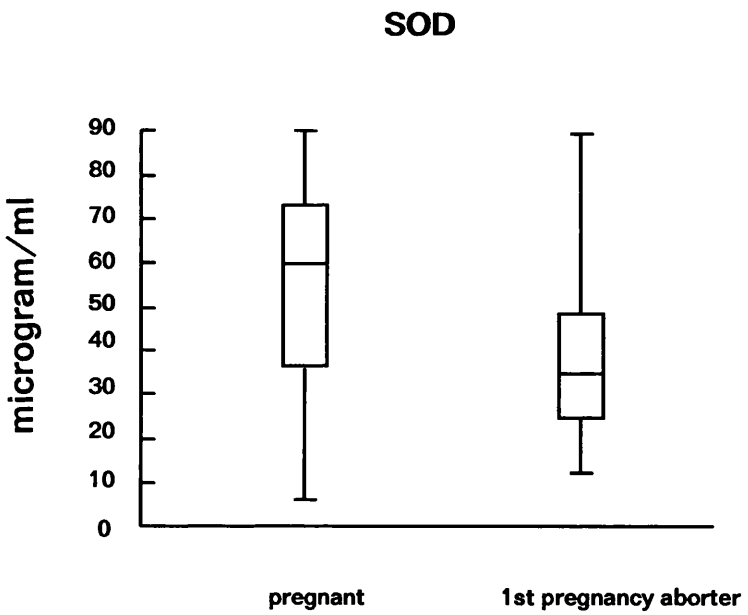


Figure 4.10. **Box and Whisker plot; SOD Pregnant v 1st Pregnancy Aborters**

4.3.6 Abortion

	PSH (U/ml)		LSH (U/ml)		Cp (mg/dl)		SOD (µg/ml)	
	mean	sd	mean	sd	mean	sd	mean	sd
1st pregnancy aborter	406.5	68.3	422.4	150.4	19.9	6.15	38.75	20.43
1st aborter regardless parity	417.1	75.8	391.4	166.7	22.07	9.4	33.28	15.61
all 1st aborters	411.4	71.1	408.0	156.9	20.87	7.75	36.29	18.4
2nd aborters	402.3	108.5	391.6	214.0	21.62	5.92	43.69	15.86
3rd aborters	330.0	206.0	344.5	68.6	21.0	2.83	44.5	27.6
4th aborters	436.0	*	230.0	*	24.0	*	38.0	*
> 1 aborter	279.0	112.9	378.1	199.8	21.68	5.48	43.46	15.93
all aborters	406.7	85.8	398.6	170.5	21.13	7.06	38.52	17.85

* denotes insufficient data to obtain these figures

Table 4.6. **Abortion Free Radicals**

No significant difference was found when comparing all the different abortion groups for any of the parameters measured.

4.3.7 Spontaneous aborters followed up

Of the 22 primigravida patients admitted with a spontaneous abortion, 13 were seen 3 months after their abortion . Of these 13 patients, 9 were seen in a later pregnancy. Comparing this group of patients at these times gave the following results (table 4.7).

	PSH (U/ml)		LSH (U/ml)		Cp (mg/dl)		SOD (µg/ml)	
	mean	sd	mean	sd	mean	sd	mean	sd
1st visit	406.5	68.3	422.4	150.4	19.9	6.15	38.75	20.43
3/12 follow-up	425.2	95.5	363.5	194.6	19.38	3.17	34.74	11.57
2nd pregnancy	497.0	88.1	274.7	123.4	24.06	6.22	46.88	21.17

Table 4.7. **Follow Up Patients Free Radicals**

There was no significant difference in these women at first admission compared to 3 months later for any of the parameters measured. For Cp and SOD there was no significant difference between results obtained on first admission compared to a following pregnancy. PSH levels for the following pregnancy were significantly higher than those at first admission (P=0.0049), while LSH levels were significantly lower in the following pregnancy than in the initial sample (P=0.0148). Analysis was made of the results from the 9 pregnant women with those of the normal pregnant group. There was no significant difference for any of the parameters measured.

The following table gives a summary of significant differences found between the various groups.	**PSH**	**LSH**	**Cp**	**SOD**
non pregnant female v male	p= 0.0207 (males↑)	nsd	nsd	nsd
menstrual cycle follicular v luteal	nsd	p= 0.0466 (follicular ↑)	nsd	nsd
non pregnant v pregnant	nsd	nsd	p= 0.0001 (preg ↑)	p= 0.0187 (preg ↑)
non pregnant v aborters	nsd	nsd	p= 0.0048 (aborters ↑)	nsd
$0+^{0}$ v 1y aborters	nsd	nsd	nsd	p= 0.0463 (prims ↑)
$0+^{0}$ v 1^{st} aborters	nsd	nsd	nsd	p= 0.0129 (prims ↑)
$0+^{0}$ v all aborters	nsd	nsd	nsd	p= 0.0339 (prims ↑)
pregnant v 1y aborters	nsd	nsd	p= 0.0323 (aborters ↓)	p= 0.0218 (aborters↓)
all pregnant v all aborters	p= 0.0310 (aborters↓)	nsd	nsd	p= 0.0023 (aborters ↓)

$0+^{0}$ = women in first pregnancy; 1y aborters = women aborting first pregnancy; 1^{st} aborters = 1y aborters + women aborting for first time following successful pregnancy

Table 4.8. **Free Radicals Summary of Results**

4.4 Discussion

I have been unable to find any previous mention in the literature of how gender affects free radical scavenger levels in the blood. The conclusion of this study is that in all but one parameter measured there is no significant difference. PSH levels were found to be significantly lower in females than in males. This difference was only detected between males and the large group of all non pregnant females, ie there was no significant difference in PSH levels between males and any of the small menstrual cycle sub groups of non pregnant women. The implications of this difference in PSH is unclear, as with lower levels of extra-cellular thiol you might expect to find raised levels of intracellular thiol, but it must be stressed that future studies must take care to sex match control and study groups.

Scavenger levels were compared throughout the menstrual cycle. LSH levels were found to be significantly lower in the luteal phase when compared to the follicular phase. Oxidative stress will result in the conversion of thiol groups to disulphide forms with a consequent fall in the observed thiol level. There is evidence supporting a role for oxidative stress in luteolysis (the regression of the corpus luteum which occurs after ovulation and where no fertilisation and nidation has taken place) (Rueda 1995). Rueda noted that levels of SOD, catalase and glutathione peroxidase increase at the time of luteal cell demise in the bovine ovary. The observed drop in LSH level may be a reflection, in the peripheral blood, of this local increase in oxidative stress.

Levels of SOD and Cp were significantly higher in pregnant women when compared to non pregnant controls. All the pregnant women were in the first trimester of pregnancy. A previous study (Wisdom 1991) found higher levels of Cp

in women in later (third trimester) pregnancy when compared to non pregnant controls. This present study indicates that Cp levels rise at an early stage in pregnancy. This concurs with an early study (Cranfield1979) where it was found that serum antioxidant activity progressively increased throughout pregnancy. Wisdom found SOD levels to be lower in pregnancy than in non pregnant controls. However, the mean SOD level quoted for non pregnants in his study is very high (62 μg/ml) compared to that found in this study (42.52 μg/ml).

It is also important to note that as stated in the methods chapter 2 the intra assay variation for the SOD assay is very high (39.5%) and therefore results of this assay must be interpreted with caution.

As mentioned in the introduction to this chapter antioxidants rise in pregnancy to offset toxic actions of lipid peroxides. This is not the case in women with pre-eclampsia in the third trimester, ie abnormal pregnancy (Walsh 1994). In this study some anti-oxidants were decreased in pregnancies failing in the first trimester.

Cp levels were significantly higher in the group of all aborters compared to non pregnant women. Sane (1992) noted elevated levels of serum lipid peroxide, i.e. evidence of increased free radical activity, in women undergoing abortion (induced or spontaneous). He attributed this rise to the high degree of stress these women were undergoing and found that the levels dropped at the end of the stress. Samples for this present study were taken from women admitted during or immediately following a spontaneous abortion. It might be concluded that samples taken earlier (i.e. *all* samples taken during abortion) might have revealed raised levels in the other parameters, and not just Cp. It might be interesting to compare free radical levels in abortion with other stressful situations, such as surgery for other reasons. It is

interesting to note that women seen three months after their abortion (stress) did not have significantly different levels of antioxidants compared to non pregnant women.

In all the comparisons made between the various groups of aborters and pregnant women, when significant differences were found, levels were found to be higher in pregnancy. Perhaps if pregnancy is to have a successful outcome free radical scavenger levels have to increase.

Chapter Five

Cytokines

5.1 Cytokines

Cytokines are a group of hormone-like polypeptide mediators that play a variety of regulatory roles in both host defence and normal and abnormal homeostatic mechanisms (Cohen 1996). As well as being potent mediators of inflammatory and immune responses they mediate a variety of non immunologic phenomena. A search of the scientific literature between 1990 and 1996 for the word "cytokine" found 25,063 documents, a reflection of the importance given to these substances. Sub groups of cytokines are variously known as lymphokines, monokines, interleukins, interferons and certain growth factors. These groups have the following characteristics in common:

They are low molecular weight proteins (< 80kDa).

They are involved in regulating the size and duration of response in immunity and inflammation.

They are usually locally produced and act in a paracrine, juxtacrine or autocrine manner (Provided that the appropriate receptors are expressed on the cell surface, cytokines can act on the cells that secrete them [autocrine growth control], immediately adjacent cells [juxtacrine growth control], or neighbouring cells [paracrine growth control]).

They are very potent, acting in picomolar concentrations.

They interact with high affinity cell surface receptors specific for each cytokine or cytokine group.

Binding of a cytokine to a cell surface leads to altered protein synthesis and to

altered cell behaviour.

They have overlapping regulatory actions.

They interact in a network, by inducing each other; by transmodulating cytokine cell surface receptors and by synergistic, additive or antagonistic interactions on cell function.

(Balkwill et al 1989).

Most cytokines have growth factor activity, while only some, such as the interferons and tumour necrosis factor (TNF), act to inhibit cell growth. The majority of cytokines act on T cells and B cells, with a combination of cytokines often required to obtain a response. Cytokine regulation of major histocompatibilty complex (MHC) expression and action on macrophages, granulocytes, natural killer cells and eosinophils appears to be more restricted.

5.2 Interleukins

Interleukins are a series of lymphocytotrophic hormones. They are a group of hormone-like polypeptide mediators that play a variety of regulatory roles in both host defence and normal and abnormal homeostatic mechanisms in much the same way as hormones and their receptors act on the endocrine system. Eighteen different interleukins have been identified to date, though the list is sure to expand.

Tables 5.1a and 5.1b summarise the interleukin family to date, their previous names and some indication of their functions.

Interleukin	Previous Names or Function
IL-1	Lymphocyte Activating Factor
	Endogenous Pyrogen (EP)
	Mitogenic Protein (MP)
	Helper Peak-1 (HP-1)
	T Cell Replacing Factor III (TRF III)
	B Cell Activating Factor (BAF)
	B Cell Differentiating Factor (BDF)
IL-2	T Cell Growth Factor (TCGF)
	T Cell Maturation/Stimulation Factor
	Killer Helper Factor (KHF)
	T cell replacing Factor (TRF)
IL-3	Colony Forming Unit-stimulating activity (CFU-SA)
	Multiple Colony Stimulating Factor (Multi- CSF)
IL-4	B Cell Stimulatory Factor-1 (BSF-1)
	T Cell Growth Factor-2 (TCGF-2)
	Mast Cell Growth Factor-2 (MCGF-2)
IL5	Eosinophil Differentiating Factor (EDF)
	B Cell Growth Factor-II (BCGF-II)
	T Cell Replacing Factor (TRF)
	IgA Enhancing Factor (IgA-EF)
	Eosinophil colony stimulating Factor (EOCSF)

Table 5.1a **Interleukins**

Interleukin	Previous Names or Function
IL-6	Plasmacytoma Growth Factor (PCT-GF) Interferon-b-2 (IFN-b_2) Monocyte derived human B cell growth factor B Cell Stimulating Factor (BSF-2) Hepatocyte Stimulating Factor (HSF) Interleukin Hybridoma/Plasmacytoma-1 (IL- HP1)
IL-7	B Cell Growth Factor
IL-8	Monocyte derived neutrophil chemotactic factor Neutrophil-Activating Peptide-1 (NAP1)
IL-9	Mast cell growth factor P40
IL-10	TH1 Cytokine Synthesis Inhibitor
IL-11	Adipogenesis Inhibitory Factor (AGIF)
IL-12	Natural Killer Cell Stimulatory Factor (NKSF) T cell stimulating factor
IL-13	Induces B cell proliferation
IL-14	High molecular weight B cell growth factor (HMW-BCGF)
IL-15	Stimulates the proliferation of T lymphocytes. Produced by a wide variety of cells and tissues, it shares many biological properties with Il-2
IL-16	Lymphocyte chemoattractant factor (LCF) All of its biological activities depend upon the cell surface expression of CD4.
IL-17	Cytotoxic T-cell lymphocyte-associated antigen-8 (CTLA-8)
IL-18	Interferon-gamma-Inducing Factor (IGIF)

Table 5.1b **Interleukins**

The following cytokines were measured in this study:

5.2.1 Interleukin 1 (IL-1)

Originally known as Lymphocyte Activating Factor, IL-1 activates T cell lymphocytes which then proliferate and secrete Interleukin 2 (IL-2) (Gery 1972). IL-1 is produced by many cell types such as fibroblasts, endothelial cells, keratinocytes and smooth muscle, but is primarily released from stimulated macrophages and monocytes and plays a key role in inflammatory and immune responses. IL-1 has been implicated as a local regulator in embryo implantation (Simon 1995). IL-1 has recently been discovered to be raised in follicular fluid taken from patients with premature luteinization (Chen, Chen et al 1995). Luteinization is the forming of the corpus luteum. Premature luteinization correlates with poor pregnancy outcome in *in vitro* fertilisation (IVF). IL-1 refers to two polypeptide hormones, interleukin-1α and interleukin-1β. Although the two forms of IL-1 are distinct gene products, they recognise the same receptor and share biological properties and for this study an ELISA kit that measured IL-1β was used.

5.2.2 Interleukin-2 Receptor (IL-2R)

IL-2 is an immunomodulatory factor produced by certain subsets of T lymphocytes, ie CD4+ve, (Smith 1988). IL-2 has been shown to promote long term growth of activated T cells, activation and proliferation of natural killer (NK) cells, induction of gamma-interferon and B cell growth factor secretion.

Proliferation of T cells is initiated by the following process. Firstly the interaction of antigen/MHC molecule and T cell receptor complex triggers the expression of a

gene, located on chromosome 4, that is responsible for IL2 production. IL2 then interacts with its homologous receptor IL2R. IL2 plays a critical role in antigen-specific immune response and the IL2/IL2R interaction determines the size and duration of response.

IL-2R mediates the action of IL-2. Although not normally detected on resting T and B cells IL-2R is found on the surface of cells stimulated by an immune challenge. When cells are stimulated by such an immune challenge and begin to divide, the expression of Il-2R changes in two ways: more molecules of Il-2R are expressed on the cell's plasma membrane and a form of the IL-2R protein is released by the activated cells into the fluid surrounding them (Nelson 1986). IL-2R has been found to be elevated in certain conditions such as ovarian cancer (Hurteau 1995), rheumatoid arthritis (Rubin 1990) and post renal transplant rejection (Colvin 1987). Raised IL-2R levels have also been found in patients with viral infections, such as AIDS and in certain autoimmune diseases including autoimmune thyroid disease (Nakanishi 1991), rheumatoid arthritis (Manoussakis 1989), systemic lupus erythematosus (Manoussakis 1989) and autoimmune chronic active hepatitis (Simpson 1995). Autoimmunity has recently been implicated as an important factor in miscarriage (Scott et al 1987) and so, for this present study, serum samples from aborters were assayed for IL-2R and compared to samples from normal pregnant and non pregnant subjects.

5.2.3 Interleukin 6 (IL-6)

IL-6 is a multifunctional 26 kD polypeptide that appears to be directly involved in the responses that occur after infection, inflammatory stimuli or injury. It acts on a variety of cells, including fibroblasts, activated T cells, B cells and

hepatocytes. IL-6 appears to interact with IL-2 in the proliferation of T lymphocytes (Nordan 1986). IL-6 levels have been found to be raised in amniotic fluids of women who aborted within 30 days after the procedure to remove the fluid, when compared to matched controls who delivered healthy babies at term (Wenstrom 1996).Wenstrom concluded that up to 12% of pregnancy losses may result from preexisting subclinical intrauterine inflammation. They found that although the overall correlation between maternal serum and amniotic fluid IL-6 levels was good, only one of the eight cases who aborted would have been identified by a maternal serum IL-6 level.

5.2.4 Interleukin 8 (IL-8)

IL-8 is an 8kD protein which exhibits chemotactic activity *in vitro* for T cells (Larsen 1988), basophils and enzymes. IL-8 was originally isolated from human monocytes and distinguished from IL-1 by its chemotactic activity (Yoshimura 1987) It is a pro-inflammatory chemokine which has also been known as neutrophil chemotactic factor (NCF), neutrophil activating protein (NAP-1), monocyte derived neutrophil chemotactic factor (MDNCF) or T-lymphocyte chemotactic factor (TCF). Il-8 is produced by monocytes, fibroblasts and keratinocytes in response to stimulation by lipopolysaccharides, Il-1 or TNF-α, or, in the case of T lymphocytes, by PHA stimulation. Il-8 has been shown to be present in the endometrium in normal pregnancy (Jones 1997). Il-1 is known to invoke Il-8 activity (Puren 1997). As levels of Il-1 were decreased in the serum of aborting women in this study, we decided to look at serum levels of Il-8.

5.3 Measurement of interleukins/cytokines

The cytokines are measured in "sandwich ELISA" assays. Detailed methods are given in chapter two. The principle of this type of assay is summarised below and in figure 5.1.

The cytokine to be measured is the antigen in this assay. Antibody to the cytokine is bound onto the wells of a microtitre plate. A set of standards of known concentration of antigen and test solutions of unknown concentration are added and allowed to bind, forming antibody/antigen complexes on the plate. The plate is now washed, removing any unbound antigen. A second population of cytokine specific antibody conjugated to an enzyme (eg peroxidase, alkaline phosphatase) is added. After incubation, unbound labelled second antibody is removed by washing. Immobilised enzyme is detected following the addition of a sensitive enzyme substrate and a coloured end product proportional to the amount of cytokine present in the standard or sample is measured.

Sandwich ELISA assay

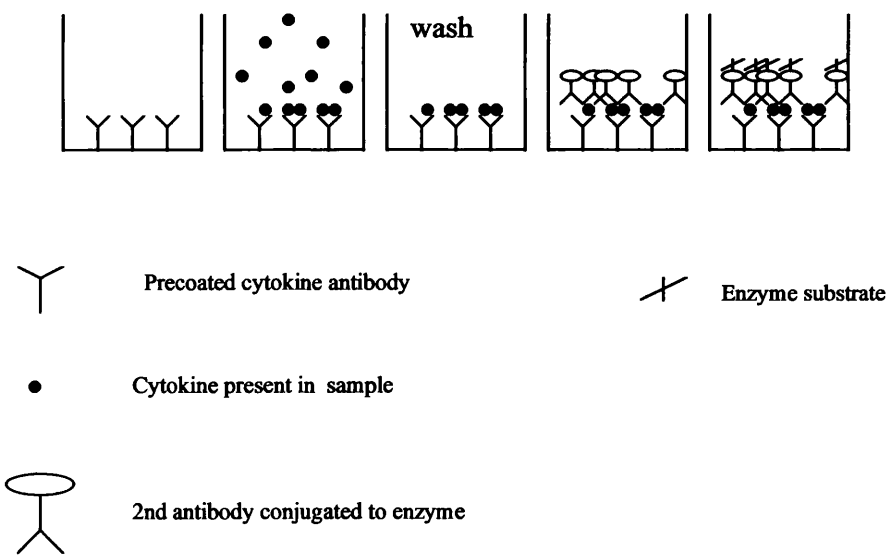


Figure 5.1 **Sandwich ELISA**

5.4 Subject Population

Interleukin-1

Il-1 estimation by ELISA was carried out on serum samples from the following groups;

18 non pregnant women not on the oral contraceptive pill, with a mean menstrual cycle of 28 plus or minus 3 days duration

18 women in the first trimester of pregnancy and with no history of miscarriage

21 women admitted to hospital suffering a spontaneous abortion.

17 women with a history of recurrent miscarriage ie two or more abortions

(rec aborter).

There was no significant age difference between the groups.

The following table summarises the results.

	Il-1β (pg/ml)	
	mean	sd
non pregnant n=18	202.4	157.2
pregnant n=18	218.8	153.6
sp aborters n=21	51.8	49.6
rec aborter n=17	242.4	137.0

Table 5.2 **Il-1β results**

Because the data were not normally distributed, a non parametric statistical analysis (Mann Whitney) was carried out.

There was no significant difference in the results of samples from non pregnant women compared to those of normal pregnant women or recurrent aborters.

Results of spontaneous aborters were found to be significantly lower than those of non pregnant ($p=0.0001$), pregnant ($p=0.0001$) and recurrent aborters ($p<0.0001$).

The following box and whiskers plot shows minimum, maximum, inter-quartile and median values for each group.

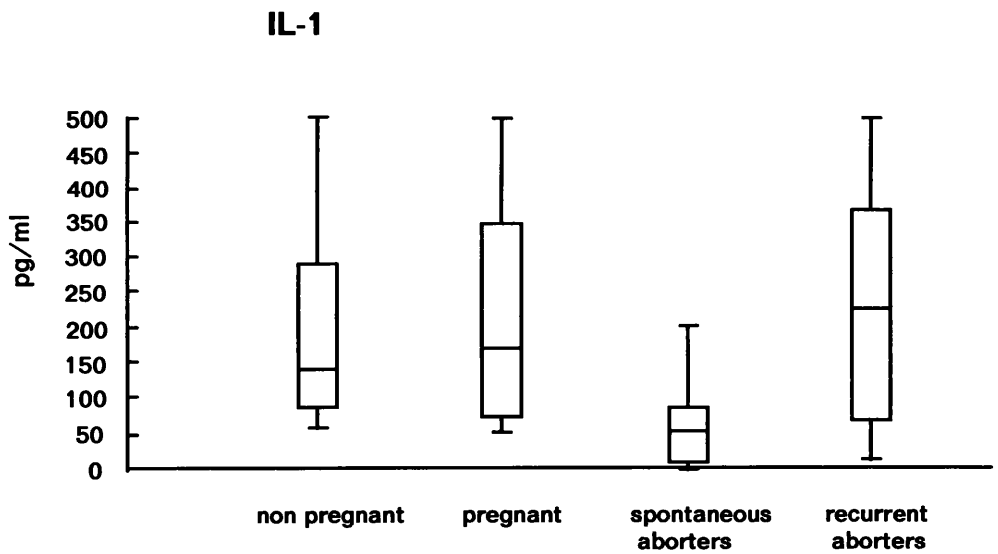


Figure 5.2. **Box and whisker plot; IL-1**

Il-6 estimation by ELISA was carried out on serum samples from the following groups;

12 non pregnant women not on the oral contraceptive pill, with a mean menstrual cycle of twenty-eight plus or minus 3 days duration (non preg)

14 women in the first trimester of pregnancy and with no history of miscarriage (preg)

8 women in the first trimester of their second pregnancy and whose first pregnancy had spontaneously aborted (f.u. preg).

18 women admitted to hospital suffering a spontaneous abortion (spont ab).

15 women with a history of recurrent miscarriage ie two or more abortions (rec ab).

10 women undergoing elective termination of pregnancy (stop).

There was no significant age difference between the three groups. Table 5.3 summarises the results.

	Il-6 (pg/ml)	
	mean	sd
non pregnant n=12	1.182	0.53
pregnant n=14	36.9	133.3
f.u. pregnancy n=8	3.53	6.04
sp aborters n=18	4.64	8.56
rec aborter n=15	3.03	4.87
stop n=10	53.4	157.1

Table 5.3. **Il-6 Results**

Because the data were not normally distributed, a non parametric statistical analysis (Mann Whitney) was carried out.

Il-6 levels were found to be significantly lower in non pregnant women when compared to women in 2nd pregnancy following spontaneous abortion (p=0.0166), spontaneous aborters (p=0.001), recurrent aborters (p=0.0097) and women undergoing elective termination (p=0.003). Mean Il-6 levels for pregnant group were high because one patient had level 500pg/ml. This same patient also had high levels of Il-1 (360 pg/ml) and Il-8 (>6000pg/ml). Although there was no reason to exclude this patient from the group of normal pregnant women it is interesting to note that removal of her results from the statistical analysis led to there being a statistical difference between the group of normal pregnant women and spontaneous aborters, aborters being higher (p=0.023).

The following box and whiskers plot shows minimum, maximum, inter-quartile and median values for each group.

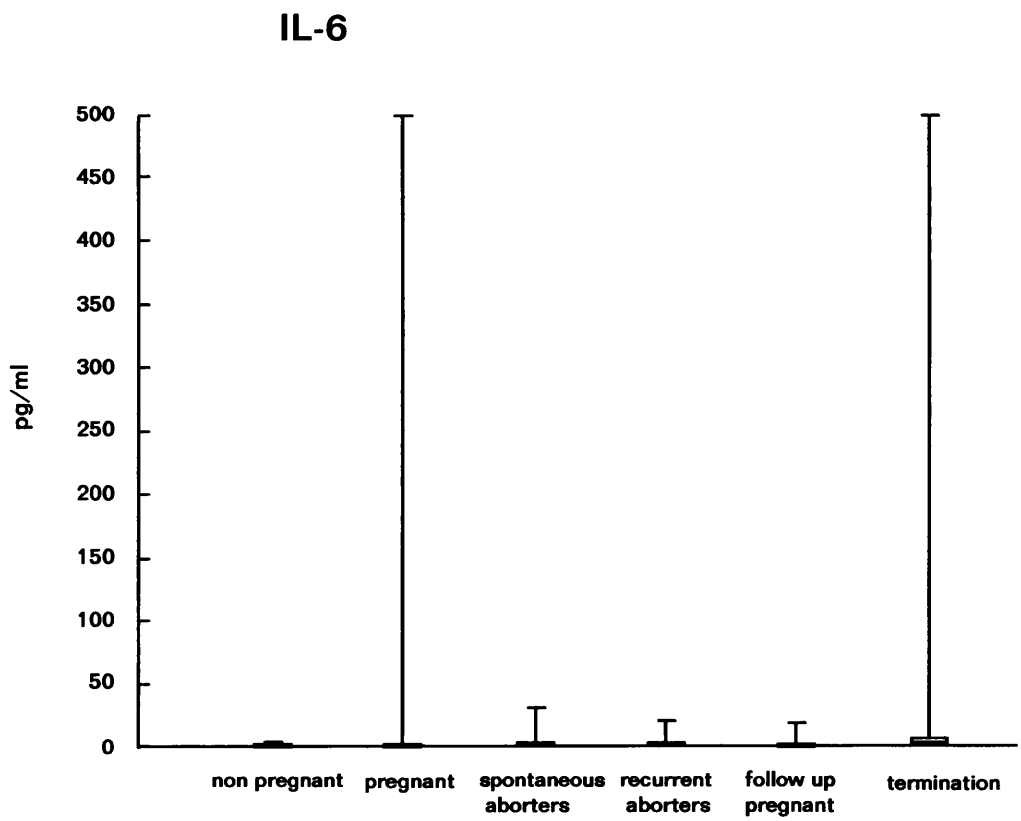


Figure 5.3. **Box and whisker plot; IL-6**

Il-8 estimation by ELISA was carried out on serum samples from the following groups;

21 non pregnant women not on the oral contraceptive pill, with a mean menstrual cycle of 28 plus or minus 3 days duration

20 women in the first trimester of pregnancy and with no history of miscarriage

20 women admitted to hospital suffering a spontaneous abortion.

15 women with a history of recurrent miscarriage ie two or more abortions

There was no significant age difference between the four groups. Table 5.4

summarises the results.

	Il-8 (pg/ml)	
	mean	sd
non pregnant n=21	0.333	1.528
pregnant n=20	600	1847
sp aborters n=19	48.1	152.9
rec aborter n=15	82.9	105.5

Table 5.4. **Il-8 Results**

Of the 21 non pregnant women 20 had undetectable levels of Il-8 and one sample had a level of 7pg/ml.

Of the 20 normal pregnant women 18 had undetectable levels of Il-8 and two samples each had grossly elevated Il-8 levels of >6000pg/ml. One of these patients also had high levels of Il-1β (360 pg/ml) and Il-6 (>500 pg/ml). These results were not available for the other patient.

Of the 20 women admitted to hospital suffering a spontaneous abortion 17 had undetectable levels of Il-8 and the 3 others had Il-8 levels of 104,153 and 656 pg/ml respectively.

Of the 15 women with a history of recurrent abortion six (40%) had raised IL-8 levels of 185,202,202,206,212 and 236 pg/ml.

Because the data were not normally distributed, a non parametric statistical analysis (Mann Whitney) was carried out.

There was no significant difference in the results of samples from non pregnant women compared to those of normal pregnant women or spontaneous aborters. There was also no significant difference in the results of samples from normal pregnant women compared to spontaneous aborters.

Mann Whitney analysis of IL-8 for non pregnants versus recurrent aborters showed a significant difference ($p=0.0071$). Chi squared analysis of the incidence of Il-8 being detected in serum of pregnant women compared to those women who were recurrent aborters showed the presence of Il-8 to be more prevalent in recurrent aborters ($p<0.05$).

Il-2R estimation by ELISA was carried out on serum samples from the following groups;

12 non pregnant women not on the oral contraceptive pill, with a mean menstrual cycle of 28 plus or minus 3 days duration

13 women in the first trimester of pregnancy and with no history of miscarriage

21 women admitted to hospital suffering a spontaneous abortion.

There was no significant age difference between the three groups. Table 5.5 summarises the results.

	Il-2R (pg/ml)	
	mean	sd
non pregnant n=12	3680	683
pregnant n=13	4003	1119
sp aborter n=21	16457	7760

Table 5.5 **Il-2R Results**

Because the data were not normally distributed, a non parametric statistical analysis (Mann Whitney) was carried out.

There was no significant difference in the results of samples from non pregnant women compared to those of normal pregnant women.

Results of spontaneous aborters were found to be significantly higher than both non pregnant and pregnant results ($p<0.0001$ in both instances).

The following box and whiskers plot shows minimum, maximum, inter-quartile and median values for each group.

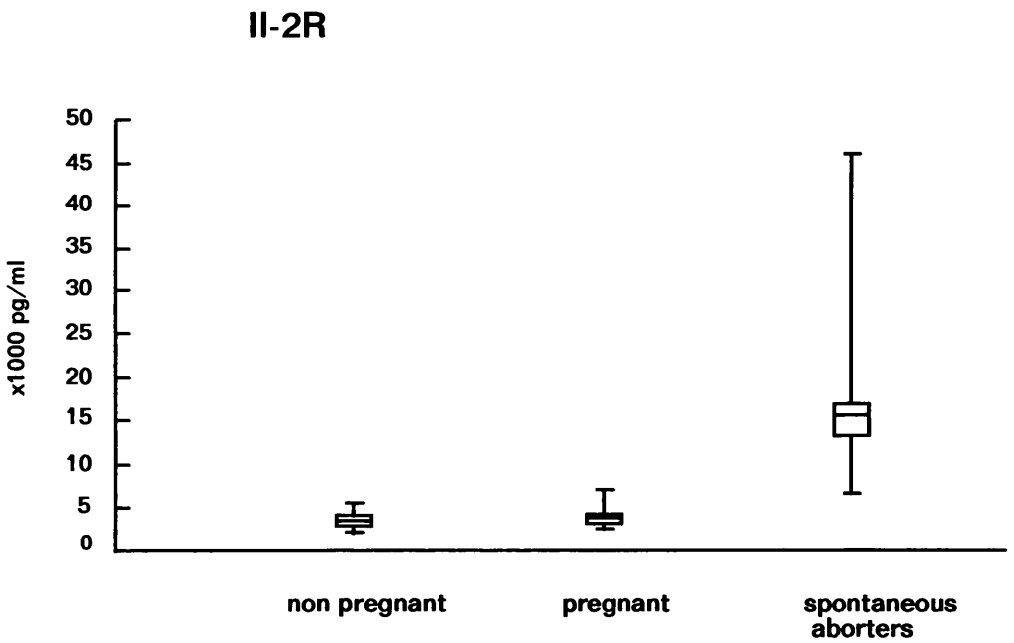


Figure 5.4. **Il-2R Inter quartile ranges**

5.5 Discussion

Table 5.6 gives a summary of significant differences found between the various groups.

	Il-1	**Il-2R**	**Il-6**	**Il-8**
non pregnant v pregnant	nsd	nsd	nsd	nsd
non pregnant v spontaneous aborters	p=0.0001 (sp ab ↓)	p<0.0001 (sp ab ↑)	p=0.001 (sp ab ↑)	nsd
non pregnant v recurrent aborter	nsd	****	p=0.0097 (rec ab ↑)	p=0.0071 (rec ab ↑)
pregnant v spontaneous aborters	p=0.0001 (sp ab ↓)	p<0.0001 (sp ab ↑)	nsd	nsd
spontaneous aborters v recurrent aborter	p<0.0001 (sp ab ↓)	****	nsd	nsd
non pregnant v follow up pregnant	****	****	p=0.016 (fu preg ↑)	****
non pregnant v termination	****	****	p=0.003 (term ↑)	****

↑ higher than ↓ lower than **** not applicable

Table 5.6. **Interleukins Summary of Results**

This study found there to be no difference in the pattern of cytokines measured between non pregnant women and healthy pregnant women. Samples from pregnant women were taken in the first trimester of pregnancy. Other studies have found that levels of cytokines increased with gestational age. Il-6 and Il-8 were

found in amniotic fluid in the latter half of gestation, with Il-8 being detected in blood plasma at this time (OlIah 1996). Il-1 and Il-6 were found to increase with gestational age (Austgulen 1994). Il-2R was found in one study to be raised in retroplacental, but not peripheral serum in normal pregnancy (Lien 1996).

Spontaneous aborters were found to have lower Il-1 levels and raised Il-2R when compared to both non pregnant and healthy pregnant women. Why this should occur is unclear, but raised Il-2R is found in cases of transplant rejection (Colvin 1987) and autoimmunity (Xiu 1994, Kroemer 1989). As discussed in chapter one the rejection of the foetal allograft and autoimmunity are two mechanisms often cited as possible causes of spontaneous abortion. Raised levels of Il-6 and Il-8 may be indicators of inflammatory response and fever or infection which are also discussed in chapter one possible mechanisms of spontaneous abortion.

Chapter Six

Thyroid Auto-antibodies

6.1 The Thyroid

The thyroid gland is an endocrine gland that sits as two lobes, connected by an isthmus, on either side of the trachea, just below the larynx (figure 6.1). It weighs approximately 20 grams and consists of cuboidal epithelial cells arranged into sacs called follicles (figure 6.2) (Mc ougal 1992a). The thyroid secretes the hormones thyroxine (T_4) and tri-iodothyronine (T_3). Calcitonin (thyrocalcitonin) is produced by the parafollicular or C-cells of the thyroid (Austin 1981). The parathyroid glands which are situated behind the thyroid, produce parathyroid hormone (PTH). Thyroid hormones affect the growth rate and metabolism of all of the body's cells. They control reflexes and regulate the rate at which the tissues produce energy. Thyroxine regulates the rate of oxygen consumption and the metabolic rate (the rate which the body burns up its fuel). Calcitonin and PTH function to help maintain homoeostasis of blood calcium (Breslau 1992).

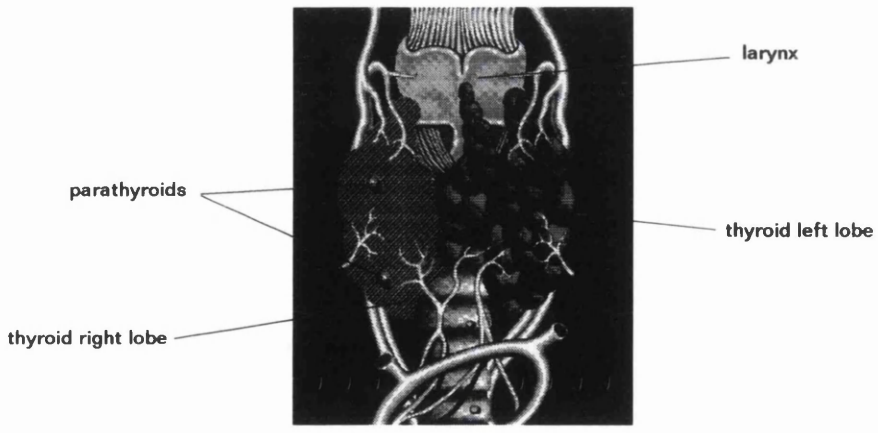


Figure 6.1 **The thyroid**

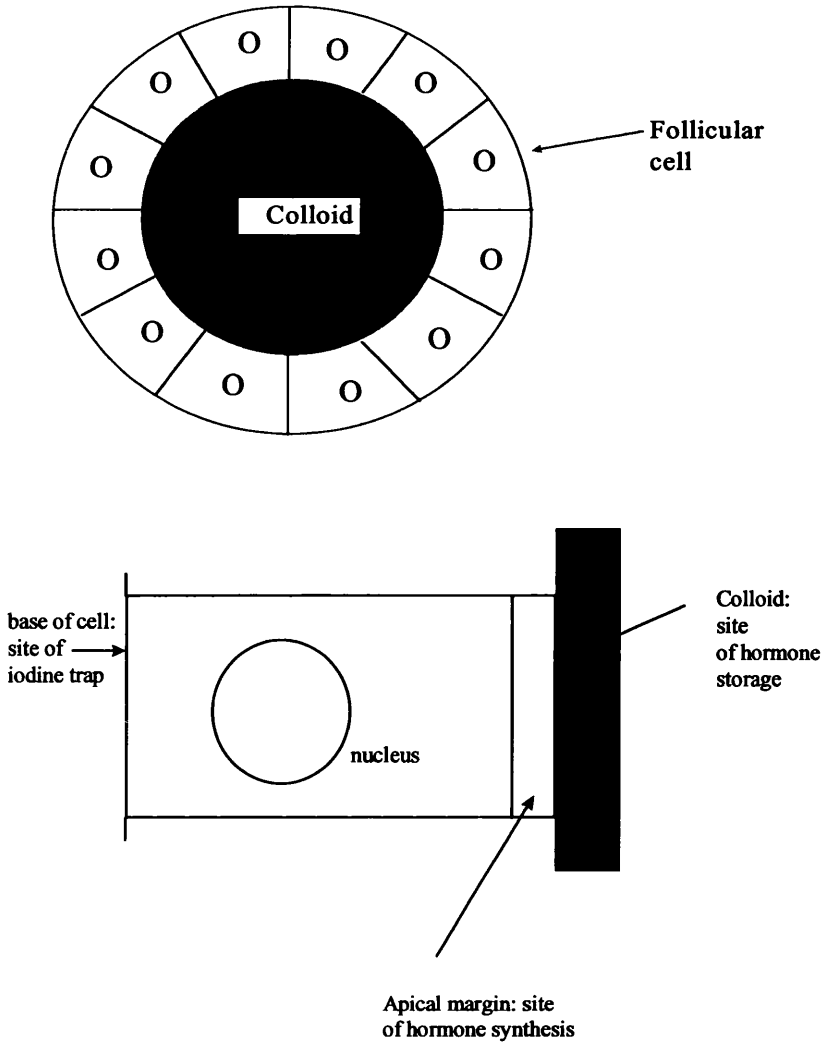


Figure 6.2 **Follicle & follicular cell**

T_3 and T_4 are composed of multiple copies of the amino acid tyrosine, bound to either three or four iodine molecules (figures 6.3 & 6.4) (McDougal 1992b). The epithelial cells of the thyroid are arranged in spherical structures called follicles. The thyroid hormones are stored in the colloid substance that fills the follicles, bound to the protein thyroglobulin (Tg). Thyroid hormones are released from the breakdown of Tg into the extracellular space and plasma, largely bound to the three transport proteins thyroxine binding globulin (TBG), thyroxine binding pre albumin (TBPA) and albumin. The small amounts of T_3 (0.3%) and T_4 (0.03%) not bound to transport proteins are called free hormones. The formation and release of

T_3 and T_4 are under the control of thyroid stimulating hormone (TSH), a glycoprotein with two peptide chains, which is produced in, and excreted from, specific cells in the anterior pituitary.

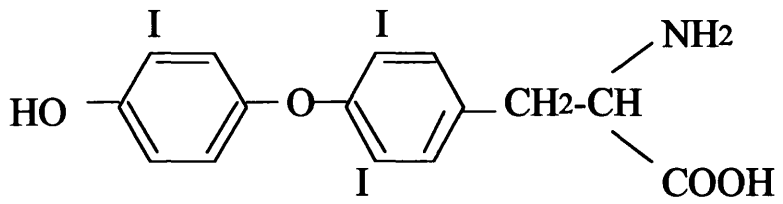


Figure 6.3. **The structure of T3**

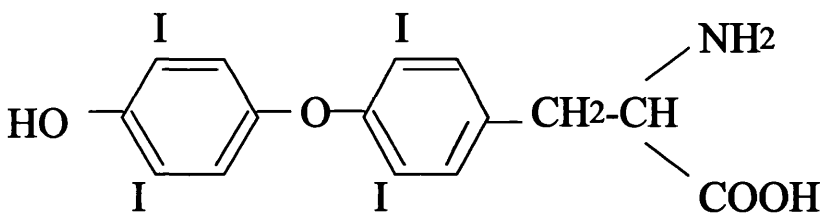


Figure 6.4. **The structure of T4**

6.2 Thyroid Disease

An individual with normal circulating levels of the thyroid hormones is said to be euthyroid.

Levels of thyroid hormones insufficient to the requirement of the body gives rise to hypothyroidism. Figures given in the literature for the incidence of this disease vary. In the UK 3.3 per 1,000 are affected (Wartofsky 1996) or up to 1% of the population (McDougal 1992b) Hypothyroidism is 5-10 times more commonly found in women than in men and increases in frequency with age so prevalence rates will vary with the age of population sampled.

Hypothyroid patients may have a disease of the thyroid (primary hypothyroidism), pituitary (secondary hypothyroidism) or hypothalamus (tertiary hypothyroidism) Of these the most common is primary hypothyroidism. In overt primary hypothyroidism serum TSH levels are invariably raised, T_4 levels are depressed and thyroid antibodies are often found, reflecting the underlying autoimmune process that is believed to be the usual cause (Tunbridge 1981). Hashimoto's thyroiditis, first described in 1912, is a disease, mostly found in women over forty, in which thyroid insufficiency is accompanied by the presence of goitre (an enlargement of the thyroid).

Excess levels of thyroid hormones is hyperthyroidism or thyrotoxicosis (McDougal 1992d). In the UK figures given in the literature for the prevalence of thyrotoxicosis in women vary from 2.5-3.0 per 1,000 (Wartofsky 1996) to around 2%, a figure which is 5-10 fold higher than that for males (Weetman 1994). The commonest syndrome to cause hyperthyroidism is diffuse toxic goitre or Graves' disease, an

organ specific autoimmune disease where there is production of anti TSH receptor antibodies. Symptoms of Graves' disease include tachycardia, nervousness, weight loss and palpitations as well as goitre. Graves' disease is associated with other autoimmune disorders, both organ specific and generalised, more frequently than by chance (McDougal 1992c).

Autoimmune thyroid disorders such as Graves' disease or Hashimoto's disease are 5 to 8 times more common in women than in men (Wartofsky 1996).

6.2.1 Thyroid Disease and Pregnancy

In normal pregnancy the thyroid undergoes changes in both structure and function. By the end of the first trimester renal tubular reabsorption of iodide falls, resulting in increased urinary excretion of iodide and a relative deficiency in plasma iodide. To compensate for this the thyroid triples its uptake of iodide from the blood (Rodin 1989). This adaptation is usually sufficient to maintain production of thyroid hormones, but where there is a dietary iodine deficiency, pregnant women may develop a goitre.

Because of oestrogen mediated increase in TBG synthesis the concentration of serum TBG doubles by the twelfth week of pregnancy. This results in increased amounts of bound T_3 and T_4 in the serum and this is reflected in measurements of total T_3 and T_4 in the serum.

Thyroid disease is estimated to complicate 0.5% of all pregnancies (Rodin 1989).

Some thyroid diseases, particularly autoimmune thyroid disease, tend to be less severe during pregnancy. During pregnancy levels of circulating lymphocyte subsets such as CD4+ve are reduced and natural killer cell activity is depressed

(Lazarus 1994). This modification of the maternal immune response appears to have a significant influence on disease activity in autoimmune disorders.

Hyperthyroidism is rare in pregnant women (prevalence 0.05-0.2%). Graves' disease is the commonest form of hyperthyroidism in pregnancy, though this disease may go into remission during pregnancy due to decreased maternal immunologic activity. In some patients the hyperthyroidism is so mild it may go unnoticed, but will relapse in the post partum period. This post partum exacerbation of the disease process must be distinguished from the condition known as post partum thyroid disease.

Hypothyroid females are relatively infertile and as a result hypothyroidism is rarely found in pregnancy. Where it does occur Hashimoto's thyroiditis is the commonest cause.

Post partum thyroid disease is a transient thyroid dysfunction associated with painless goitre which occurs after childbirth in 7% of previously healthy women with no personal or family history of thyroid disease (Barnett 1994). Anti thyroid peroxidase (anti-TPO) antibodies are found in the serum and there is a marked lymphocytic infiltration of the thyroid gland. A recent study (Lazarus 1996) found that 73 women out of 152 with anti TPO antibodies in the serum while pregnant, went on to suffer from post partum thyroiditis, compared to a group of 239 controls, none of whom showed symptoms of the disease.

6.3 Thyroid Auto-antibodies

Thyroid cells have three main antigens against which antibodies are produced. These are thyroid stimulating hormone receptor, thyroid microsomes (or

thyroid peroxidase, TPO) and thyroglobulin (Tg). The antibodies produced to these antigens are respectively called thyrotrophin receptor antibody (TRAb), anti-TPO and anti Tg.

Anti Tg and anti TPO have been implicated as potentially important factors in miscarriage (Stagnaro-Green et al 1990). Acceptance of the foetal allograft is a major physiologic response to pregnancy. The immune system is thought to be responsible for many miscarriages, with autoimmunity recently implicated as an important factor (Scott et al 1987). Stagnaro-Green et al found that the tendency to secrete detectable levels of anti Tg and anti TPO was significantly correlated with an increased rate of miscarriage. They concluded that anti Tg and anti TPO were independent markers of "at risk" pregnancy.

6.4 Subject Population

Using ELISA kits, with methods as described in chapter two, anti Tg and anti TPO levels were measured in the following groups of patients.

Group 1. 10 healthy, non pregnant women not on the oral contraceptive pill, with a mean menstrual cycle of 28 plus or minus 3 days duration.

Group 2. 11 healthy women in the first trimester of an ongoing pregnancy.

Group 3. 11 women admitted with spontaneous abortion. Blood was obtained from these women prior to them going to surgery for evacuation of the retained products of conception.

Group 4. 10 healthy women admitted to hospital for termination of pregnancy.

Blood was taken from these women 2-3 hours after termination.

Group5. 11 women with a history of recurrent miscarriage ie more than three miscarriages, no live births. All were pregnant at time of sampling.

Samples were sent to the haematology laboratories in GRI for estimation of anticardiolipin and antinuclear antibodies as there an association has been reported between these antibodies and pregnancy loss (MacLean 1994). All patients were negative for these antibodies.

All blood samples were separated and stored as serum in -20°C freezer till the day of the assay when they were removed and allowed to come to room temperature.

Routine thyroid function tests (T_4 and TSH) were carried out in the GRI biochemistry department on samples from all groups. The normal reference ranges for these tests are:

	T_4	TSH
non pregnant	55-144 nmol/l	<5 mU/l
pregnant	55-158 nmol/l	<5 mU/l

Table 6.1. **Normal reference ranges for T_4 and TSH**

In accordance with the literature received with the kits anti Tg values greater than 8U/ml and anti TPO values greater than 1U/ml are taken to be positive.

6.5 Results

The results are shown in table 6.2 as mean ± standard deviation. Statistical significance was tested for using the Mann Whitney U test. Chi-squared analysis was performed on the data from antibody analysis.

Group	**Gestation** (weeks)	**n**	**mean age** (years)	**T_4** (nmol/l)	**TSH** (U/ml)	**antibody +ve**	
						Tg	TPO
1 non preg		10	28.4 ± 3.2	99 ±29	1.1 ± 0.5	0	0
2 pregnant	9.4 ± 1.1	11	24.5 ± 4.1	143 ± 39	1.3 ± 0.3	0	0
3 sp. ab.	9.3±2.0	11	25.5 ± 3.8	110 ± 32	1.0 ± 0.5	0	0
4 termin.	6.8 ± 2.2	10	25.7 ± 6.9	114 ± 27	2.0 ± 2.2	1	1
5 rec. ab.	7.8 ± 1.4	11	30.4 ± 4.8	94 ± 9	1.7 ± 0.9	1	3

Table 6.2. **Thyroid Test Results**

Total T_4 levels were significantly lower in groups 4 and 5 (induced and recurrent abortion) when compared to group 2 (healthy pregnant) with P values < 0.001 and < 0.03 respectively.

No thyroid antibodies were detected in groups 1, 2 or 3 (healthy, non pregnant, healthy pregnant and spontaneous aborters). 2 out of 10 termination patients and 4 out of 11 recurrent aborters had detectable thyroid antibodies. Chi-squared analysis

of this data showed the prevalence of thyroid antibodies to be significantly higher ($p < 0.01$) in women with a history of recurrent abortion.

6.6 Discussion

Recently there have been a number of studies looking at thyroid antibodies in pregnancy. In the study undertaken by Stagnaro-Green et al (1990) 552 first trimester women were involved. They found 20% to be thyroid antibody positive. Of these 17% miscarried compared with only 8.4% of the antibody negative women. This study did not determine whether the women were first time aborters or whether they had a history of unsuccessful pregnancy. This present study, although much smaller than Stagnaro-Green's has attempted to look at abortion sub groups and shows increased incidence of thyroid antibodies in the group of recurrent , but not spontaneous, aborters.

Although women in the child-bearing age range have a higher incidence of circulating thyroid antibodies than is found in the general population (Vargas et al 1988) no thyroid antibodies were detected in groups 2 and 3, the healthy pregnant and spontaneous aborters. A recent study (Pratt et al 1993) found that the incidence of anti-thyroid antibodies in women who have had recurrent abortions was not significantly increased compared to a normal random control population. However, they found that anti-thyroid antibodies do occur with greater frequency in women with a history of recurrent spontaneous abortion than non-organ-specific-autoantibodies.

A role for thyroid hormones in the maintenance of early pregnancy has been implied in a recent study (Maruo et al 1992). This group looked at thyroid function

results in patients with threatened abortion. They found serum levels of total T_3 and T_4 and free T_3 and T_4 to be lower in patients who subsequently went on to abort than in those who had a successful pregnancy outcome. They found that T_3 and T_4 levels were higher at the onset of threatened abortion compared to pre pregnancy for those women who did not abort, but for those who aborted T_3 and T_4 levels did not rise from the pre pregnancy levels. They concluded from this that raised levels of maternal thyroid hormones may be required to maintain early pregnancy. In this present study total T_4 levels were significantly lower in women with a history of miscarriage ($p < 0.03$) and those undergoing induced abortion ($p < 0.001$) when compared to healthy pregnant women. All T_4 values were within the normal euthyroid range for women in the first trimester of pregnancy. The reduced gestation for these groups compared to the healthy pregnant group may explain in part the lower T_4 levels.

The findings of this chapter were published in the European Journal of Endocrinology (Roberts 1996)

Chapter Seven

Discussion

7.1 Effect of Variation in Menstrual Cycle

The statistical findings with respect to variations in the menstrual cycle are summarised in table 7.1.

Immunoglobulins			
IgG	**IgM**		
nsd	nsd		
Mitogens			
PHA	**ConA**	**PWM**	
nsd	nsd	nsd	
Free radical scavengers			
PSH	**LSH**	**Cp.**	**SOD**
nsd	p= 0.0466 (follicular ↑ than luteal)	nsd	nsd

Table 7.1. **Summary of statistical analyses: variation in menstrual cycle**

This study has shown that B cell production of antibodies or stimulation of lymphocytes by mitogens did not significantly differ throughout the menstrual cycle in non pregnant women. The time of sampling within the menstrual cycle would thus appear to be an unimportant factor when measuring these parameters. This has important implications for other studies.

Free radical scavenger levels were found to remain constant throughout the menstrual cycle, with the exception of lysate thiol levels which were significantly

higher in the follicular phase than in the luteal phase (p=0.0467).

7.2 Effect of Normal Pregnancy

The statistical findings with respect the effect of normal pregnancy are summarised in table 7.2.

Immunoglobulins			
non-pregnant vs. pregnant			
IgG	**IgM**		
p=0.0355 (preg ↓)	nsd		
Mitogens			
non-pregnant vs. pregnant			
PHA	**ConA**	**PWM**	
nsd	nsd	nsd	
Free radical scavengers			
non-pregnant vs. pregnant			
PSH	**LSH**	**Cp.**	**SOD**
nsd	nsd	p= 0.0001 (preg ↑)	p= 0.0187 (preg ↑)
Cytokines			
non-pregnant vs. pregnant			
IL-1	**IL-2R**	**IL-6**	**IL-8**
nsd	nsd	nsd	nsd

Table 7.2. **Summary of statistical analyses: effect of normal pregnancy.**

There were no patients with detectable thyroid antibodies in either group.

Normal pregnancy is associated with changes in immune function (as can be seen in lower IgG levels) and in anti-oxidant levels (as seen in raised caeruloplasmin and SOD levels). As discussed in Chapter one, extracellular proteins with a large proportion of disulphide bridges such as IgG or albumin appear to be particularly susceptible to hydroxyl and peroxy radical attack. The results in this thesis concur with this.

If the T_H2 bias of pregnancy postulated by Wegmann (1993) and discussed in the introduction of this thesis is correct then B-cells taken from pregnant woman will be up-regulated and producing large amounts of antibody. Stimulation of these, already up regulated cells, might not result in the production of large amounts of antibody and the stimulation index would therefore be smaller as was found when comparing pregnant with non pregnant women.

These changes were detected in the first trimester of pregnancy when compared to non pregnant women. Whether the immune response causes changes in antioxidant levels or vice versa is not clear.

This study has shown changes in anti-oxidant levels in the peripheral circulation of healthy pregnant women. Whether these changes are a direct result of events in the uterus is not clear. However, previous workers have shown that free radicals can effect certain immune processes. Increased levels of Il-2 are known to inhibit free radical production (Hoffield 1981). Normal pregnancy has been associated with a bias towards antibody associated immunity and away from cell mediated immunity (the T_H2 bias of pregnancy) (Wegmann 1993). As a result of this reduced levels of the abortogenic cytokine Il-2 are found. Reduced levels of Il-2 could explain the increased anti-oxidant activity noted in this study. I found lower levels of anti-oxidants in spontaneous aborters when compared to normal pregnant women.

This may be an indication that a failure to mount this T_H2 bias of pregnancy was the cause of miscarriage for some of these women. These findings suggest that events in the uterus influence events in the peripheral circulation.

Cytokine levels were not significantly different in pregnant women when compared to non pregnant women. This concurs with findings of other workers (Austgulen 1994, Lien 1996) looking at peripheral blood in early pregnancy.

Thyroid antibodies were not detected in the serum of either non pregnant or pregnant women. Other workers, in a larger study, found that 20% of pregnant women had thyroid antibodies (Stagnaro-Green 1990). These women's past pregnancy history was not indicated, while the small group of pregnant women in this present study were primigravidae.

This study looked at peripheral blood. Changes found in peripheral blood may reflect what happens in the uterus or indeed may be driven by events in the uterus.

7.3 Effect of Abortion

The statistical findings with respect the effect of abortion are summarised in table 7.3.

Immunoglobulins			
non-pregnant vs. abortion			
IgG	**IgM**		
nsd	nsd		
Mitogens			
non-pregnant vs. abortion			
PHA	**ConA**	**PWM**	
nsd	nsd	nsd	
Free radical scavengers			
non-pregnant vs. abortion			
PSH	**LSH**	**Cp.**	**SOD**
nsd	nsd	p= 0.0048 (aborters ↑)	nsd
Cytokines			
non-pregnant vs. abortion			
IL-1	**IL-2R**	**IL-6**	**IL-8**
p=0.0001 (sp ab ↓)	p<0.0001 (sp ab ↑)	p=0.001 (sp ab ↑)	nsd

Table 7.3. **Summary of statistical analyses: effect of abortion.**

There were no thyroid antibodies in the non-pregnant group, 4 out of 11 recurrent aborters were positive for thyroid antibodies.

Cp levels were higher in women following spontaneous abortion compared to non pregnant women. This concurs with another study (Sane 1991) which reported evidence of increased free radical activity in women undergoing abortion (induced or spontaneous).

An autoimmune cause for spontaneous abortion is indicated by presence of thyroid antibodies and raised Il-2R. Raised Il-6 may indicate the presence of an infection being the cause of abortion.

Free radical scavengers pregnant vs. abortion				
	PSH	**LSH**	**Cp**	**SOD**
$0+^{0}$ vs. 1y aborters	nsd	nsd	nsd	P= 0.0463 (aborters↓)
$0+^{0}$ vs. 1^{st} aborters	nsd	nsd	nsd	P= 0.0129 (aborters↓)
$0+^{0}$ vs. all aborters	nsd	nsd	nsd	P= 0.0339 (aborters↓)
pregnant vs. 1y aborters	nsd	nsd	p= 0.0323 (aborters ↓)	p= 0.0218 (aborters↓)
all pregnant vs. all aborters	P- 0.0310 (aborters ↓)	nsd	nsd	P= 0.0023 (aborters↓)
Cytokines pregnant vs. abortion				
	Il-1	**Il-2R**	**Il-6**	**Il-8**
	p=0.0001 (sp ab ↓)	p<0.0001 (sp ab ↑)	nsd	nsd

Table 7.4. **Summary of statistical analyses: effect of abortion.**

Spontaneous aborters generally had lower levels of SOD than pregnant women. Although there were limited numbers in this study it appears that these abortions were influenced by neither parity nor previous abortions. This information had not previously been available.

7.4 T_H2 bias of pregnancy

As discussed in the introduction of this thesis T-helper cells can be sub-grouped as follows;

> T_H1 cells produce cytokines detrimental to pregnancy such as Il-2, IFN-γ and TNF-β and induce NK activity.
>
> T_H2 cells produce cytokines beneficial to pregnancy such as Il-3, Il-4, Il-5 and Il-10 and induce B-cell production.

The hypothesis that normal pregnancy is allowed to continue because the body mounts a response in favour of humoral (antibody-mediated) and away from cellular (T-cell-mediated) immunity is known as the T_H2 bias of pregnancy. Much of the work in this area has been carried out on mice (Chaouat 1995) and in ruminants (Martal 1997). Other work on human trophoblast cell lines has been reported. In this study peripheral blood has been studied. Although any changes found may or may not reflect what is happening *in utero,* ease of sampling was the main criteria for looking at peripheral blood. Modulations of cytokine expression in the peripheral blood of third trimester women have been noted recently (Tranchot-

Diallo 1997).

Some of the findings discussed in this thesis concur with the T_H2 bias of pregnancy. These are:

The lower stimulation index found in IgG production of pregnant women compared to non pregnant women suggests that the B-cells of pregnant women are already in an up regulated state.

Levels of some anti-oxidants were found to be lower in aborters than levels found in blood of pregnant women. The cytokine Il-2 is known to be raised in cases of abortion and it is known that raised Il-2 levels cause a drop in levels of anti-oxidants.

Il-2R was raised in samples from aborters when compared to samples from pregnant women. Il-2R mediates the action of Il-2 and is found on the surface of cells stimulated by immune challenge.

It would be of interest for future studies to look at the presence in pregnancy and abortion of other cytokines implicated in the T_H2 bias of pregnancy such as Il-10 which is said to inhibit T_H1 cells. Another interesting area of research would be to look at trophoblast cell lines and the release of cytokines from these cells on the addition of blood from pregnant or aborting women.

The case for an immune cause of abortion might be stronger had karyotyping of all abortuses been carried out. Around 50% of all aborted foetuses are reported to be chromosomally abnormal (Simpson, 1980). Karyotyping of aborted material would have enabled me to verify this finding and, importantly, to discount those found to be abnormal from the study. Unfortunately, due to the expense of this technique,

and because of current clinical practice, karyotyping was not performed. Had samples from women with abnormal foetuses been discarded from the study this would possibly have had the effect of augmenting the prevalence of abortion caused by immunological abnormalities among those women left in the study. Thus, for example, anti thyroid antibodies were detected in 2 out of 10 women who suffered spontaneous abortion. If 5 of these 10 were taken out of the study because of chromosomal abnormality then this leaves 2 out of the remaining 5 to be antibody positive.

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