STANDARDIZATION OF FLOW CYTOMETRIC CROSSMATCH (FCXM) FOR INVESTIGATION OF UNEXPLAINED HABITUAL ABORTION

by

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Abstract

Recurrent pregnancy loss (RPL), defined as two or more pregnancy losses at any gestational age, affects many couples who are trying to establish a family. Research in the broad field of reproduction has focussed classically upon infertility, that is, the inability to conceive. The inability to maintain pregnancies, that is, recurrent pregnancy loss, remains an enigma, both on a pathophysiological and psychological level. Consequently, many couples who suffer from repeated pregnancy wastage are left without answers, without appropriate therapy and, consequently, often without a family.

This Master's thesis will provide an overview of the current knowledge about recurrent pregnancy loss. In particular, the immunological aspects of recurrent pregnancy loss will be discussed. Flow cytometry, as an investigative tool in reproductive immunology, will be described and clinically evaluated as a technique to detect maternal allosensitization to paternal mononuclear cells following immunization.

There are many intriguing questions to be answered in regard to the normal and abnormal immune response to pregnancy. With the thorough evaluation of the flow cytometric crossmatch methodology as a clinical tool to assess maternal allosensitization, as described in this Master's thesis, it is hoped that a collaborative approach will be undertaken to answer some of the fundamental questions about the maternal immune response to the fetal allograft. Hopefully this flow cytometric crossmatch methodology will eventually serve

a small role in the answering of Sir Peter Medewar's thought-provoking question of 1953, which was, "why does the fetus not habitually provoke an immunological reaction from its mother".

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Introduction

Recurrent pregnancy loss is a prevalent health problem in our country, affecting many couples who are trying to establish a family. The inability to maintain pregnancies, that is, recurrent pregnancy loss, remains an enigma, both on a pathophysiological and psychological level. Consequently, many couples who suffer from repeated pregnancy wastage are left without answers, without appropriate therapy and, consequently, often without a family.

This Master's thesis will provide an overview of the current knowledge about recurrent pregnancy loss, in particular, about potential mechanisms by which recurrent pregnancy loss may occur on an immunological basis. As well, current immunological investigations and treatment options will be discussed. The basic principles of flow cytometry and its use in reproductive immunology will be reviewed. A flow cytometric crossmatch methodology for use in the investigation of unexplained, possible alloimmune habitual abortion will be critically appraised and the results of a reference range study will be presented. Finally, the flow cytometric crossmatch will be assessed as a clinical tool to detect maternal allosensitization following paternal mononuclear cell immunization in couples with a history of primary unexplained, possible alloimmune habitual abortion.

With the standardization of the flow cytometric crossmatch, as presented in this Master's thesis, the clinical significance of maternal alloantibodies and the effect of maternal

allosensitization following immunotherapy in couples with unexplained habitual abortion could be evaluated collaboratively in multicentre studies, which would hopefully result in a better understanding of the normal and abnormal maternal immune responses to pregnancy.

Chapter 1

Recurrent Pregnancy Loss, an Overview

Recurrent pregnancy loss is a prevalent health problem in our country, affecting up to 5% of couples who are trying to establish a family[1]. Research in the broad field of reproduction has focussed primarily on the inability to conceive, that is, infertility. The inability to maintain pregnancies, that is, recurrent pregnancy loss, remains an enigma, both on a pathophysiological and psychological level. Consequently, many couples who suffer from repeated pregnancy wastage are left without answers, therapy and often without a family.

Definitions

"Recurrent pregnancy loss" is defined as the occurrence of two or more pregnancy losses at any gestational age. Other terms are often used interchangeably, but each differs slightly in meaning. "Recurrent spontaneous abortion" is a rather confusing term, since it has been defined as two, or three or more consecutive spontaneous abortions[2,3]. Furthermore, the World Health Organization's definition of an abortion, "the expulsion or extraction from its mother of an embryo or fetus weighing 500 grams or less"[4] (which corresponds to a gestational age between 20 and 22 weeks), is often not strictly adhered to. For example, in the European literature, a pregnancy loss up to 28 weeks's gestation is considered an abortion.

The term "habitual abortion" is more strictly defined, which therefore decreases some of the ambiguity in the literature. It has been defined as three or more consecutive spontaneous pregnancy losses, at less than 20 weeks' gestation, or, of a fetus weighing under 500 grams[5]. Habitual abortion has been further divided into primary and secondary habitual abortion, according to pattern of presentation. The classic definition of primary habitual abortion is three or more consecutive spontaneous abortions without an antecedent pregnancy which progressed beyond 20 weeks' gestation. As well, secondary habitual abortion is defined as three or more consecutive spontaneous abortions with an antecedent pregnancy which progressed beyond 20 weeks' gestation.

With the ready availability of karyotype analysis in the province of British Columbia, the term "habitual abortion" excludes spontaneous abortions in which karyotype analysis revealed aneuploidy, that is, an abnormal number of chromosomes, such as monosomy, trisomy or polyploidy, since this occurs randomly. In other words, the chance of aneuploidy in the next pregnancy does not increase if the prior spontaneous abortion was found to have aneuploidy by karyotype analysis.

Spontaneous abortion has been estimated to occur in 15% of all clinical pregnancies[6]; half of which are due to aneuploidy, although most of the data was obtained from women with $\leq$2 abortions[7]. By excluding spontaneous abortions in which karyotype analysis revealed aneuploidy, the cohort of patients being studied should be more likely to have an underlying factor associated with their history of habitual abortion.

As well as excluding aneuploidy, habitual abortion is divided into primary, secondary and unclassified habitual abortion, based on the woman's partner-specific obstetrical history. Primary habitual abortion is strictly defined as three or more consecutive, spontaneous abortions excluding any with documented aneuploidy without an antecedent pregnancy which progressed beyond 20 weeks' gestation with the woman's present reproductive partner. Secondary habitual abortion is strictly defined as three or more consecutive spontaneous abortions excluding any with documented aneuploidy with an antecedent pregnancy, or pregnancies, which progressed beyond 20 weeks' gestation with the woman's present reproductive partner. The term "unclassified habitual abortion" is used for couples who do not meet the definitions of either the primary or secondary habitual abortion, predominantly due to more than one reproductive partner in the woman's history of habitual abortion. Some treatment, such as paternal mononuclear cell immunization, appears to be efficacious for primary, but not secondary or unclassified habitual abortion.

In clinical practice it is very important to explain the terminology carefully to patients. For example, patients may regard "habitual" as a term which implies it was their own doing, thereby assigning blame. The term "abortion" is generally thought of as an elective termination of pregnancy. "Recurrent pregnancy loss" is a more understandable and socially acceptable term for general use in the physician's office. For scientific purposes, "habitual abortion" should be used since it is a term that has been strictly defined in the literature.

Frequency of Recurrent Pregnancy Loss

Naylor and Warburton retrospectively analysed reproductive histories from over 14,000 women and estimated the risk of a subsequent spontaneous abortion after 1 spontaneous abortion to be 23%, after 2 spontaneous abortions to be 29% and after 3 spontaneous abortions to be 33%[8]. In British Columbia, Poland et al. studied the morphology of spontaneously aborted embryos and fetuses and correlated their observations with factors from the maternal history, focussing primarily on the preceding reproductive history[9]. In all, 472 patients with 638 subsequent pregnancies were followed. The results of Poland et al. confirmed the observations made by Naylor and Warburton. The risk of a subsequent spontaneous abortion after 1 spontaneous abortion appeared not to change from the baseline rate of 19% seen in the B.C. population. After 3 consecutive spontaneous abortions without an antecedent live birth, the risk of a subsequent spontaneous abortion was found by Poland et al. to approach 50%.

Evaluation

Couples with a history of habitual abortion, defined as 3 or more consecutive spontaneous abortions excluding documented aneuploidic abortuses, should be offered a thorough evaluation to detect possible genetic, endocrine, infectious, anatomical and autoimmune factors. A decision to investigate earlier, for example, a woman over 35 years of age with two unexplained, consecutive spontaneous abortions, should be at the physician's

discretion, based on the clinical presentation and the couple's medical history.

An obstetrical history must be extensively documented, specifically in regard to the gestational age at which the spontaneous abortions occurred and their clinical presentations. Previous ultrasound reports are useful to differentiate between anembryonic and embryonic gestations. An anembryonic spontaneous abortion, often referred to as a "blighted ovum", occurs prior to six weeks' gestation and is characterized by an empty gestational sac on ultrasound. Since the pregnancy ceased so early in gestation, it is generally accepted that an anembryonic spontaneous abortion is due to aneuploidy. Conversely, an embryonic spontaneous abortion, more commonly referred to as an intrauterine fetal demise, is characterized by the presence of embryonic structures on ultrasound, including a yolk sac, fetal pole or a previously documented fetal heart. Despite the availability of endovaginal ultrasound, a thorough ultrasound evaluation for fetal structures is often not undertaken, thus limiting the usefulness of the procedure.

Complications associated with any previously successful pregnancies need to be well documented. A change in reproductive partners is important to delineate since some factors associated with habitual abortion appear to be partner specific.

Infertility, defined as unprotected intercourse for more than a year without conception, is often associated with a history of habitual abortion[11]. Premenstrual spotting or a change in menstrual cycle length may be suggestive of a luteal phase deficiency. A history of

intrauterine exposure to diethylstilbestrol is associated with both infertility and habitual abortion[12]. The association of endometriosis and habitual abortion remains controversial[13]. Repeated or vigorous uterine curettage may result in significant intrauterine adhesion formation, which has been previously estimated to be associated with habitual abortion in 10-15% of cases[5].

A medical history of an underlying autoimmune disease, such as systemic lupus erythematosus, hypothyroidism or diabetes mellitis, is occasionally associated with a history of habitual abortion. The history of a thromboembolic event could be associated with elevated antiphospholipid antibody levels, suggesting an autoimmune origin. It is important to evaluate migraines headaches, Raynaud's phenomenon and joint pain, since they are clinical features which may be related through their underlying cause to habitual abortion.

The association of smoking, alcohol or illegal drug use and habitual abortion has not been established clearly. Recent animal research has supported an association between stress and habitual abortion, possibly through the action of transforming growth factor beta[14].

A thorough physical examination of the woman is required. For example, skin or hair changes, thyroid enlargement, joint swelling or pain, livedo reticularis and peripheral vasculitis are all suggestive of an underlying autoimmune factor. A broad uterine fundus or duplication of the lower genital tract is suggestive of an anatomical factor. Hirsutism,

obesity and/or an unusual body habitus may be suggestive of an endocrinopathy.

Initial investigations include a complete blood count, a thyroid stimulating hormone (TSH) assay and a prolactin level. A structural genetic factor, such as a balanced Robertsonian translocation, is determined by karyotype analysis of both partners. Evaluation of the luteal phase by endometrial biopsy is required. The endometrial biopsy can also be used as the screening test for endometritis. The presence of plasma cells, infiltrates and giant cells are all suggestive of endometritis. Anatomical factors are classically assessed by an x-ray hysterosalpingography. If there is no associated history of infertility, other diagnostic procedures such as hysteroscopy or hysterosonography may be used as an alternative.

The use of screening tests for autoimmune factors varies widely from site to site, but usually consists of sensitive clotting-based assays and enzyme-linked immunosorbent (ELISA) assays to measure antiphospholipid antibody levels in the maternal serum. Presently, antibodies to cardiolipin IgG and IgM are the most extensively studied antiphospholipid antibodies in patients with a history of habitual abortion.

If endometriosis is suspected by history and physical, a diagnostic laparoscopy is warranted. The recent finding of elevated antiphospholipid antibody levels in women with endometriosis has certainly raised the question as to whether or not endometriosis is of an autoimmune origin[15].

Management

Once the investigation of a couple with habitual abortion is completed, the question of management is then posed. If an abnormality is identified, and it correlates with the clinical history, treatment should be offered. Empiric treatment, such as progesterone vaginal suppositories before completion of the investigations, will often delay evaluation of the couple with a history of habitual abortion, thereby deferring appropriate care.

Genetic counselling is required when a structural chromosome abnormality is identified in one of the partners. Hormonal abnormalities due to thyroid or pituitary disease require appropriate endocrine treatment. Presently, the luteal phase deficiency is being treated with either clomiphene citrate, which improves folliculogenesis, or progesterone vaginal suppositories, which increases progesterone locally. It is imperative that a repeat endometrial biopsy is done during the first treatment cycle, to confirm that the luteal phase deficiency has been corrected.

Anatomical factors are usually corrected surgically. An uterine septum is presently excised hysteroscopically, replacing the classic metroplasty through a laparotomy incision. Intrauterine synechiae can be treated hysteroscopically, using laser, scissors or more recently, an intrauterine balloon[16].

Patients with the Antiphospholipid Antibody (APA) Syndrome were previously treated

with low-dose acetylsalicylic acid (ASA) and prednisone. Alternatives to prednisone were sought because of its associated significant maternal and neonatal morbidity. A combination of low-dose ASA and subcutaneous heparin administration has met with significant success, with fewer complications than ASA and prednisone[17]. Intravenous immunoglobulin is also being evaluated in couples treated unsuccessfully with low-dose ASA and heparin[18].

Frequency of unexplained recurrent pregnancy loss

The first comprehensive paper assessing frequency of factors associated with recurrent pregnancy loss was published in 1984 by Stray-Pedersen and Stray-Pedersen[10]. They used a diagnostic screening protocol to evaluate 195 couples with a history of habitual abortion. Endocrine, anatomical, infectious and male factors were investigated, with abnormalities being identified in 56% of the couples. The other 44% of the couples were classified as having "habitual abortion of unknown etiology". Immunological factors were not evaluated in the Stray-Pedersen et al. diagnostic screening protocol.

With the establishment of a provincial Recurrent Pregnancy Loss Program in June 1992, located in British Columbia's Women's Hospital and Health Centre, a unique opportunity was created to study the prevalence of recurrent pregnancy loss in British Columbia and to assess the various factors associated with this condition. Upon reviewing data from 197 couples with a history of habitual abortion who completed the investigations cited above,

one or more factors, including genetic, endocrine, infectious, anatomical or autoimmune factors, appeared to be causative in approximately 57% of the cases[19]. The remaining 43% of couples were therefore classified as having "unexplained habitual abortion". Coulam et al. suggested that over 80% of couples with unexplained habitual abortion may have an alloimmune factor, that is, a lack or inappropriate maternal immune response to the pregnancy[20]. Unfortunately, defining an alloimmune factor has been difficult to date because of the limited usefulness of available alloimmune investigations and the lack of control data. For these reasons, the use of the term "unexplained habitual abortion" may be more appropriate than "alloimmune-associated habitual abortion"at the present time.

The laboratory component of this Master's thesis was undertaken to standardize the flow cytometric crossmatch (FCXM) methodology which is used in the "alloimmune" evaluation of couples with a history of unexplained habitual abortion. Since there had been no standardized FCXM methodology for reproductive immunology published in the North American literature prior to this research project, it had been difficult to reproduce FCXM results published previously. If the FCXM methodology presented in this Master's thesis is accepted by other reproductive immunology centres, collaborative research should flourish in the future.

Recurrent pregnancy loss is a prevalent health problem which requires a multidisciplinary approach to its investigation and management. With further development of investigative tools such as the flow cytometric crossmatch, it is proposed that evaluation and treatment

of couples who suffer from unexplained, possible alloimmune-associated habitual abortion will improve, resulting in subsequent deliveries of healthy newborns.

Chapter 2

Immunological Investigations and Treatments of Habitual Abortion

The diagnostic screening protocol published by Stray-Pedersen and Stray-Pedersen in 1984 evaluated genetic, endocrine, anatomical, infectious, and male factors which were historically associated with a history of habitual abortion[10]. In their study of 195 couples, one or more of these factors were identified in 56% of the couples. The other 44% of the couples were classified as having "habitual abortion of unknown etiology". Since the publishing of this landmark paper, it has been hypothesized that up to 80% of couples with a history of habitual abortion may have an underlying auto- or allo-immune factor which is causative[20].

Since the original paper of Stray-Pedersen et al., the evaluation of couples with a history of habitual abortion has changed significantly. Male and infectious factors are not considered to be causative, therefore they are not routinely investigated. Autoimmune testing is commonly performed, although the type of tests and the normal ranges differ considerably from site to site. Alloimmune testing is not available universally throughout this country and, where available, the testing varies considerably. Therefore, the frequency of immunological factors associated with a history of habitual abortion is very difficult to estimate. Perhaps with the establishment of a national registry and guidelines for investigations, the frequency of immunological-associated habitual abortion could be more accurately determined.

In 1992, Makino et al. published a survey of 1,120 Japanese women with two or more spontaneous abortions, not necessarily consecutive[22]. Of the 1,120 women, only 148 were evaluated for an autoimmune factor. ELISAs for anticardiolipin antibodies were used as the only diagnostic test, and of the women tested, 16% had elevated results. No alloimmune testing was undertaken in this survey and since evaluations were not offered universally, no estimate of the couples with "habitual abortion of unknown etiology" could be given.

In 1993, Tulppala et al. published a prospective study of 63 couples with a history of recurrent spontaneous abortion[23]. The study population consisted of 34 women with three consecutive spontaneous abortions and 29 women with four to eight spontaneous abortions which were not necessarily consecutive. Using ELISAs for anticardiolipin IgG and IgM, and the recalcification-time test to detect the lupus anticoagulant, an autoimmune factor was identified in 10% of the women. Fifty-five percent of the couples were found to have "recurrent spontaneous abortion of unknown etiology".

The frequency of factors associated with a history of habitual abortion in 197 consecutive couples seen in the Recurrent Pregnancy Loss Program, located in the B.C. Women's Hospital and Health Centre, was recently reported by Stephenson[19]. Habitual abortion was defined as three or more documented consecutive spontaneous pregnancy losses less than 20 weeks gestation, excluding any spontaneous abortions with documented aneuploidy by karyotype analysis. This strict inclusion criteria should have improved the identification

of truely affected couples with factor-associated habitual abortion and excluded many couples with pregnancy losses due to aneuploidy, which is known to occur randomly. Autoimmune-associated habitual abortion was found in 20% of the women. Forty-three percent of the couples were classified as having unexplained habitual abortion. Of this latter group, 65% (n=55) were subgrouped as primary, 27% (n=23) were subgrouped as secondary and 7% (n=6) were subgrouped as unclassified unexplained habitual abortion. The results of this prospective case series are summarized in Table 1.

Autoimmunity and habitual abortion

The association between autoimmunity and habitual abortion was initially described in the early 1950s when a relationship between systemic lupus erythematosus and spontaneous abortion was noted[24], but it was not until the late 1980s that the term "Antiphospholipid Antibody (APA) Syndrome" was derived[25]. The recent literature continues strongly to associate autoimmunity and habitual abortion, although the exact pathophysiology remains controversial. Elevated levels of antiphospholipid antibodies in women with habitual abortion are associated with placental thrombosis which may subsequently lead to fetal death[26]. Proposed mechanisms leading to thrombosis include; (1) inhibition of prostacyclin production by endothelial cells resulting in enhanced thromboxane release leading to platelet aggregation[27], (2) decreased thrombomodulin-dependent activation of protein C[28] and (3) binding to antithrombin III[29] thereby interfering with the anticoagulation pathway.

Table I

Frequency of Factors Associated with Habitual Abortion in 197 Couples[19,*]

Factor	Number of Couples	Frequency
Genetic	7	3.5%
Endocrine	39	20%
Infectious	1	0.5%
Anatomical	31	16%
Autoimmune	40	20%
Unexplained, possible alloimmune	84	43%
primary: 55 couples		
secondary: 23 couples		
unclassified: 6 couples		

*couples evaluated at the Recurrent Pregnancy Loss Program, B.C. Women's Hospital and Health Centre, between June 1992 and December 1994.

Since pregnancy itself results in an increased production of coagulation factors, and, the placenta is rich in thrombomodulin, which is important in the activation of protein C, alterations in the thromboxane:prostacyclin ratio and inhibition of the anticoagulation pathway, makes the placenta especially susceptible to thrombotic events[30].

Women with habitual abortion and persistent elevation of antiphospholipid antibodies often present with other clinical features and laboratory findings suggestive of an underlying autoimmune disease. It must be remembered that women with a history of habitual abortion in association with the Antiphospholipid Antibody Syndrome have an increased risk of venous and/or arterial thrombosis throughout their lifetime, which is even higher in pregnancy. Therefore, these women require extensive counselling in regard to the use of medication, such as estrogen-based oral contraceptives, and smoking, which could also increase their risk of a thrombotic event in the nonpregnant state.

Antiphospholipid antibody testing classically consists of two types of assays; the coagulation-based assays to detect the "lupus anticoagulant" and the enzyme-linked immunosorbent assays (ELISA) to detect specific antiphospholipid antibodies. Women may test repeatedly positive to one of the tests but not the other in up to one-third of cases[31]. Therefore, both types of assays are required as screening tools.

In vitro, there are three phospholipid-dependent steps in the coagulation pathways where the lupus anticoagulant may interfere, therefore, either the intrinsic or extrinsic pathways

may be affected. There are several coagulation-based assays available for clinical use to assess the presence of the lupus anticoagulant, including the sensitive activated partial thromboplastin time, the dilute Russell viper venom test ratio (DRVVT) and the kaolin clotting time. Confirmatory tests, such as mixing studies and the platelet neutralization test are necessary to differentiate the lupus anticoagulant from factor deficiencies or serum inhibitors of in vitro coagulation.

Presently, anticardiolipin IgG and IgM antibodies are the most widely used ELISAs for the investigation of the APA syndrome. Enzyme-linked immunosorbent assays to identify other APAs including antiphosphatidylserine, antiphosphatidylinositol and anti-phosphatidylethanolamine antibodies are available in some sites, although the relationship between these other APAs and habitual abortion needs further evaluation.

The measurement of antinuclear antibodies (ANA) in the woman's serum is commonly included in the evaluation of recurrent pregnancy loss. The significance of an elevated ANA level in this clinical setting is still difficult to determine, especially when there is no evidence of an underlying autoimmune disease.

Lubbe et al. was the first to report the use of low dose acetylsalicylic acid (ASA) and prednisone (40 mg/day) in women with a history of recurrent pregnancy loss and laboratory evidence of a lupus anticoagulant[32]. Although this combination of medication has improved subsequent pregnancy outcomes in women with the APA Syndrome, chronic

administration of steroids is fraught with side effects, including the cushingoid habitus, osteoporosis, myopathy, poor wound healing, infection, necrosis of the femoral head, pregnancy-induced hypertension and gestational diabetes. Long-term steroid administration is also associated with fetal complications including intrauterine growth restriction and premature rupture of membranes.

As an alternative to the use of steroids, subcutaneous heparin has been used alone or in combination with low dose ASA for treatment of the APA Syndrome. Prolonged use of heparin is associated with osteoporosis and thrombocytopenia, so careful monitoring is required with the use of this medication. Cowchock et al. published a collaborative randomized trial which compared the use of 40 mg prednisone to subcutaneous heparin, both with 80 mg ASA, for treatment of women with two or more unexplained fetal losses and persistent elevation of either the lupus anticoagulant or anticardiolipin antibodies[17]. Both treatment arms had similar live birth rates, 6/8 for the prednisone arm vs 9/12 for the heparin arm. However, patients treated with prednisone and ASA had a higher incidence of pregnancy induced hypertension, gestational diabetes, preterm delivery, premature rupture of membranes and intrauterine growth restriction. Following the interim data analysis, the study was abandoned because of these statiscally significant complications associated with the prednisone treatment.

Acetylsalicylic acid alone has recently been evaluated for the treatment of women with habitual abortion associated with persistently elevated anticardiolipin IgG[33]. Sbracia et al.

reported a successful pregnancy outcome of 85% in the treatment arm (ASA 50 mg/day) and 52.6% in the placebo arm ($p < 0.02$).

Kutteh subsequently published a prospective, "pseudo-randomized" clinical trial comparing heparin and ASA (81mg/day) to ASA alone in women with a history of habitual abortion and positive antiphospholipid antibodies (anticardiolipin or antiphosphatidylserine IgG or IgM)[34]. In the heparin/ASA arm, 80% (20/25) had viable infants delivered compared with 44% (11/25) in the ASA alone arm ($p < 0.05$). There were no significant differences in regard to gestational age at delivery, number of caesarean sections or complications.

Intravenous immunoglobulin (IVIG) has also been used, initially in combination with ASA and prednisone or ASA alone in women with previous APA Syndrome treatment failures[17,35]. More recently, IVIG has been used alone, although no randomized clinical trials have been published to date[36].

Alloimmunity and habitual abortion

The understanding of how the pregnant woman tolerates the allogeneic fetus remains an enigma. The immune system normally recognizes and rejects nonself antigens, yet pregnancy seems to represent an exception to this rule. The uterus appears to be an immunologically "privileged site", where the maternal immune system mounts a protective

immune response to nonself, paternally derived antigens expressed in early pregnancy. Even at the blastocyst stage, immunosuppressor factors can be isolated from the growth medium in vitro[37]. Implantation and early embryo growth results from communication between the embryo and the decidualized endometrium, through mediators such as cytokines and growth factors, and suppression of natural killer (NK) cells in the decidua.

The presence of maternal suppressor cells, blocking antibodies and specific cytokines in the decidua protect the fetus against other immune cells such as cytotoxic T cells, lymphokine-activated killer (LAK) cells and macrophages. Pregnancy modulates the maternal local uterine and systemic immune responses, which results in a decrease in cell-mediated immunity and an increase in humoral immunity. Wegman et al. hypothesized that this dichotomy between cell-mediated and humoral immune responses is mediated by inhibitory subsets of T-helper (Th) cells at the level of the uterine decidua[38]. They proposed that Th2 cell cytokine production, specifically interleukin 4 (IL-4), IL-5 and IL-10, is favoured over Th1 cell cytokine production, IL-2 and interferon gamma (IFN-γ). These Th2 cytokines, which stimulate B cell proliferation and antibody production, dampen inflammation and suppress NK cell activation, with the net effect being immunotolerance of the feto-placental unit. A Th1 cytokine response, which promotes cytotoxicity and local inflammatory reactions, has been reported in patients with unexplained habitual abortion in vitro[39]. Normally NK cells cannot kill trophoblast cells in vitro, but if activated by IL-2, which is a Th1 cytokine, NK cells can be cytotoxic to trophoblasts[40].

Another pathway leading to immunosuppression of the feto-placental unit involves the interaction between trophoblast cells and lymphocytes in the uterine decidua. As well as producing immunosuppressive factors, trophoblast cells also activate a population of small lymphocytes with cytoplasmic granules, neither of T or B origin but bone marrow-derived[41]. These cells, presently termed natural suppressor (NS) cells, are similiar but not identical to peripheral NK cells (CD56+16+), with the phenotype CD56+16-. The NS cells release transforming growth factor beta type 2 (TGF-β2). Decidual-derived TGF-β2 is a potent local suppressor of cytotoxic cells. It is interesting to note that the activity of TGF-β2 is boosted by allogeneic mononuclear cell immunization in the murine model[42].

The interaction between gestational hormones and the immune response to pregnancy has recently been described by Szekeres-Bartho et al[43]. It is well-known that estradiol promotes the expression of progesterone receptors on a variety of cells, and that progesterone is necessary for the maintenance of pregnancy. In the initial weeks of pregnancy, the corpus luteum is the major site of progesterone production, after being "rescued" by syncytiotrophoblast-derived beta human chorionic gonadotropin (βHCG), which is produced as early as 9-13 days after ovulation. Endogenous βHCG maintains steroidogenesis in the corpus luteum until the placental steroidogenesis is established by 9-10 weeks of gestation.

Szekeres-Bartho et al. have shown also that pregnancy in both the murine and human model activates CD8+ T cells to express progesterone receptors on their cell surface[44].

In response to progesterone, these "activated CD8+ T cells" secrete a factor, termed "progesterone-induced blocking factor" (PIBF) which suppresses cytotoxicity, specifically the cytolytic activity of NK cells[45]. Dampening of NK cytolytic activity has been proposed to be crucial to a successful pregnancy outcome[46]. Women with recurrent unexplained spontaneous abortions have been found to have subnormal levels of progesterone receptor CD8+ T cells in early pregnancy[47]. It is plausible that active immunization with allogeneic mononuclear cells may activate these CD8+ T cells to produce progesterone receptors and subsequently progesterone induced blocking factor, which results in the inhibition of cytotoxic immune responses to pregnancy in patients with a history of uexplained habitual abortion.

As our understanding of the immune mechanisms involved in normal and abnormal pregnancies improves, more specific immuno-modulatory treatment protocols can be developed which will hopefully help couples who presently suffer from unexplained, possible alloimmune habitual abortion.

Despite the recent strides made in the understanding of some of the potential mechanisms involved in maternal immunotolerance of the fetoplacental unit, the availability of tests for clinical purposes are presently limited and of questionable use. In the 1980s, alloimmune testing classically consisted of; (1) human leukocyte antigen (HLA) tissue typing of both partners, to assess the amount of histocompatibility between the male and female partner, (2) the one-way mixed lymphocyte culture (MLC), to assess the maternal cell-mediated

response to her partner as well as the presence or absence of a maternal serum "blocking factor" and (3) the complement-dependent cytotoxicity (CDCC) assay, to assess for the presence or absence of complement-dependent cytotoxic antipaternal antibodies in the maternal serum.

The HLA tissue typing of couples with unexplained habitual abortion is still being performed in some centres but the significance of sharing is still of questionable importance[48]. The MLC and the CDCC assay are both bioassays, and are therefore prone to significant intra- and interobserver variability. The MLC was abandoned following the control data published by Coulam which showed that the presence of "blocking factor" was directly dependent on the total number of weeks in a lifetime that a woman was pregnant[48].

The CDCC assay is still used in many centres despite its limitations. The assay consists of adding paternal lymphocytes to maternal serum, followed by the addition of complement, which binds to the Fc portion of the maternal immunoglobulin bound to paternal lymphocytes. The fixation of complement results in the loss of cell membrane integrity and subsequent cell death. Fluoroscein dye is taken up by the dead cells and the amount of cell death is subjectively determined microscopically.

In the 1990s, several alloimmune tests have surfaced for clinical use based upon the recent advances in the research setting, but unfortunately, little control data has been published

to date. Many of the clinical tests used today are extensions of the basic science research which was previously summarized.

To assess maternal systemic cell-mediated immunity, the concentration of peripheral blood natural killer (NK) cells is measured clinically in women with a history of unexplained habitual abortion. Aoki et al. published their results that showed that an increased percentage of NK cells in peripheral blood prior to pregnancy correlates with an increased subsequent spontaneous abortion rate[49]. In another study, Coulam et al. concluded that an increased percentage of peripheral blood NK cells in pregnancy is predictive of a karyotypically normal spontaneous abortion, though the study was small, with a total of 42 patients with two or more consecutive losses with the same reproductive partner[50]. Makido et al. studied peripheral blood NK cell activity before and after paternal mononuclear cell immunotherapy in women with a history of recurrent spontaneous abortion[51]. In their study, all women in whom NK activity decreased after immunotherapy became pregnant and had healthy babies.

In 1984, Chavez and McIntyre published a description of a mouse blastocyst assay used to assess the presence of cytotoxic antibodies, possibly antitrophoblast IgG, in the maternal serum in couples with histories of unexplained habitual abortion[52]. Unfortunately, the bioassay was labour intensive, expensive and lacked reproducibility. Since then, many other researchers have published refinements to this original assay of Chavez and McIntyre. Zigril et al. showed a reversibility of the embryotoxic effect of serum in

women with a history of recurrent pregnancy loss following immunotherapy[53].

With the theory of Th2 predominance in the maternal-fetal relationship, as proposed by Wegmann et al. in 1993, cytokines, such as IL-2 and IFN-γ, produced by Th1 cells, have now been implicated as cytotoxic factors, rather than immunoglobulin as previously proposed[39]. Roussev et al. recently published a validated embryotoxicity assay[54] with a specificity of 95% and a positive predictive value of 83% ($p < 0.001$). This research group proposed that they could use this embryotoxicity assay to identify a subgroup of patients with alloimmune recurrent spontaneous abortion. Despite the many publications to date, the embryotoxicity assay continues to be a controversial test, due to the inherent problems associated with this bioassay.

To assess maternal humoral immunity, the flow cytometric crossmatch (FCXM) has been used recently on a clinical basis to assess couples with histories of unexplained habitual abortion. The FCXM is superior to the CDCC for many reasons; 1) the FCXM detects the binding of either both complement-dependent and complement-independent immunoglobulins, while the CDCC assay indirectly detects the binding of only complement-dependent immunoglobulin, 2) the FCXM can differentiate between IgG and IgM bound to either T or B lymphocytes while the CDCC assay can not differentiate, 3) the FCXM is a quantitative test while the CDCC assay is only qualitative, 4) the FCXM is more sensitive than the CDCC assay, and 5) the FCXM is less labour intensive than the CDCC assay. The major disadvantages to the FCXM are twofold. The first is the initial

cost of hardware, which includes a flow cytometer and computer. The second is that prior to this Master's thesis, there was no publication which described a standardized FCXM methodology for use in reproductive immunology. Thus, the FCXM methodology was evaluated and clinically used to assess maternal allosensitization in treated couples with a history of unexplained habitual abortion.

Management of unexplained habitual abortion

Management of unexplained, possible alloimmune habitual abortion remains controversial. Paternal mononuclear cell (PMC) immunization, commonly called "white cell immunization", has been used for over a decade, though published randomized trials to date have revealed conflicting results. In 1985, Mowbray et al. published the first randomized, double-blinded, controlled trial assessing PMC immunization[55]. Women enrolled had at least three abortions with their current partner and no more than one prior live birth. Exclusion criteria included identification of a known factor associated with habitual abortion, including infectious, metabolic, anatomical or genetic factors, or, a positive CDCC assay. A successful pregnancy outcome occurred in 17/22 couples in the PMC immunization arm and 10/27 couples in the control arm, which consisted of an autologous immunization ($p=0.01$). There were no other published randomized controlled trials which assessed the efficacy of PMC immunization in couples with unexplained, possible alloimmune recurrent pregnancy loss until Ho. et al. and Cauchi et al. both published in 1991[56,57].

The randomized, non-blinded, controlled trial by Ho et al. included couples with three or more consecutive spontaneous abortions (primary and secondary habitual abortion) in whom no genetic, endocrine, anatomical, autoimmune or sperm factor was found[56]. A successful pregnancy outcome was defined as a viable pregnancy >5 months gestation. Of the 39 women who had PMC immunization, 31 had a successful pregnancy outcome compared to 32 out of 49 in the control arm and 8 out of 11 in the third party mononuclear cell immunization arm ($p=0.84$).

The other trial by Cauchi et al., which was randomized, double-blinded and placebo-controlled, included couples with a history of three or more consecutive first trimester spontaneous abortions (both primary and secondary habitual abortion), after excluding genetic, endocrine, anatomical and autoimmune factors[57]. As in the trial by Mowbray et al., a positive CDCC against paternal lymphocytes was an exclusion criterion. A successful outcome was defined as a pregnancy that continued beyond 20 weeks gestation. Of the 21 women who underwent PMC immunization, 13 had a successful pregnancy outcome. Of the 25 women in the control arm, which was normal saline rather than autologous immunization, 13 were successful ($p=0.30$).

To address the conflicting results from these three clinical trials, an Ethics Committee of the American Society of Reproductive Immunology was formed. This committee organized a worldwide collaborative observational study and meta-analysis to determine the potential value and risks of paternal mononuclear cell immunization for couples with

unexplained recurrent spontaneous abortion[58]. A total of fifteen clinical centres with controlled raw data were identified worldwide. Of these, nine centres had undertaken randomized controlled trials, seven of which were double-blinded. The raw data from these nine trials were evaluated independently by two separate data analysis teams, located at McMaster University in Hamilton, Ontario and University of Utah Medical Center in Salt Lake City, Utah.

One of the data analysis team used the raw data from 430 patients, of which 180 had PMC immunization, 51 had third party mononuclear cell immunization and 199 were controls. The other data analysis team used the raw data from 449 patients, of which 240 were immunized with either partner or third party mononuclear cells and 209 were controls. The results did show an absolute difference in live birth rates between treatment and control groups of 8% and 10%, as determined by the two separate analysis teams. In other words, the number needed to treat (number of patients that have to be immunized to achieve one additional live birth) was estimated as 11. Live birth rates were lower with older female partners, more than five abortions, with a positive ANA or with positive anticardiolipin antibodies.

Daya et al. performed a subgroup analysis on the data obtained from this worldwide collaborative observational study, including only patients with three or more spontaneous abortions and no previous pregnancy beyond 20 weeks' gestation with her current partner, no identifiable cause for the abortions, no evidence of antipaternal antibodies and no

simultaneous cointervention[59]. A total of 285 women met the inclusion criteria, of whom 150 received paternal or third party mononuclear cell immunization and 135 served as controls. Immunotherapy significantly improved the probability of live birth (relative risk=1.46, 95% CI, 1.19 to 1.69). The number needed to treat in this subgroup was six. Although PMC immunization was shown to be effective in the original worldwide collaborative observational study, it appears that treatment efficacy was improved when limited to women with primary unexplained, possible alloimmune habitual abortion with a negative CDCC prior to treatment.

Presently there are two large, prospective, randomized, double blinded, placebo controlled, multicentered clinical trials in progress, with Coordinating Centers in Chicago, USA and Oxford, UK. These two trials should finally settle the controversy surrounding paternal mononuclear cell immunization for treatment of unexplained habitual abortion. The Canadian site of the Chicago-based trial is in Vancouver, at B.C. Women's Hospital[60].

As an alternative to active immunization with allogeneic mononuclear cells, passive immunization with intravenous immunoglobulin (IVIG) is currently being evaluated for couples with a history of either primary or secondary recurrent spontaneous abortion. In 1995, Coulam et al. published the results of a randomized controlled trial which evaluated the efficacy of IVIG in this patient population[61]. Eighteen of the 29 women (62%) who conceived in the IVIG arm had a successful pregnancy outcome. Eleven of the 33 women (33%) who conceived in the control arm had a successful pregnancy outcome (p=0.04,

odds ratio 3.1). Presently, a similiar trial, which is prospective, randomized, double blinded and placebo-controlled, is being conducted at the B.C. Women's Hospital, the results of which will be published in 1997[62].

Conclusions

Research over the past decade has shown increasing evidence that immunological factors are important in the evaluation of couples with a history of habitual abortion. Criteria for diagnosis require further development, including guidelines for investigation and treatment management. The Antiphospholipid Antibody Syndrome is a well recognized cause of autoimmune-associated habitual abortion and should be treated aggressively to give a reasonable chance of pregnancy success, bearing in mind the potential side effects of the medications prescribed and the associated maternal and fetal morbidity in the antenatal period. Long term follow-up is required because of the lifelong risk of thrombosis in patients with the APA Syndrome. Management of women with other clinical or serological evidence of autoimmunity requires further investigation. Further prospective, randomized trials are necessary to delineate the best approach for treatment of autoimmune-associated habitual abortion.

The concept of how the pregnant woman immunologically tolerates the fetoplacental unit is still poorly understood in the 1990s. Despite the active ongoing research in the field of reproductive immunology, many of the recent hypotheses cannot be proven because of the

limited availability of clinically useful alloimmune tests. Natural killer cells are presently being measured in the peripheral blood to assess grossly maternal cell-mediated immunity. Cytotoxic factors in maternal serum are presently being assessed using an embryotoxicity assay. With further advances in cytokine research, serum levels of these and other soluble mediators will probably be available clinically in the not too distant future.

To assess maternal humoral immunity to pregnancy, the FCXM has a potentially significant role as a reliable investigative tool. With the standardization of the FCXM methodology, as presented in this Master's thesis, results should be reproducible between different centres, which will facilitate collaborative research in the field of reproductive immunology. With the FCXM methodology being strictly defined, the significance of maternal alloantibodies in regard to pregnancy will, it is hoped, be better understood in the next few years. Once the normal and abnormal immunological responses to pregnancy are understood better, we will be able to develop more specific treatment protocols for couples who suffer from unexplained habitual abortion.

Chapter 3

The Advent of Flow Cytometry in Reproductive Immunology

The use of flow cytometry, a sensitive, quantitative, cytological technique, is being explored presently as an investigative tool in reproductive immunology. Flow cytometry has been used more extensively in the field of organ transplantation. It is well known that the success or failure of an organ transplant is dependent on the tissue histocompatibility between the donor and recipient. With increasing similarity between donor and recipient human leukocyte antigens (HLA), transplantation survival improves. Conversely, HLA dissimilarity is associated with the development of antigraft antibodies. The development of anti-HLA antibodies can result in early graft loss (3 - 6 months)[63].

Measurement of anti-HLA antibodies in the recipient's serum, both pre- and post-transplantation was previously performed using the complement-dependent cytotoxicity (CDCC) assay[64]. Cytotoxicity was determined microscopically with the use of stains such as eosin or more recently, acridine orange and ethidium bromide. The CDCC assay is unfortunately prone to error, primarily due to the variable activity of complement, which is added to the mixture of recipient's serum and donor's cells.

Flow cytometry has become popular recently as a technique to evaluate anti-HLA antibodies in potential transplant recipients. The lymphocyte flow cytometric crossmatch (FCXM), is complement-independent, and has been found to be more sensitive than the

CDCC assay[65,66,67]. In 1983, Garovoy et al. published their assessment of flow cytometric analysis as a new investigational technique to detect clinically relevant antibodies in recipient's serum prior to transplantation that are missed by the standard CDCC assay[68]. Their results indicated that the FCXM can not only detect low levels of anti-leukocyte antibodies, but also can distinguish between anti-T and anti-B lymphocyte antibodies in a heterogeneous leukocyte cell population. Garovoy et al. found that there was a correlation between the presence of anti-lymphocyte antibodies prior to transplantation and subsequent acute graft rejection. Four out of 14 cadaveric recipients who were FCXM positive prior to transplantation subsequently rejected their grafts within 3 months, while 5 of the remaining 10 recipients rejected their grafts by 1 year. Iwaki et al. confirmed that the FCXM could be used clinically to improve the selection of donors and recipients, thereby reducing the risk of early rejection of transplanted kidneys[69].

In 1989, Bray et al. published a FCXM methodology for assessment of potential transplant recipients[70]. Since there was no prior FCXM methodology published, Bray et al. proposed that their methodology should be used widely so that results could be reproduced at different sites. They examined several parameters of the FCXM, including Fc-specific and nonspecific binding of secondary antibodies, dual- and single-colour analysis, and assessment of a positive FCXM. Bray et al. also showed that the preincubation of the cell culture with goat IgG prevented non-specific binding of human IgG to Fc receptors. Therefore, when human serum was added subsequently, only antibodies that specifically bound to surface markers on the lymphocytes would be identified with the secondary

antibody, such as fluorescein isothiocyanate (FITC)-conjugated goat anti-human IgG, Fc-specific.

Bray et al. strictly defined what a positive FCXM result was[70]. Peripheral blood lymphocytes from 20 individuals were analyzed with negative (autologous or normal human serum) and pooled positive serum. With the negative serum, the mean linear channel fluorescence varied by as much as five channels for the T lymphocytes and as much as 10 channels for the B lymphocytes. They also compared shifts in the mean channel fluorescence for a 20% increase in positive cells by adjusting the photomultiplier setting from 1250 to 1050. Their conclusions were that a positive FCXM for T lymphocytes corresponds to a channel shift above the negative control serum of more than 10; and a positive FCXM for B lymphocytes corresponds to a channel shift above the negative control serum of more than 25.

It is important to remember that if one is going to use another site's determination of a positive FCXM, the same FCXM methodology must be used, including the same reagents. Bray et al. designed a standardized FCXM methodology for use in renal allograft recipients[70]. Since then, there have been further improvements in flow cytometry as well as further refinements in commercially prepared secondary antibodies.

The use of surface markers, to identify lymphocyte subsets and/or bound antibodies, has flourished in the past decade, almost in parallel with advances in flow cytometry.

Biological assays, such as the sheep erythrocyte rosette and the surface immunoglobulin assays, were used previously to detect T and B lymphocytes in peripheral blood. Biological assays were fraught with difficulties, for example, stored erythrocytes formed rosettes poorly and nonspecific binding of IgG to Fc receptors on other leukocytes resulted in falsely high estimates of B cell levels in peripheral blood. Refinements in separation of mononuclear cells, using density gradient centrifugation, improved the specificity of the cell population. The use of fluorochrome-conjugated $F(ab')_2$ fragments prevented binding of the commercially prepared antibodies to the Fc receptors as well as each other. Analysis with immunofluorescence microscopy or enzyme histochemistry was also too subjective and time-consuming. The addition of flow cytometry allowed rapid, objective analysis of large number of cells (usually 10,000 cells) in a relatively heterogeneous cell population.

With the rapid advancement of surface marker technology, and sophisticated computer programming, flow cytometry has become a "state of the art" technique to analyze cell types, development and function. Flow cytometry plays a key role in the evaluation of organ transplantation recipients, in the identification of neoplastic cells, and in the study of immune deficiency states. It is also being used as an innovative technique in the field of reproductive immunology. Flow cytometry technology will help advance understanding of the maternal immune response to pregnancy. Recently, flow cytometric analysis of endometrial and peritoneal tissues has been reported. With further refinement of monoclonal antibodies, other immune cells besides T and B lymphocytes, such as

$CD56^{+}CD16^{-}$ lymphoid cells in the first trimester decidua, have been recently studied[41].

Flow cytometry has obviously become a powerful tool for the study of cell biology. Its further potential is vast, however, standardization of the methodology is important so that results are reproducible from site to site. In the field of reproductive immunology, careful evaluation of methodology will allow us to work in a collaborative manner to answer some important questions about the significance of alloantibodies in the maintenance of pregnancy.

Chapter 4

Basic Principles of Flow Cytometry

Flow cytometry is the measurement of cellular properties as they move in a fluid past a set of detectors. In 1938, Caspersson et al. published absorption measurements for intracellular constituents such as nucleic acids[71]. Shortly thereafter, Papanicolaou and Traut published their work using nuclear chemistry morphology to diagnose uterine malignancy from vaginal smears[72]. Coons et al. were the first to describe the use of a flow cytometer using fluorochrome-conjugated antibodies to measure intracellular constituents and sort cells[73]. It was not until the mid 1950s that flow cytometers became available for clinical use[74]. With optical improvements and the development of more sophisticated computer software to sort cells, flow cytometers are becoming more readily available for clinical use, at less cost.

The technique of flow cytometry relies on the use of commercially manufactured fluorochrome-conjugated antibodies. These antibodies can be monoclonal, such as phycoerythrin-conjugated (PE) mouse anti-human CD3 (Cluster of Differentiation) antibodies which identifies the CD3 antigen which is present on the surface of all T cells, or, they can be polyclonal, such as fluorescein-conjugated (FITC) goat anti-human IgM antibodies which identifies IgM bound to cells.

Often $F(ab')_2$ (Fraction that is antigen-binding) fragments of antibodies are prepared

commercially. These fragments are prepared by adding pepsin to the manufacturing process. Pepsin cleaves an individual IgG molecule into one $F(ab')_2$ fragment and one Fc (Fraction that Crystallizes or is constant) fragment. By using this $F(ab')_2$ fragment, agglutination of the commercial antibody is inhibited and Fc receptor binding on T lymphocytes is avoided.

The diversity of commercially prepared fluorochrome-conjugated monoclonal and polyclonal antibody fragments is rapidly expanding. Any known cell constituent, either intra- or extracellular, will probably be identifiable with the use of this technology soon.

Fluorochromes are molecules which can absorb and re-emit light of a specific wavelength. Fluorescein isothiocyanate (FITC) and phycoerythrin (PE) are common fluorochromes used in flow cytometry. Both of these fluorochromes absorb light emitted from an argon laser, which is of a blue to blue-green wavelength. Fluorescein isothiocyanate and PE absorb the laser light and emit light of a longer wavelength. The FITC fluorochrome emits green light at 530 nm and the PE fluorochrome emits red light at 580 nm, which allows optical separation of the two fluorochromes. This optical separation allows for concomitant quantitative analysis of two different cellular markers. As well, individual cell types in a heterogeneous sample can be physically sorted using these fluorochrome-conjugated antibodies.

The key components of a flow cytometer are: the laser light source, the sample chamber,

the detection system and the signal processing and data analysis systems. Each of these components will be described briefly.

A flow cytometer is depicted in Figure 1. Photons of predetermined energy are emitted from the laser light source and are either scattered or absorbed and emitted by the cells in a monodispersed sample, as they pass through an "interrogation point", one cell at a time. The scattered and emitted wavelengths are separated by a series of filters and mirrors and collected in detectors, either photodiodes for forward light scatter or photomultipliers for side light scatter and emitted light from the fluorochromes. The photons of lights which impinge upon the detectors are converted to an electrical impulse, which is called an analog signal and is measured in millivolts. The analog signals are proportional in magnitude to the number of photons emitted from each individual cell which passes through the interrogation point. The analog signal is converted to a digital (number) signal with an analog-to-digital converter (ADC). Most commonly, flow cytometers produce digital signals with 256-channel resolution, meaning that a number from 0 to 255 is produced following the analog-to digital conversion. Higher-resolution flow cytometers produce digital signals with 1024-channel resolution.

Digital signals are stored in the computer in a "list" or listmode. The collected data can be processed immediately or at a later date. By selecting one or more parameters, histograms can be displayed.

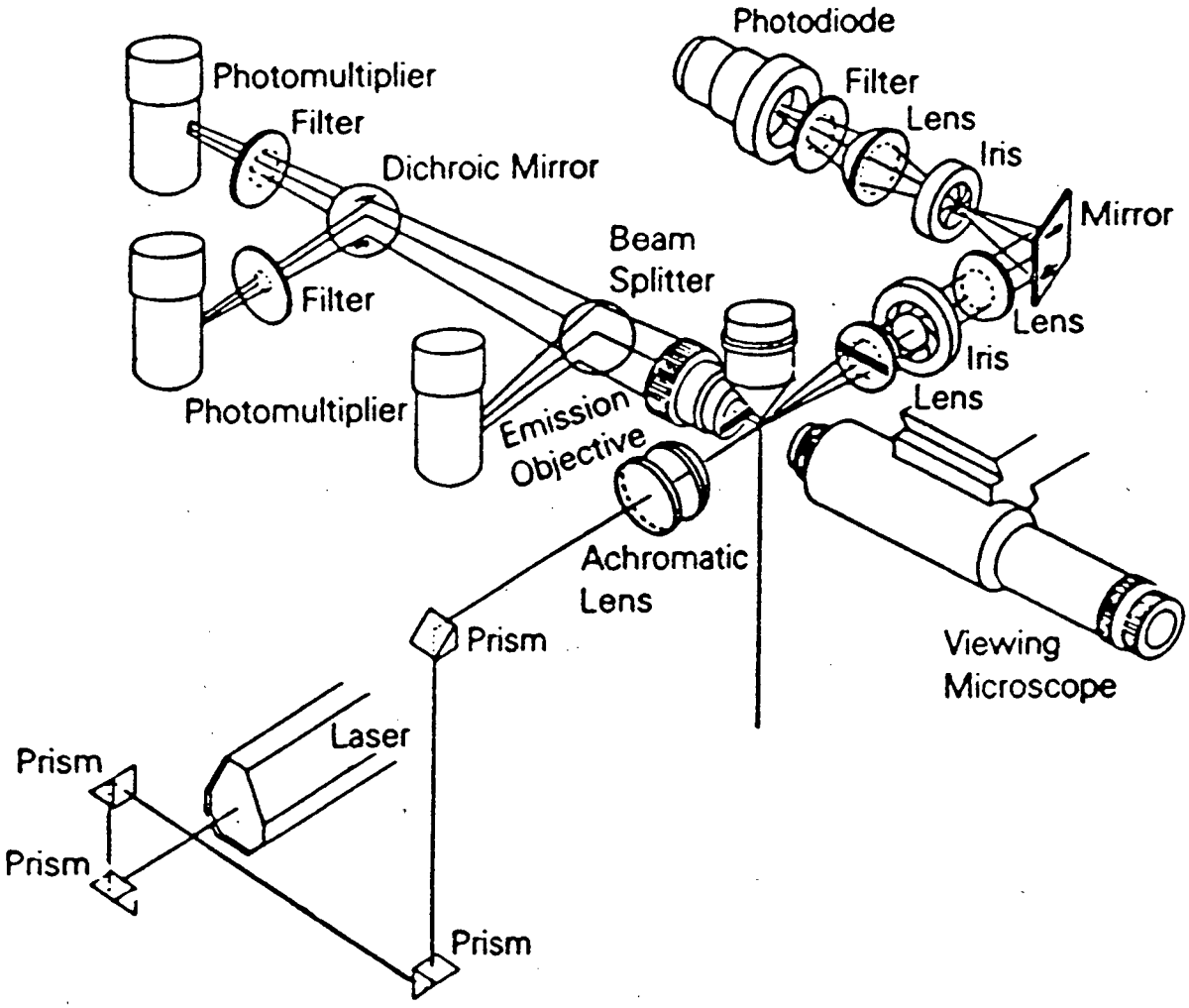


Figure 1: Features of a Flow Cytometer

(Reproduced from Becton Dickinson, Mountain View, CA)

Light Source

Laser light is used for flow cytometric analysis because it is coherent (parallel) and monochromatic (of a single wavelength). The type of laser light is chosen based upon its emission wavelength. Primarily gas lasers are used, such as the argon laser with an emission wavelength of 488 nm. The argon gas atoms in the laser tube are excited to a higher energy state by electrical energy. Photons of light with similar wavelengths are released when the atoms return to their ground state. The laser assembly needs to be cooled because of the large amount of excess heat which is given off with the high voltage of electricity required for the excitation of the atoms.

The photons pass through an optical system that concentrates the photons to a fine spot. The photons of light intersect with the individual cells of the sample at the interrogation point and light is either scattered through 360° or absorbed and emitted by fluorochrome-conjugated antibodies which are bound to the cells in the sample.

By convention, scattered light collected along the 0° axis is termed forward light scatter, and along the 90° axis is termed side scatter. Forward light scatter is known to be proportional to the size of the cell, if one assumes that the cell is a homogeneous sphere. Side scatter is known to correlate proportionally with cell granularity. Alignment of the laser must be done prior to its use on a daily basis to ensure that results from forward and side scatter are reproducible. The use of commercial beads with standard size and

granularity, such as DNA-Check (Coulter Corp., Miami, FL) are used for alignment.

A histogram displaying light scattering characteristics of white blood cells is shown in Figure 2. Lymphocytes are smaller and less granular than monocytes and granulocytes, therefore exhibit the lowest forward light scatter and side scatter.

Although fluorescent light, which is light emitted by fluorochromes, is scattered through 360°, by convention it is also collected along the 90° axis. The number of photons emitted from cells is proportional to the amount of fluorochrome bound internally or externally to the cell. The flow cytometer must be adjusted on a day-to-day basis to ensure that the intensity of fluorescent light is reproducible. The intensity of fluorescent light is standardized on a daily basis by using commercial fluorescent beads, Standard Brite (Coulter Corp., Miami, FL) and adjusting the laser and/or the photomultiplier.

Sample Chamber

A sample chamber is shown in Figure 3. It consists of a sample insertion tube within a sheath. Both the sample insertion tube and sheath narrow into a conical nozzle assembly which is designed to produce laminar flow. The monodispersed sample solution is introduced into the sample insertion tube at a higher differential pressure than the sheath fluid, which is a commercial balanced electrolyte solution. Laminar flow focusses the monodispersed sample solution into the center of the sheath fluid, called a coaxial stream.

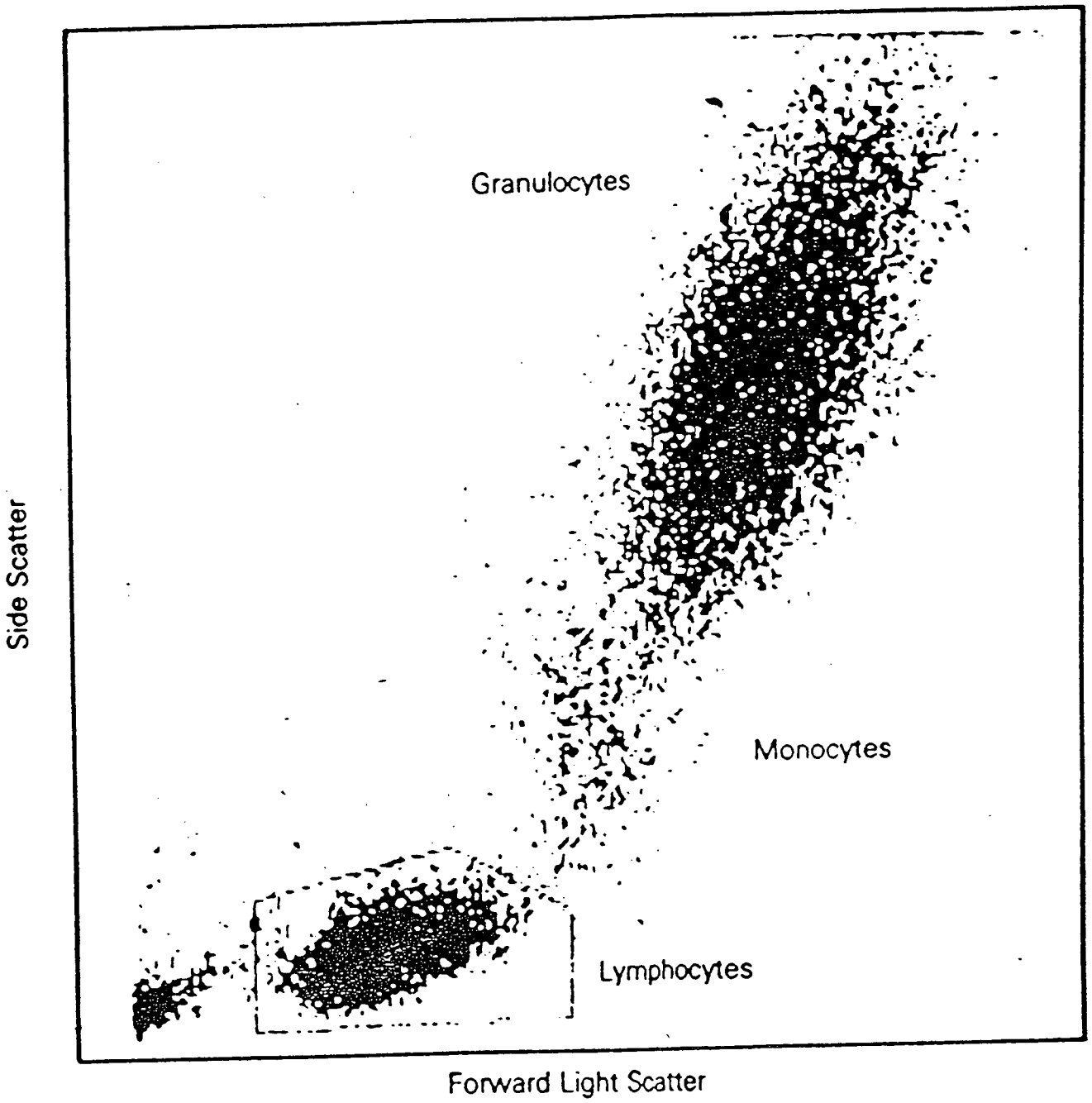


Figure 2: Two Parameter Histogram with a Bitmap drawn around the Lymphocyte Population

(Reproduced from Becton Dickinson, Mountain View, CA)

The inner concentric stream that is created, called the sample stream, is one cell wide. After a cell in the sample stream has passed through the interrogation point, which is the point at which the laser light intersects with the cell, it is collected into a hose for disposal.

Some flow cytometers have a sorting apparatus which consists of a piezoelectric crystal surrounding the sheath. Electrical pulses are applied to the crystal which causes the sheath to oscillate. In this way, cells can be sorted according to the preprogrammed parameters set by the operator.

The sample must be in a monodispersed suspension, which means that the heterogeneous cell population must be uniformly distributed in the sample solution, to prevent blockage and technical errors. The size of the cells must be known so that they will not cause a blockage in the narrow sample insertion tube or at the interrogation point. The flow of both the sample and sheath streams must be controlled to create the coaxial stream.

Detection and Analysis

The 90° side scatter and fluorescent light is collected in a detection chamber, called a photomultiplier. The re-emitted light is separated into various wavelengths by filters or mirrors which selectively allow only certain wavelengths of light to pass. The filters absorb wavelengths of light which are not of interest, while mirrors reflect them. A dichroic (doubly refracting) mirror can allow two specific wavelengths of light to pass

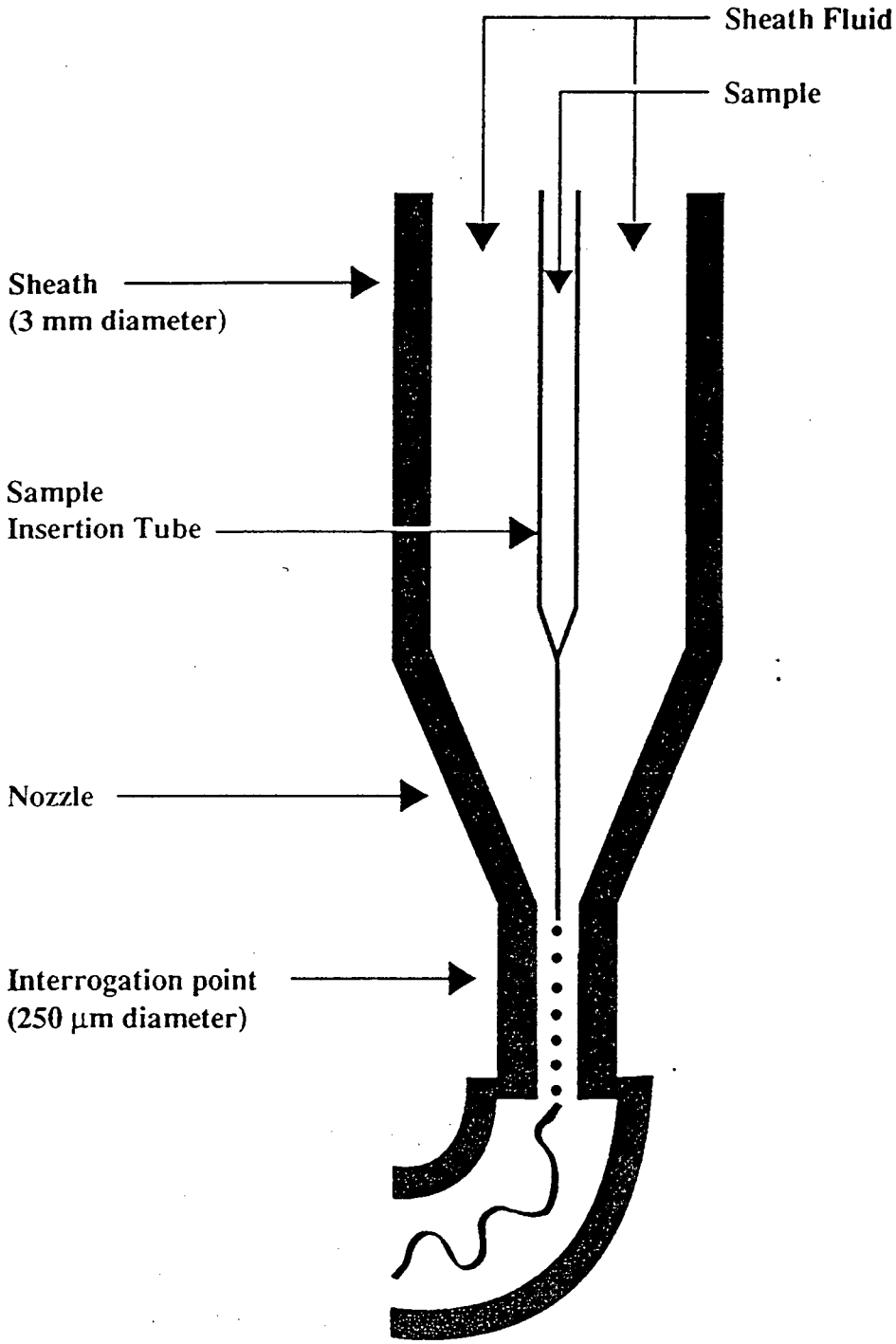


Figure 3: Sample Chamber in a Flow Cytometer

simultaneously, such as green light from FITC and red light from PE.

The photons of light that are allowed to pass through the filters, or, are not reflected by the mirrors, are directed towards detector chambers called photomultipliers. Each photomultiplier detects light of a specific wavelength, for example, green light re-emitted from the FITC fluorochrome. A dichroic mirror can separate the re-emitted fluorescent light from two different fluorochromes, simultaneously, to two different photomultipliers. A series of detectors within the photomultiplier convert the photon energy re-emitted from each cell which intersects with the laser beam into an electrical impulse. The electric impulse, or signal intensity, generated is proportional to the total number of photons received from an individual cell. In flow cytometry, a total of 10,000 cells are usually analysed in each sample.

The electrical impulses generated from the cells are collected with logarithmic amplification for two reasons; to improve the sensitivity of cells re-emitting lesser amounts of photons, and, to detect a broader range of total photons re-emitted. The electrical signals are converted to digital signals, which are stored in the computer, called listmode. The listmode is used to form histograms once the operator has selected the parameters of interest.

Histograms can display a single parameter or multiple parameters. Figure 4 is an example of a single parameter histogram, with PE fluorescence intensity on the x-axis and number

of cells on the y-axis. The digital signals are usually displayed using low resolution histograms, which means that the range of the digital signals, using logarithmic amplification, is spread over 256-channels. Some newer flow cytometers use high resolution histograms which display the range of log digital signals over 1024-channels.

Multiparameter analysis allows further separation of cells by their constituent characteristics. Three-dimensional analysis is available on the newest models of flow cytometers. It is often easier to subdivide the parameters and analyze the results sequentially, as displayed in Figures 5 and 6.

Gating of cells, which means that cells with certain forward and side scatter properties are selected for evaluation, takes advantage of multiparameter analysis. For example, in Figure 2, an electronic gate or "bitmap" has been manually drawn around the lymphocyte population of cells, based upon their forward and side scatter properties as displayed in the histogram. The bitmap is drawn electronically by the flow cytometric operator once the histogram displaying the light scattering characteristics of the cells in the sample has been created. Following the gating of a certain cell population of interest, the digital signals of cells from only within the gate are displayed. Gating is a powerful way of improving both the sensitivity and the specificity of flow cytometric analysis.

Computerization has rapidly advanced the technology of flow cytometry. Computerization has resulted in rapid analysis of cells, presently at a rate of 10,000 cells per second. With

the rapid cell sorting technology, cells can be separated while maintaining their viability for future use. Multiparameter gating allows the analysis of rare cells in a heterogeneous sample as well as the elimination of artifacts from within the sample. Flow cytometry has therefore become a very valuable tool with far reaching capabilities for the advancement of medical research.

Chapter 5

Materials and Methods

In organ transplantation, the flow cytometric lymphocyte crossmatch (FCXM) is known to be a sensitive test for the detection of alloantibodies in human serum. In reproductive immunology, the FCXM has recently been used to detect maternal alloantibodies (IgG and IgM) in maternal serum to paternal T and B lymphocytes, in couples with a history of unexplained habitual abortion, that is, in whom genetic, endocrine, infectious and autoimmune factors associated with habitual abortion have been ruled out[75]. As with the use of FCXM in transplantation immunology, standardization of the FCXM methodology for use in reproductive immunology is essential in order to ensure reproducibility of results between different laboratories.

The FCXM methodologies used in both the reproductive immunology and transplantation literature were reviewed[65,66,67,68,69,70,75,76,77]. There appeared to be two methods of isolating mononuclear cells from peripheral blood: by lysing whole blood and by density gradient centrifugation. Since there were advantages and disadvantages to both methods, the two methods were compared prospectively. As well, there appeared to be two methods of preincubating mononuclear cells: by adding fetal calf serum and by adding goat IgG. These two methods were also compared prospectively. Following this, a reference range study was undertaken to determine what defined a positive FCXM for maternal serum IgG and IgM against paternal T and B lymphocytes. This standardized FCXM assay was then

used clinically to monitor maternal allosensitization in nine patients with a history of primary unexplained habitual abortion who had undergone a modified method of paternal mononuclear cell immunization prior to their next pregnancy.

Comparison of Two Methods to Isolate Mononuclear Cells: Whole Blood Lysis vs. Density Gradient Centrifugation

The preparation of mononuclear cells for use in flow cytometry is performed by either the whole blood lysis method or by density gradient centrifugation. The advantage of the whole blood lysis method is that it requires less technical time to complete (30 minutes vs. 60 minutes), less blood is required (1:3) and it is significantly less expensive. It was therefore important that the two methods of mononuclear cell isolation be compared.

Preparation of Mononuclear Cells

Peripheral blood mononuclear cells were obtained from two healthy volunteers from the Immunology Laboratory, Vancouver Hospital & Health Sciences Centre. Twenty-eight milliliters (mls) of peripheral blood was drawn from each by venipuncture into sterile, sodium heparinized glass test tubes at room temperature. From each volunteer, 7 mls was used for whole blood lysis and 21 mls was used for density gradient centrifugation.

i) Whole Blood Lysis Method

Whole blood aliquots of 0.1 mls were placed in 12 polystyrene test tubes at room temperature for each volunteer. Two mls of the lysing solution (10 ml phosphate buffered saline pH 7.22-7.28 (PBS), 90 ml distilled H_2O and 0.1 ml glacial acetic acid) was added to each test tube, vortexed for 20 seconds then neutralized with 0.2 ml of 10x PBS. The

test tubes were then centrifuged at 400x gravity (g) at room temperature for 3 minutes (min). The supernatant was decanted and the pellet resuspended in 1 ml of Medium 199 (Hank's salt with L-glutamine) containing 2% Fetal Calf Serum (FCS). The concentration of mononuclear cells was adjusted to 1-2 x 10^6 per tube, using a cell counting chamber. The test tubes were then centrifuged at 400xg at room temperature for 10 min and the supernatant decanted leaving a dry cell pellet at the bottom of each test tube.

ii) Density Gradient Centrifugation Method

The 21 mls of whole blood for each volunteer were diluted 1:2 with PBS and then layered and separated through a Ficoll-Hypaque (Pharmacia Biotech, Uppsala, Sweden) density gradient at 400xg at room temperature for 30 min. The mononuclear cell layer was then removed by pipette, washed once in 40 mls of PBS, centrifuged at 400xg at room temperature for 10 min and then resuspended in 1 ml of Medium 199 containing 2% FCS. Twelve test tubes were prepared for each volunteer, adjusting the concentration of mononuclear cells to 1-2 x 10^6 per tube, using the cell counting chamber. The test tubes were then centrifuged at 400xg at room temperature for 10 min and the supernatant decanted leaving a dry cell pellet at the bottom of each test tube.

Preincubation

To inhibit nonspecific Fc binding of human IgG to T lymphocytes, 50 μl of 1/200 dilution

Goat IgG (Cedarlane Laboratories Ltd., Hornby, Ontario),, was added to all of the test tubes from both methods of mononuclear cell isolation. The test tubes were then preincubated at 4°C for 15 min.

Preparation of Serum

The negative control serum used in this project was commercially prepared, MLC AB serum (RJO Biologicals, Inc., Kansas City, MO). This commercial product consists of pooled sera, collected from 30-40 healthy, blood group AB, nontransfused male donors whose serum is free of cytotoxic HLA-A, B or C loci antibodies. It is capable of supporting mononuclear cells in a mixed lymphocyte culture (MLC).

The positive IgG control serum was obtained by pooling sera from seven renal patients, each with detectable HLA antibodies to a screening panel of lymphocytes from 30 known donors. Each serum had panel reactive antibodies (PRA) greater than 90% . Each serum sample was treated with dithiothreitol (Sigma Chemical Co., St. Louis, MO) to determine which immunoglobulin isotype predominated. Dithiothreitol (DTT) cleaves intersubunit disulfide bonds of IgM, but leaves IgG intact. If the PRA remained elevated after DTT treatment, the sample was considered to be predominantly IgG.

The positive IgM control serum was obtained from a single renal allograft patient. Her serum showed a PRA >90%. With DTT treatment, the PRA dropped to 0%, indicating

the presence of predominantly IgM.

The individual AB sera from was obtained from anonymous,nontransfused, blood group AB males who donated blood to the Vancouver centre of the Canadian Red Cross Blood Services. A total of 32 sera was required for the project. Two specimens were used in the section comparing mononuclear cell isolation methodologies, two specimens were used in the section comparing preincubation of mononuclear cell methodologies and twenty-eight specimens were used in the reference range study.

Primary Antibody Incubation

One hundred microliters (μls) of negative control serum, positive control serum (IgG or IgM) and individual AB serum was added to the four of the twelve test tubes containing mononuclear cells, as shown in Table II. The tubes were then incubated for 30 min at 4°C, and twice washed with 2 mls of PBS containing 0.1% sodium azide (NaN_3) and 1% FCS followed by centrifugation at 400xg at room temperature for 10 min. The supernatant was decanted leaving a dry cell pellet at the bottom of each test tube.

Secondary Antibody Incubation

To identify the T lymphocytes, 10 μls of an optimal dilution (twofold serial titrations of each lot) of the phycoerythrin-conjugated (PE) mouse anti-human CD3 monoclonal

Table II

Configuration of Test Tubes for FCXM

Tube	Primary Incubation (serum)	Secondary Incubation	
		P/E	$F(ab')_2$ FITC
1	Negative control serum	CD3 (T cell)	IgG-Fc
2	IgG positive control serum	CD3	IgG-Fc
3	Individual AB serum	CD3	IgG-Fc
4	Negative control serum	CD3	IgM
5	IgM positive control serum	CD3	IgM
6	Individual AB serum	CD3	IgM
7	Negative control serum	CD20 (B cell)	IgG-Fc
8	IgG positive control serum	CD20	IgG-Fc
9	Individual AB serum	CD20	IgG-Fc
10	Negative control serum	CD20	IgM
11	IgM positive control serum	CD20	IgM
12	Individual AB serum	CD20	IgM

antibodies (Becton Dickinson, San Jose, CA), abbreviated CD3 PE, was added to six of the twelve test tubes in each group, as shown in Table IV. To identify the B lymphocytes, 5 μls of an optimal dilution of PE mouse anti-human CD20 monoclonal antibodies (Becton Dickinson, San Jose, CA), abbreviated CD20 PE, was added to the other six test tubes in each group.

To identify the binding of human IgG to lymphocytes, 100 μls of an optimal dilution of fluorescein-conjugated (FITC) AffiniPure $F(ab')_2$ fragment goat anti-human IgG (Fc specific) antibodies (Jackson Immunoresearch Lab Inc., West Grove, PA), abbreviated IgG-Fc $F(ab')_2$ FITC, was added to six of the test tubes (three tubes with CD3 PE and three test tubes with CD20 PE) in each group, as shown in Table II.

To identify the binding of human IgM to lymphocytes, 50 μls of an optimal dilution of FITC $F(ab')_2$ fragment goat anti-human IgM antibodies (Tagoimmunol, Camarillo, CA), abbreviated IgM $F(ab')_2$ FITC, was added to the other six test tubes in each group.

The twelve tubes were then incubated for 30 min at 4°C. The cells were then twice washed with 2 mls of PBS containing 0.1% NaN_3 and 1% FCS followed by centrifugation, at 400xg at room temperature for 10 min. The supernatant was decanted leaving a dry cell pellet at the bottom of each test tube. The cells were then fixed in 0.3 mls of 1% paraformaldehyde.

Dual-Colour Flow Cytometric Analysis

The samples were analyzed using an EPICS Profile I (Coulter Electronics, Miami, FL) flow cytometer, equipped with a 15mW argon laser (488 nm excitation, 250 mW emission). The argon laser was aligned with DNA-Check (Coulter Corp., Miami, FL) beads. Fluorescence was standardized using Standard Brite (Coulter Corp., Miami, FL) beads. Data was collected with logarithmic amplification, and fluorescence intensity was displayed on a 256-channels, four decade log scale.

For each test tube, an electronic gate, called a bit map, was manually drawn around the lymphocyte population, based on their forward scatter (FS) and side scatter (SS) properties, as illustrated in Histogram 1 (Figure 4).

Phycoerythrin (PE) fluorescence (CD3 or CD20) of 10,000 cells from within the bit map was plotted on a single parameter histogram, on a 256 channel, four decade log scale, as shown in Histogram 2 (Figure 5). A window gate was drawn across the CD3 PE- or CD20 PE-positive cell population. FITC fluorescence of cells within the CD3 PE or CD20 PE window gate was plotted to a single parameter histogram, on a 256 channel, four decade log scale, as shown in Histogram 3 (Figure 6). A cursor was drawn across the x-axis of Histogram 3, to determine the logarithmic mean channel fluorescence of FITC. Using the table supplied by the manufacturer, the logarithic mean channel fluorescence was converted to the linear mean channel fluorescence.

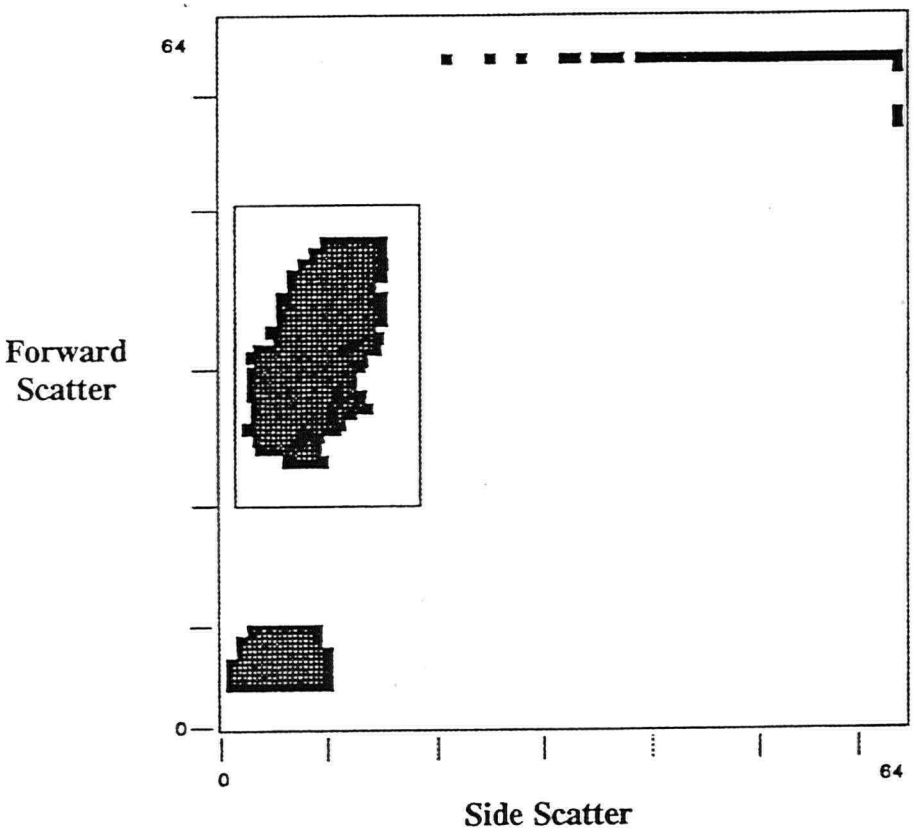


Figure 4: Histogram 1: Identifying Lymphocyte Population Using Flow Cytometry

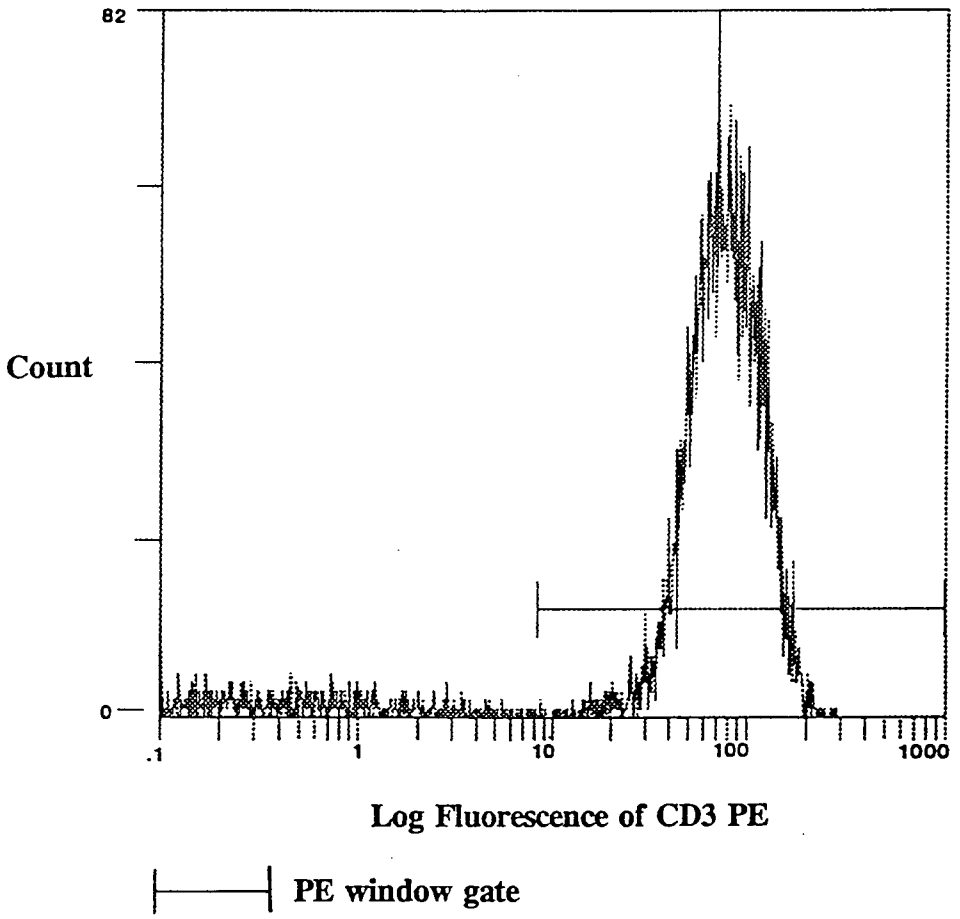


Figure 5: Histogram 2: PE fluorescence (CD3 or CD20)

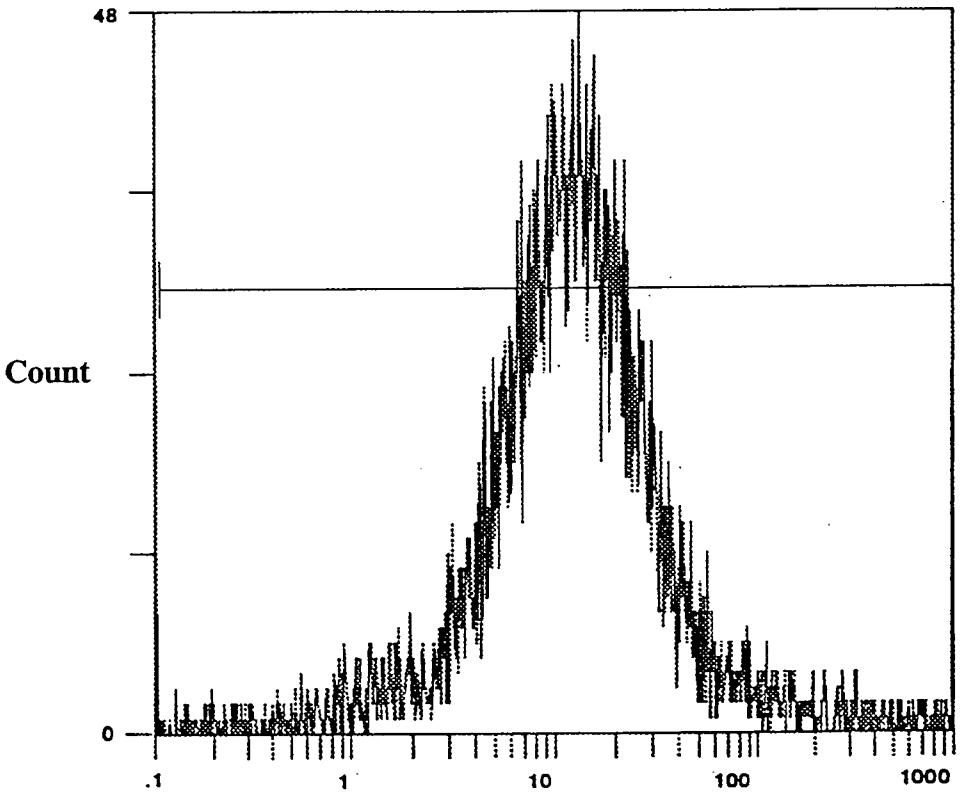


Figure 6: Histogram 3: FITC Fluorescence (IgG-Fc or IgM) Within PE Window Gate

Comparison of Two Methods to Preincubate Mononuclear Cells: Goat IgG vs Fetal Calf Serum

Since the flow cytometric crossmatch utilizes indirect immunofluorescence, nonspecific binding of human IgG to Fc receptors, would result in erroneous mean channel shifts. Inhibition of nonspecific human IgG binding to Fc receptors can be achieved by preincubating the cells with either goat IgG or fetal calf serum. Since fetal calf serum was used previously in the Immunology Laboratory at Vancouver Hospital, it was necessary to compare the two methods of inhibiting nonspecific binding of human IgG to Fc receptors.

Goat IgG is isolated from goat serum using size exclusion column chromatography. The goat IgG binds to the Fc receptors, blocking nonspecific binding of the human IgG, added in the subsequent step.

Fetal calf serum (10%) has been used previously in both tissue typing and flow cytometric procedures in the laboratory. The exact mechanism by which fetal calf serum inhibits nonspecific binding is unclear, since there is little or no immunoglobulin in the product.

Preparation of Mononuclear Cells

Forty-two mls of peripheral blood was drawn from two healthy volunteers by venipuncture

into sterile, sodium heparinized glass test tubes at room temperature. The blood was diluted 1:2 with PBS and then the mononuclear cells were separated using the density gradient centrifugation method as described above. Twenty-four test tubes were prepared for each volunteer, adjusting the concentration of mononuclear cells to 1-2 x 10^6 per tube, using the cell counting chamber. The test tubes were then centrifuged at 400xg at room temperature for 10 min and the supernatant decanted leaving a dry cell pellet at the bottom of each test tube.

Preincubation of the Mononuclear Cells

For each of the two volunteers, twelve of the twenty-four test tubes were preincubated with goat IgG. The other twelve test tubes were preincubated with FCS, as described below.

i) Preincubation using Goat IgG

Goat IgG (Cedarlane Laboratories Ltd., Hornby, Ontario), 50 ul of a 1/200 dilution, was added to twelve test tubes for each of the volunteers. The tubes were incubated at 4°C for 15 min.

ii) Preincubation using Fetal Calf Serum

Four mls of 10% FCS (Fetal Bovine Serum, Qualified, Gibco Laboratories), was added to the other twelve test tubes for each volunteer. After one hour at 37°C, the test tubes were centrifuged at 400xg at room temperature for 10 min and the supernatant decanted leaving a dry cell pellet at the bottom of each test tube. The cells were then twice washed with 2 mls of PBS containing 0.1% sodium azide (NaN_3) and 1% FCS followed by centrifugation at 400xg at room temperature for 10 min. The supernatant was decanted leaving a dry cell pellet at the bottom of each test tubes.

Primary and Secondary Antibody Incubation

Primary and secondary antibody incubation was performed as described above.

Dual-Colour Flow Cytometric Analysis

Dual-colour flow cytometric analysis was performed as described above.

FCXM: Reference Range Study

After comparing methods to isolate and subsequently preincubate mononuclear cells, a reference range study was undertaken to determine what a positive FCXM was for maternal serum IgG and IgM against paternal T and B lymphocytes, in couples being investigated for unexplained habitual abortion.

Preparation of Mononuclear Cells

Peripheral blood was obtained from twenty-eight healthy male and female volunteers from the Immunology Laboratory, Vancouver Hospital & Health Sciences Centre. Fourteen milliliters of peripheral blood was drawn by venipuncture into sterile, sodium heparinized glass test tubes at room temperature. The blood was diluted 1:2 with PBS and then separated through a Ficoll-Hypaque (Pharmacia Biotech, Uppsala, Sweden) density gradient at 400xg at room temperature for 30 min. The mononuclear cell layer was then removed by pipette, washed once in 40 mls of PBS and then resuspended in Medium 199 containing 2% FCS. Twelve test tubes were prepared for each volunteer, adjusting the concentration of mononuclear cells to 1-2 x 10^6 per tube, using a cell counting chamber. The test tubes were then centrifuged at 400xg at room temperature for 10 min and the supernatant decanted leaving a dry cell pellet at the bottom of each test tube.

Preincubation of the Mononuclear Cells

Goat IgG (Cedarlane Laboratories Ltd., Hornby, Ontario), 50 μl of 1/200 dilution, was then added to each of the twelve test tubes. The test tubes were then preincubated at 4°C for 15 min. This was repeated for each of the 28 volunteers.

Configuration of the Test Tubes

Twelve test tubes were prepared for each of the twenty-eight volunteers who donated blood for mononuclear cell isolation. For each of the volunteers, an individual AB serum, obtained from the Vancouver centre of the Canadian Red Cross Blood Services, was used. The configuration of the test tubes for each set of donated AB serum and mononuclear cells is illustrated in Table II.

Primary and Secondary Antibody Incubation

Primary and secondary antibody incubation was performed as described above.

Dual-Colour Flow Cytometric Analysis

The flow cytometric analysis was performed as described.

Monitoring Maternal Allosensitization using the FCXM Methodology

To assess the FCXM as a clinical tool, a prospective case series was designed, using women with a history of primary unexplained habitual abortion who had consented to receive paternal mononuclear cell (PMC) immunization as treatment. As previously described, primary unexplained habitual abortion was defined as three or more consecutive spontaneous abortions without an antecedent pregnancy which progressed beyond 20 weeks' gestation with the same reproductive partner and no evidence of genetic, endocrine, infectious, anatomical or autoimmune factors which are associated with habitual abortion.

Only women with a negative FCXM to their partner were offered immunization, since this indicated that there was no detectable maternal allosensitization to her partner's mononuclear cells. Prior to immunization, both partners were screened for hepatitis and HIV, the woman's rhesus (Rh) and platelet (PL)-A^1 antigen status were also determined. Exclusion criteria included evidence of hepatitis or HIV in either partner, or Rh negative or PL-A^1 antigen negative in the female partner, to avoid maternal allosensitization to the fetus. Two days prior to immunization, a serum qualitative βhCG was ordered. If the result indicated pregnancy, the couple was excluded.

Preparation of the paternal mononuclear cells

Peripheral blood mononuclear cells were obtained from the male partner. One hundred

mls of peripheral blood was drawn by venipuncture into 15 x 7 mls sterile, sodium heparinized glass test tubes at room temperature. The blood was diluted with 1:2 with PBS and layered over Ficoll-Hypaque in 8 x 50 mls test tubes. The test tubes were then centrifuged at 400xg at room temperature for 30 minutes. The mononuclear cell layer from each test tube was removed by pipette and washed with 50 mls of sterile normal saline, followed by centrifugation at 400xg at room temperature for 10 minutes and the supernatant decanted. The cell pellet was washed once in 40 mls of PBS, centrifuged at 400xg at room temperature for 10 min and the supernatant decanted. The cells in one of the test tubes was resuspended in 25 mls of sterile normal saline and added to the next test tube. This was repeated with all of the eight test tubes. The mononuclear cell suspension was was topped up to 50 mls with sterile normal saline, centrifuged at 400xg at room temperature for 10 min and the supernatant decanted. The cells were resuspended in 1-1.5 ml sterile normal saline and a cell count was obtained, using the cell counting chamber. An optimum count was over 100 x 10^6 cells. The suspension was drawn up into a 3 ml syringe. The sample was irradiated twice at 25 grays.

Immunization protocol and monitoring of subsequent pregnancies

The PMC immunization involved injecting the 1-1.5 ml suspension subcutaneously, using a 26-gauge needle, into several sites on the woman's forearm. The woman was observed for 1/2 hour and then discharged.

The FCXM was repeated six weeks later. If negative, the woman was reimmunized and retested at six week intervals until a positive FCXM was obtained. When the FCXM became positive, the couple was encouraged to conceive. If pregnancy did not occur in six months, the FCXM was repeated and if negative, the woman was reimmunized and retested. Close monitoring, consisting of endovaginal ultrasonography and physician visits at 6, 9 and 12 weeks' gestation, was offered in the first trimester in the pregnancies following the immunization. Routine prenatal care was given in the second and third trimesters. Pregnancy outcome data was obtained on all patients from the referring doctors' and/or hospital records.

Chapter 6

Results

The results will be presented as separated in the previous chapter. The results from the monitoring of maternal allosensitization following paternal mononuclear cell immunization will include pregnancy outcome data from all documented pregnancies to date.

Comparison of Two Methods to Isolate Mononuclear Cells: Whole Blood Lysis vs. Density Gradient Centrifugation

The linear mean channel fluorescence for each of the test tubes has been tabulated in Table III. Analysis of variance was used to compare the two methods: whole blood lysis and density gradient centrifugation, adjusting for the two volunteers and the four combinations of fluorochrome-conjugated antibodies. The difference between the methods was not significant, $p=0.13$.

Table III

Comparison of Two Methods to Isolate Mononuclear Cells: Whole Blood Lysis vs. Density Gradient Centrifugation

Fluorochrome-conjugated Antibodies Serum	Linear Mean Channel Fluorescence			
	Cells from volunteer #1		Cells from volunteer #2	
	Whole Blood Lysis	Density Gradient[1]	Whole Blood Lysis	Density Gradient
CD3 PE/IgG-Fc F(ab')$_2$ FITC				
Negative Control[2]	55	53	58	50
IgG Positive Control[3]	77	69	74	75
Individual AB[4]	66	56	57	50
CD3 PE/IgM F(ab')$_2$ FITC				
Negative Control	36	34	34	32
IgM Positive Control[5]	75	85	78	70
Individual AB	40	35	37	36
CD20 PE/IgG-Fc F(ab')$_2$FITC				
Negative Control	62	63	66	61
IgG Positive Control	82	82	74	90
Individual AB	63	154	66	63
CD20 PE/IgM F(ab')$_2$ FITC				
Negative Control	57	52	55	58
IgM Positive Control	106	111	119	118
Individual AB	61	57	88	70

[1]Ficoll-Hypaque (Pharmacia Biotech, Uppsala, Sweden)
[2]MLC AB Serum (RJO Biologicals, Inc., Kansas City, MO)
[3]Pooled positive IgG serum from seven renal patients, each with PRA >90%
[4]Anonymous, nontransfused male blood donor
[5]Positive IgM serum from a single renal allograft patient with a PRS >90% prior to DTT treatment, and 0% following DTT treatment

Comparison of Two Methods to Preincubate Mononuclear Cells: Goat IgG vs Fetal Calf Serum

The linear mean channel fluorescence for each of the test tubes has been tabulated in Table IV. Analysis of variance was used to compare the two methods of preincubation: goat IgG and vs 10% fetal calf serum, adjusting for the two volunteers and the four combinations of fluorochrome-conjugated antibodies. The difference between the methods was not significant, $p=0.32$.

Table IV

Comparison of Two Methods to Preincubate Mononuclear Cells: Goat IgG vs Fetal Calf Serum

Fluorochome-conjugated Antibodies Serum	Linear Mean Channel Fluorescence			
	Cells from volunteer #1		Cells from volunteer #2	
	Goat IgG[1]	Fetal Calf Serum[2]	Goat IgG	Fetal Calf serum
CD3 PE/IgG-Fc F(ab')$_2$ FITC				
Negative Control[3]	64	83	119	69
IgG Positive Control[4]	152	153	151	161
Individual AB[5]	54	69	58	76
CD3 PE/IgM F(ab')$_2$ FITC				
Negative Control	35	40	34	35
IgM Positive Control[6]	117	128	122	131
Individual AB	35	40	34	34
CD20PE/IgG-Fc F(ab')$_2$ FITC				
Negative Control	80	78	81	78
IgG Positive Control	185	169	182	173
Individual AB	94	104	65	80
CD20 PE/IgM F(ab')$_2$ FITC				
Negative ControI	111	117	130	149
IgM Positive Control	133	149	147	149
Individual AB	115	107	137	147

[1]Normal Goat IgG (Cedarlane Laboratories Ltd., Hornby, Ontario), 1/200 dilution
[2]10% Fetal Bovine Serum, Qualified (Gibco Laboratories)
[3]MLC AB Serum (RJO Biologicals, Inc.; Kansas City, MO)
[4]Pooled positive IgG serum serum from seven renal patients, each with PRA >90%
[5]Anonymous, nontransfused male blood donor donor serum
[6]Positive IgM serum from a single renal allograft patient with a PRA >90% prior to DTT treatment, and 0% following DTT treatment

FCXM: Reference Range Study

The linear mean channel fluorescence was determined for each set of twelve test tubes; a total of twenty-eight sets of test tubes were tabulated. Tables V, VI, VII, VIII list the results from the twenty-eight sets, for each combination of fluorochrome-conjugated antibodies; CD3 PE/IgG-Fc $F(ab')_2$ FITC, CD3 PE/IgM FITC, CD20 PE/IgG-Fc $F(ab')_2$ FITC and CD20 PE/IgM FITC. Channel shifts were computed by subtracting the linear mean channel fluorescence of the negative control serum (marked A) from the individual AB serum (marked B), as illustrated in Histogram 4 (Figure 7). Each of the four data sets were trimmed of its maximum and minimum channel shifts, as indicated by the **.

Table V

CD3 PE/IgG-Fc F(ab′)$_2$ FITC

Donated Serum	Mononuclear Cells (volunteers)	Linear Mean Channel Fluorescence of IgG-Fc F(ab′)$_2$ FITC			Channel Shift (B-A)
		Negative Control Serum (A)	Positive Control Serum	Donated Serum (B)	
AB-1	1	71	184	63	-8
AB-2	2	76	161	68	-8
AB-3	3	71	178	75	4
AB-4	4	58	205	63	5
AB-5	5	82	212	73	-9
AB-6**	6	77	198	54	-23
AB-7	7	71	194	63	-8
AB-8	8	77	201	57	-20
AB-9	9	75	205	60	-15
AB-10	10	70	195	70	0
AB-11	11	70	209	70	0
AB-12	12	57	203	59	2
AB-13	13	27	198	33	6
AB-14	14	32	180	32	0
AB-15	15	55	186	52	-3
AB-16	16	54	167	56	2

Table V (cont.)

AB-17	17	50	192	41	-9
AB-18	18	44	177	42	-2
AB-19	19	61	189	51	-10
AB-20	20	50	147	54	4
AB-21	21	51	154	51	0
AB-22	22	56	147	53	-3
AB-23	23	69	163	58	-11
AB-24	24	66	166	59	-7
AB-25	25	67	150	60	-7
AB-26**	26	54	150	63	9
AB-27	27	82	152	91	9
AB-28	28	90	159	97	7

**trimmed from data set (maximum and minimum channel shifts)

Table VI

CD3 PE/IgM F(ab')$_2$ FITC

Donated Serum	Mononuclear Cells (volunteers)	Linear Mean Channel Fluorescence of IgM F(ab')$_2$ FITC			Channel Shift (B-A)
		Negative Control Serum (A)	Positive Control Serum	Donated Serum (B)	
AB-1	1	26	101	28	2
AB-2	2	35	76	42	7
AB-3	3	33	67	30	-3
AB-4	4	17	96	17	0
AB-5	5	18	104	17	-1
AB-6	6	27	72	27	0
AB-7	7	26	93	34	8
AB-8	8	29	94	25	-4
AB-9	9	24	95	25	1
AB-10	10	26	96	28	2
AB-11**	11	74	134	69	-5
AB-12	12	68	152	65	-3
AB-13	13	27	104	27	0
AB-14	14	25	102	25	0
AB-15	15	27	82	27	0
AB-16	16	23	89	31	8

Table VI (cont.)

AB-17	17	25	88	25	0
AB-18	18	25	87	24	-1
AB-19	19	23	88	23	0
AB-20	20	24	98	23	-1
AB-21	21	24	83	24	0
AB-22	22	38	109	38	0
AB-23	23	38	128	43	5
AB-24	24	36	126	36	0
AB-25	25	36	110	36	0
AB-26	26	35	79	33	-2
AB-27	27	35	149	43	8
AB-28**	28	40	155	52	12

**trimmed from data set (maximum and minimum channel shifts)

Table VII

CD20 PE/IgG-Fc F(ab')$_2$ FITC

Donated Serum	Mono-nuclear Cells (volunteers)	Linear Mean Channel Fluoresence of IgG-Fc F(ab')$_2$ FITC			Channel Shift (B-A)
		Negative Control Serum (A)	Positive Control Serum	Donated Serum (B)	
AB-1	1	108	210	101	-7
AB-2	2	94	160	82	-12
AB-3	3	99	205	95	-4
AB-4	4	107	199	108	1
AB-5	5	116	190	129	13
AB-6	6	103	220	91	-12
AB-7	7	121	222	106	-15
AB-8	8	116	220	108	-8
AB-9	9	123	227	113	-10
AB-10	10	111	226	111	0
AB-11	11	110	231	138	28
AB-12	12	105	224	101	-4
AB-13	13	94	223	95	1
AB-14	14	84	197	107	23
AB-15	15	65	216	70	5
AB-16	16	79	193	84	5

Table VII (cont.)

AB-17	17	95	227	105	10
AB-18	18	113	214	116	3
AB-19	19	86	206	84	-2
AB-20	20	97	192	95	-2
AB-21**	21	97	204	79	-18
AB-22	22	86	158	84	-2
AB-23	23	95	167	85	-10
AB-24	24	102	172	87	-15
AB-25	25	81	162	76	-5
AB-26	26	88	162	104	16
AB-27**	27	94	158	123	29
AB-28	28	103	162	107	4

**trimmed from data set (maximum and minimum channel shifts)

Table VIII

CD20 PE/IgM F(ab′)$_2$ FITC

Donated Serum	Mono-nuclear Cells (volunteers)	Linear Mean Channel Fluorescence of IgM F(ab′)$_2$ FITC			Channel Shift (B-A)
		Negative Control Serum (A)	Positive Control Serum	Donated Serum (B)	
AB-1**	1	119	127	174	55
AB-2	2	95	135	98	3
AB-3	3	104	135	109	5
AB-4**	4	125	150	111	-14
AB-5	5	118	144	113	-5
AB-6	6	119	156	110	-9
AB-7	7	116	133	109	-7
AB-8	8	136	155	136	-6
AB-9	9	128	165	141	13
AB-10	10	122	162	126	4
AB-11	11	162	192	174	12
AB-12	12	153	207	183	30
AB-13	13	124	167	134	10
AB-14	14	125	184	135	10
AB-15	15	113	161	113	0
AB-16	16	114	167	118	4

Table VIII (cont.)

AB-17	17	113	161	121	8
AB-18	18	115	186	132	17
AB-19	19	103	169	118	15
AB-20	20	121	168	123	2
AB-21	21	116	165	104	-12
AB-22	22	105	147	120	15
AB-23	23	109	153	118	9
AB-24	24	116	157	120	4
AB-25	25	103	155	118	15
AB-26	26	108	151	111	3
AB-27	27	133	189	135	2
AB-28	28	137	175	142	5

**trimmed from data set (maximum and minimum channel shifts)

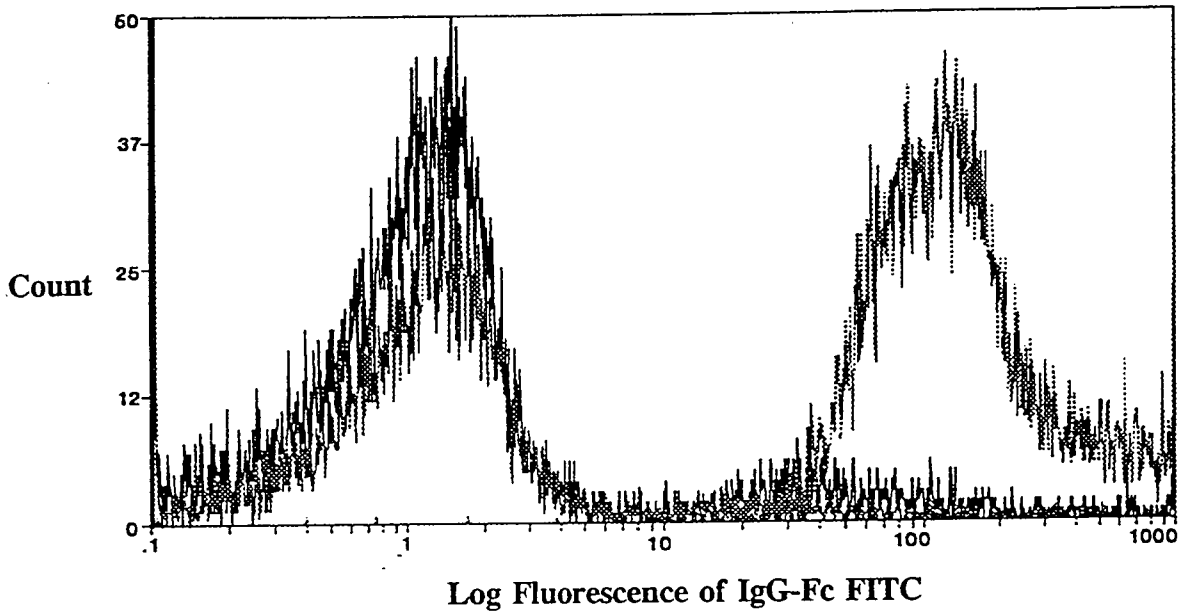


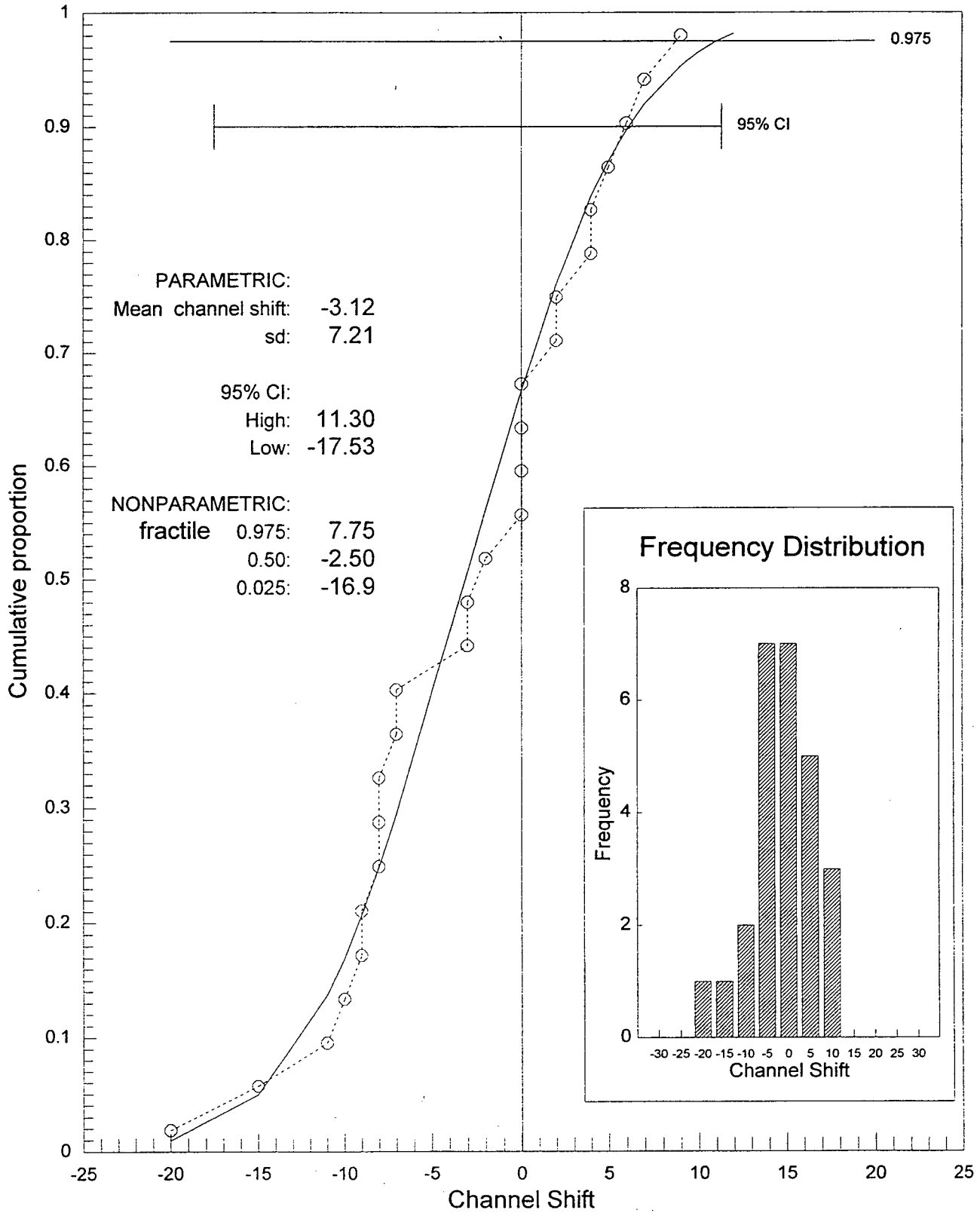
Figure 7: Histogram 4: Illustration of Channel Shift

The frequency distribution of the trimmed channel shifts for each of the data sets was plotted in Figures 8, 9, 10 and 11 (see inserts). The upper reference limits for the negative control serum were determined for each of the data sets, as shown in Table IX, using the formula,

$$\text{upper reference limit} = \text{mean}_{\text{channel shift}} + [\text{sd}_{\text{channel shift}} * t_{(0.05,n-1)} * \sqrt{(1+1/n)}]$$

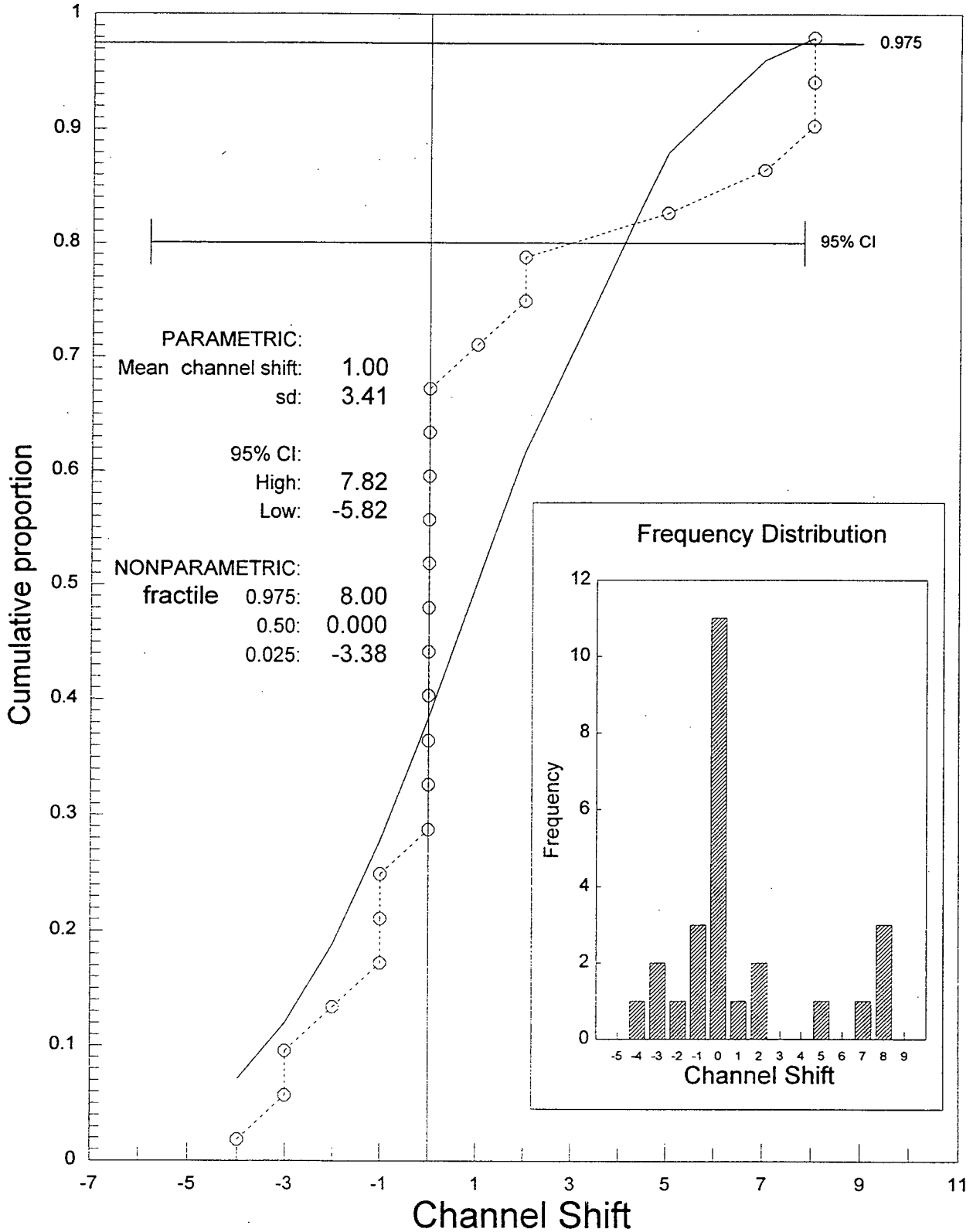
This formula is a normal distribution approximation of the 95th percentile of the distribution of trimmed channel shifts[75] (n=26) when $t_{(0.05,n-1)}$ is the 95th percentage point of the Student-t distribution. The cumulative density curves, the frequency distributions, the means, standard deviations and the nonparametric 0.025 to 0.975 interfractile ranges for each of the data sets are illustrated in Figures 8, 9, 10 and 11.

Figure 8: Normal Reference Interval of CD3 PE/IgG-Fc F(ab')$_2$ FITC



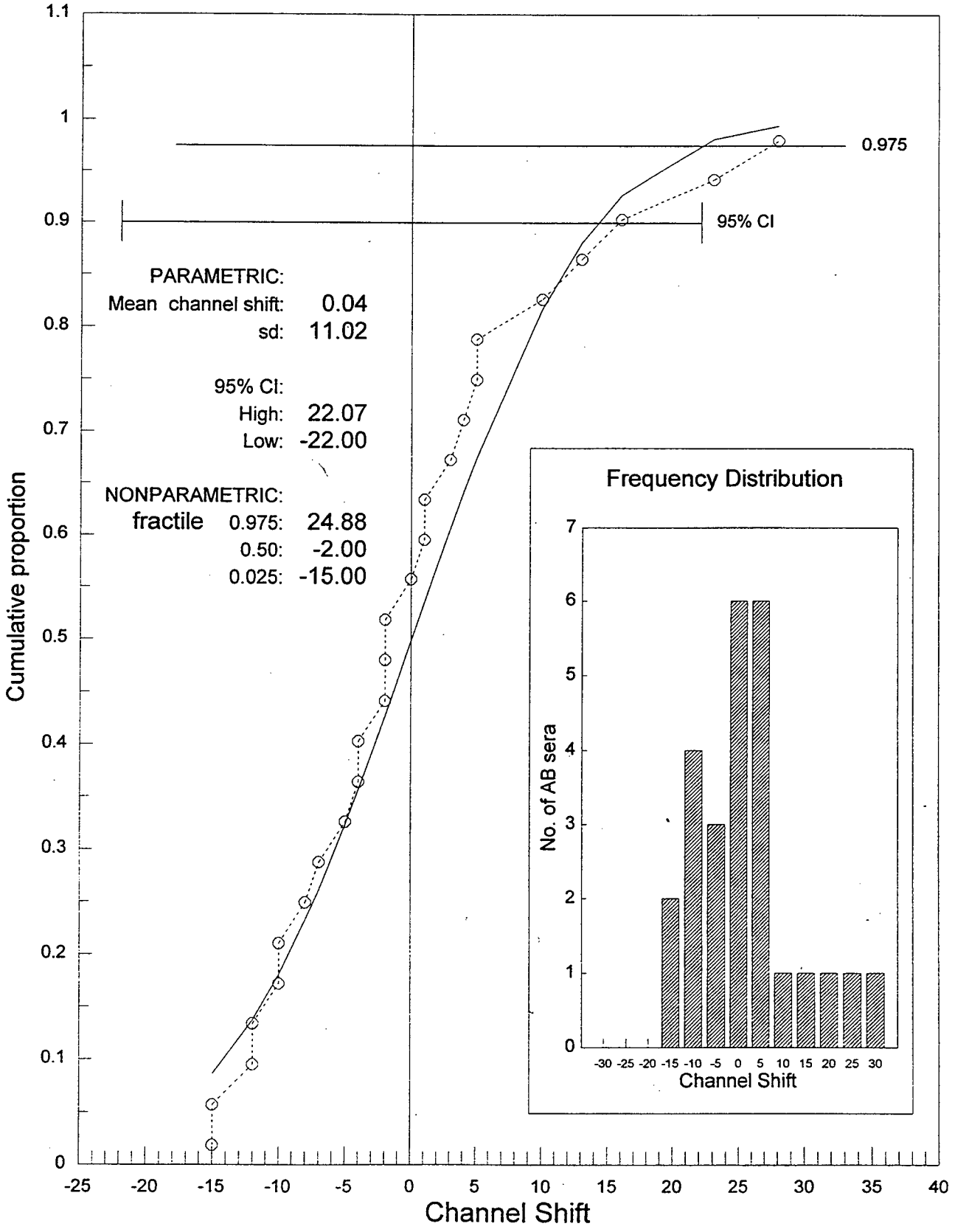
Solid curve: Cumulative normal density given mean = -3.12, sd = 7.207 with 95% confidence interval (95% CI)

Figure 9: Normal Reference Interval of CD3 PE/IgM F(ab')$_2$ FITC



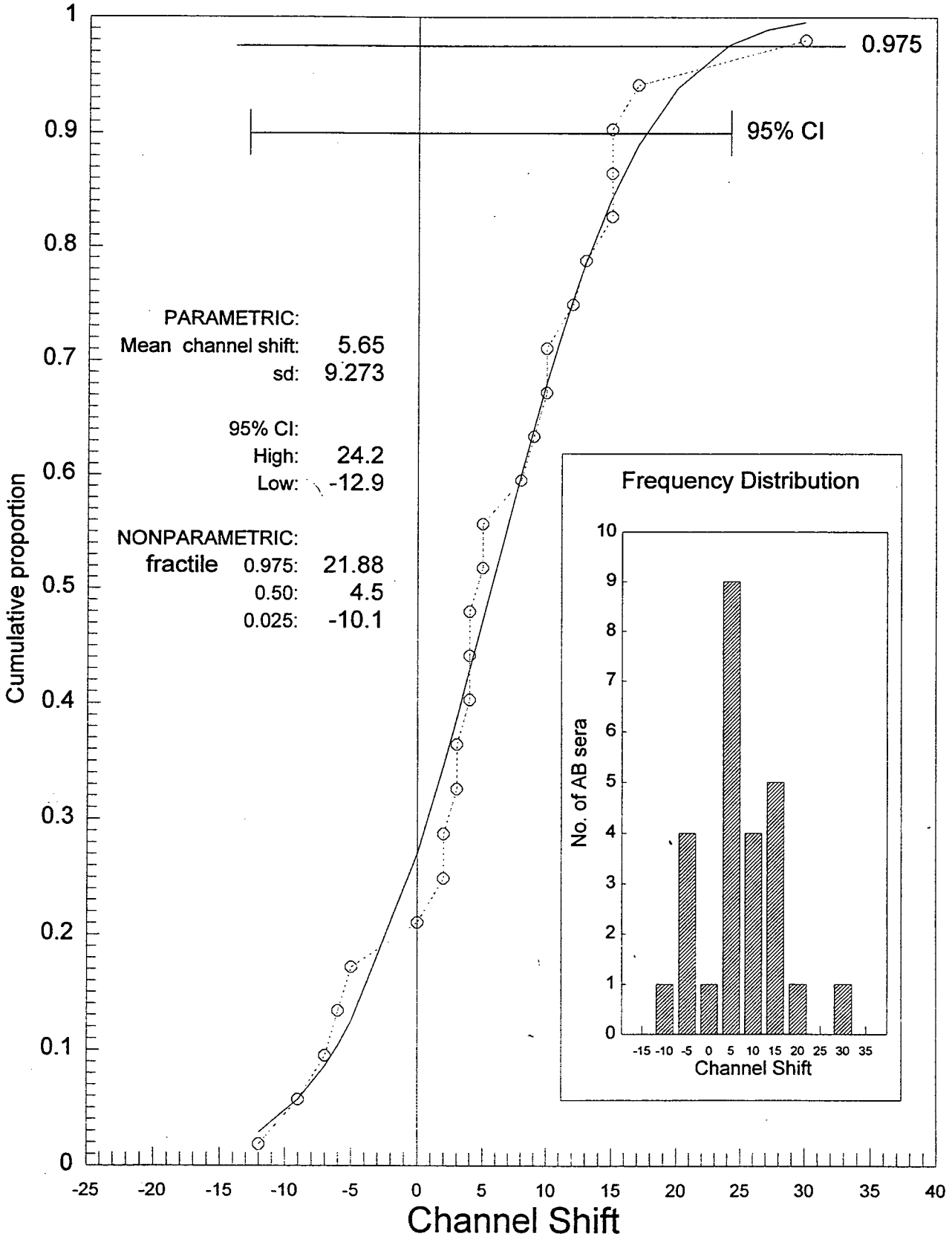
solid curve: cumulative normal density given mean = 1.00, sd = 3.41 with 95% confidence interval (95% CI)

Figure 10: Normal Reference Interval of CD20 PE/IgG-Fc F(ab')$_2$ FITC



Solid curve: cumulative normal density given mean = 0.04, sd = 11.02 with 95% confidence interval (95% CI)

Figure 11: Normal Reference Interval of CD20 PE/IgM F(ab'), FITC



Solid curve: cumulative normal density given mean = 5.65, sd = 9.27 with 95% confidence interval (95% CI)

Table IX

Upper Reference Limits of the Negative Control Serum (n=26)

Fluorochome-Conjugated Antibodies		Channel Shift		
PE	**F(ab′)$_2$FITC**	**Mean**	**sd**	**Upper Reference Limit**
CD3	IgG-Fc	-3.1	7.2	9.4
CD3	IgM	1.0	3.4	6.9
CD20	IgG-Fc	0	11.0	19.2
CD20	IgM	5.6	9.3	21.8

A positive flow cytometric crossmatch (FCXM) was defined by the upper reference limit for the negative control sera, for each of the data sets. For clinical use, the upper reference limits were rounded up to a channel shift of 10 or more for CD3 PE/IgG-Fc $F(ab')_2$ FITC and 7 or more for CD3 PE/IgM FITC. The upper reference limits were rounded up to a channel shift of 20 or more for CD20 PE/IgG-Fc $F(ab')_2$ FITC and 22 or more for CD20 PE/IgM FITC, as shown in Table X.

Table X

Positive Flow Cytometric Crossmatch (FCXM) of the Negative Control Serum

Fluorochrome - Conjugated Antibodies		Channel Shift
PE	**$F(ab')_2$FITC**	
CD3 (T lymphocytes)	IgG-Fc (bound IgG)	≥10
CD3	IgM (bound IgM)	≥7
CD20 (B lymphocytes)	IgG-Fc	≥20
CD20	IgM	≥22

Monitoring Maternal Allosensitization using the FCXM Methodology

Between September 1992 and September 1994, nine couples completed the paternal mononuclear cell immunization protocol. To date, all of the couples conceived at least once. The results of their subsequent pregnancies are summarized in Table XI. The mean age of the women at time of immunization was 34 years, with a range of 27 to 39 years. The mean number of consecutive spontaneous abortions prior to PMC immunization was 5, with a range of 3 to 12. The mean number of immunizations required prior to a positive FCXM was 3, with a range of 2 to 4. The mean number of mononuclear cells in the immunizations was 118 x 10^6 with a range of 77 - 378 x 10^6. Table XI summarizes the subsequent pregnancy outcomes.

Two of the women, patients 3 and 4, received either empiric clomiphene citrate and/or progesterone in their index (first) pregnancy following PMC immunization. Patient 7 required therapeutic heparin during her index pregnancy because of a deep vein thrombosis.

Four of the nine index pregnancies were not successful. Three ended in first trimester spontaneous abortions; one had an abnormal karyotype analysis (45,X) and the other two did not have karyotype analysis performed. The other unsuccessful pregnancy resulted in a spontaneous abortion at 18 weeks' gestation, following silent dilatation of the cervix. The autopsy confirmed chorioamnionitis.

Table XI

Pregnancy Outcome Following PMC Immunization Monitored by FCXM in Couples with Primary Unexplained, Possible Alloimmune Habitual Abortion

ID	Age	Prior #Cons SAs[1]	#PMCI to +ve FCXM[2]	Concurrent Tx	Subsequent Pregnancy Outcome
1	39	4	3		#5 NT[3] 3231 gm male #6 TD[4] 3320 gm male with single kidney, APH with marginal previa
2	28	4	3	#6 clomiphene, cerclage	#5 SA[5] 18 wks, 46XX, cervical incompetence chorioamnionitis #6 NTD 4480 gm male
3	38	4	2	#5 prog supps #6 clomiphene #7 clomiphene	#5 NTD 3755 gm male #6 SA 7 wks, 47,XX+15 #7 NTD 3910 gm male
4	35	4	3	#5 prog supps #6 clomiphene	#5 SA 5 wks #6 NPD[6] 36 wks, 3118 gm female, GD, PTL, pre-eclampsia
5	32	12	4		#13 TD 2800 gm female
6	36	5	3		#6 SA 8 wks, 45,X
7	27	3	2	#5 heparin #6 heparin	#4 NTD 3061 gm female, deep vein thrombosis #5 NPD 36 wks, 3620 gm male #6 NTD 3700 gm female
8	30	3	2	#5 empiric ASA	#4 NTD 3425 gm male #5 NTD 4020 gm male
9	39	9	2		#10 SA 8 wks

[1]Prior number of consecutive spontaneous abortions [2]Number of paternal mononuclear cell immunizations to convert the FCXM from negative to positive [3]Normal preterm delivery [4]Term delivery [5]Spontaneous abortion [6]Normal preterm delivery

Of the five that had a successful index pregnancy, four of them delivered at term and one delivered 36 weeks' gestation following the development of preeclampsia. The mean weight of the successful index pregnancies was 3254 grams, with a range of 2800 - 3755 grams.

By excluding the patient with the aneuploidic spontaneous abortion, the corrected live birth rate was five out of eight index pregnancies. The expected live birth rate, based on the mean number of prior consecutive spontaneous abortions, was 25%, or two live births out of eight index pregnancies[59].

Considering all seventeen documented subsequent pregnancies to date, twelve have been successful. With the exclusion of the two documented aneuploidic spontaneous abortions, the corrected live birth rate was 12/15, which equates to 80%. All women that had a successful index pregnancy went on to have another successful pregnancy. No further boosters were given between pregnancies because the FCXM remained positive after the successful index pregnancy.

Chapter 7

Discussion

Prior to performing the reference range study, two methods of isolating mononuclear cells from peripheral blood were compared, specifically, whole blood lysis and density gradient centrifugation. When the FCXM results were compared, using these two different methods of mononuclear cell isolation, the p value was 0.13.

Two explanations can be given for the p value of 0.13. The first explanation is that there is no difference in the mononuclear cells isolated. The second explanation is that the sample size was not large enough, which is probably more likely since the comparison was done with cells from only two volunteers. We subsequently decided to abandon the whole blood lysis method because of a practical problem: when the samples were left overnight for dual-colour flow cytometric analysis, incomplete lysis of the unwanted peripheral cells was found using the whole blood lysis method. As well, significant background noise was documented when the samples were analysed. Therefore, density gradient centrifugation was concluded to be the superior method for mononuclear cell isolation in our laboratory.

Two methods of preincubating mononuclear cells were also compared prior to the reference range study: preincubation with goat IgG and preincubation with fetal calf serum. When the FCXM results were compared, using these two different methods of

preincubation, the p value was 0.32.

Two explanations can be given for the p value of 0.32. The first explanation is that there was no difference between the two methods to inhibit nonspecific binding of human IgG to Fc receptors. The second explanation is that the sample size was not large enough, which is probably more likely since the comparison was done with cells from only two volunteers. Since preincubation with goat IgG is now the standard method cited in the literature to inhibit nonspecifc binding of human IgG to Fc receptors, it has now replaced the fetal calf serum method in our laboratory.

Another potential method we could have evaluated to inhibit nonspecific binding is preincubation using a secondary FITC antibody specific for the Fc region of immunoglobulins, ie. IgG-Fc or IgM-Fc $F(ab')_2$ FITC. Bray et al.[41] compared a human IgG-Fc specific $F(ab')_2$ FITC antibody and a nonspecific $F(ab')_2$ FITC antibody reactive with heavy and light chains of all human immunoglobulin isotypes. Two-colour analysis revealed that the use of nonspecific $F(ab')_2$ FITC antibody resulted in higher background noise.

Clinically, the objective of the reference range study was to determine what constitutes a positive FCXM in the assessment of couples with a history of unexplained habitual abortion. Upper reference limits of negative control serum were calculated for four data sets (n=28). The four combinations of fluorochrome-conjugated antibodies used in this

reference range study were CD3 PE/IgG-Fc $F(ab')_2$ FITC, CD3 PE/IgM FITC, CD20 PE/IgG-Fc $F(ab')_2$ FITC and CD20 PE/IgM FITC. Using the FCXM methodology described in this thesis, a channel shift of ≥ 10 above the negative control serum can now be considered as a positive test result for maternal serum antipaternal T lymphocyte IgG and ≥ 7 for maternal serum antipaternal T lymphocyte IgM. A channel shift of ≥ 20 can now be considered as a positive result for maternal serum paternal B lymphocyte IgG and ≥ 22 for maternal serum antipaternal B lymphocyte IgM.

In the reference range study, the linear mean channel fluorescence of the commercial negative control serum was subtracted from individual AB sera (n=28), obtained from the Canadian Red Cross Blood Services. Theoretically, the individual AB sera should have been obtained from women who had never been pregnant. Since many pregnancy losses occur very early in gestation and are commonly unrecognized,and obstetrical histories are not taken at time of blood donation, obtaining sera from truly nulliravid women would have been difficult, if not impossible. Instead, sera from anonymous, nontransfused, blood group AB males was used. This sera should theoretically be free of cytotoxic HLA antibodies. We did assume that this male sera was otherwise very similar to nongravida female sera, an assumption which needs to be validated in the future.

The mononuclear cells used in this project were obtained from male and female volunteers from the Immunology Laboratory. The assumption was that these volunteers

were healthy, as attested by the individual volunteer. We again assumed that mononuclear cells from both males and females behave similarly, which again needs to be addressed at a later date. As stated earlier in this thesis, there is preliminary evidence that immune cells, such as CD8+ T lymphocytes, may be functionally influenced by the endocrine environment[45]. If so, it may have been more appropriate to use mononuclear cells obtained from healthy male volunteers only, since the endocrine environment in females who are ovulatory varies significantly throughout the menstrual cycle.

Despite these issues, the results obtained from the reference range study are clinically very important. Although the FCXM has been used in a number of laboratories worldwide to assesss couples with histories of unexplained habitual abortion, a reference range study has not been previously published. Gilman-Sachs et al. used the negative control serum arbitrarily to set the cursors on the dual-colour fluorescence histogram so that the double positive quadrant contained less than 1% of the lymphocytes[78,79]. When maternal serum was tested, any double positive lymphocytes in that quadrant were considered positive. When the number of double positive T or B lymphocytes was ≥50% of the total number of T or B lymphocytes, the FCXM was considered positive. No explanation was given as to why 50% was chosen as the cut-off.

Maruyama et al. defined a positive FCXM as a channel shift of ≥20 above the negative control serum for anti-T lymphocyte antibodies and ≥14 above the negative control serum for anti-B lymphocyte antibodies, using a flow cytometer with 256-channels and a four

decade log scale[81]. These cut-offs were based upon the mean plus the standard deviation (mean + SD) of the fluorescence intensity increase between the preimmunization and the control sera, in couples with a history of recurrent spontaneous abortion, defined as two or more unexplained, consecutive, first-trimester spontaneous abortions with the same partner. This assumed that little, if any, maternal allosensitization occurred in couples with a history of unexplained recurrent spontaneous abortion, be it primary, secondary or unclassified. This assumption has not been verified. Furthermore, the women were used as their own controls, which is not an acceptable study design.

Although pregnancy outcome data from only nine patients studied prospectively was reported, the FCXM methodology used, based on the comparative and reference range studies performed in this thesis, appears to be a potentially useful method of assessing maternal allosensitization following paternal mononuclear cell immunization. The corrected live birth rate of five out of eight index pregnancies is significantly higher than the expected live birth rate of 25%[59]. The 80% corrected live birth rate of all subsequent pregnancies (n=15) is also higher than expected.

These encouraging results could be attributed to the strict eligibility criteria used for enrolment as well as the boosting with paternal mononuclear cells until maternal allosensitization was documented by the FCXM. Based on this pilot study, a prospective, randomized, double blinded, placebo-controlled trial has been proposed, to assess further this modified method of paternal mononuclear cell immunization with boosting until the

FCXM is positive, for couples with primary unexplained habitual abortion. The FCXM, as described in this thesis, appears to be a clinically useful test to determine maternal allosensitization following paternal mononuclear cell immunization.

Chapter 8

Conclusions

Recurrent pregnancy loss is a prevalent health problem in our country, affecting up to 5% of couples trying to establish a family[1]. Habitual abortion, defined as three or more consecutive spontaneous abortions, is a newer term used to define more strictly couples with a similiar pattern of pregnancy loss. Since the 1984 landmark paper of Stray-Pedersen et al.[10], several pregnancy loss centres have attempted to determine the frequency of factors associated with a history of habitual abortion[19,22,23]. In the a prospective case series of 197 couples with habitual abortion, Stephenson reported that a genetic factor was identified in 3.5%, an endocrine factor in 20%, infectious factor in 0.5%, anatomical factor in 16% and autoimmune factor in 20% of couples with a history of habitual abortion[19]. The remaining 43% of couples were classified as having unexplained habitual abortion.

To this day, the understanding of how the pregnant woman tolerates the allogeneic fetus is not complete. Many mechanisms have been proposed over the past several decades to explain the paradox by which the genetically and immunologically foreign fetus survives in utero. Following World War II, Peter Medewar changed his focus of research from tissue transplantation to reproductive immunology and subsequently published a landmark paper in 1953, in which he gave three possible reasons for "why the fetus does not habitually provoke an immunological reaction from its mother"[76]. Medewar proposed

that the fetus and mother were anatomically separated, the fetus was antigenically immature and there was immunological indolence or inertness of the mother. Subsequently his theories have been refuted, but his research is still highly regarded and Medewar was subsequently knighted for his pioneering work in the field of reproductive immunology.

The maternal immune response to pregnancy appears to be very complex. Even at the blastocyst stage, there is evidence of immunosuppressor factors being produced locally to suppress the maternal immune response to the allogeneic blastocyst[37]. Implantation and early embryo growth is dependent on the production of cytokines and growth factors produced by both the pregnancy itself and the local uterine environment. Local and systemic changes result in dampened cell-mediated and increased "protective" humoral immune responses to the fetal allograft. Since it appears that the maternal immune response to pregnancy is complex, there may be many mechanisms by which pregnancies fail, resulting in habitual abortion.

Thomas Wegman introduced the term "immunotropism" to describe the maternal immune response to pregnancy. Wegman et al. hypothesized that the dichotomy between cell-mediated and humoral immune responses is mediated by inhibitory subsets of T-helper (Th) cells at the level of the uterine decidua[38]. If Th2 cells predominate over Th1 cells, production of IL-4, IL-5 and IL-10 cytokines promotes antibody formation, dampens inflammation and supresses natural killer cell activation, resulting in immunotolerance of

the pregnancy. On the other hand, if Th1 predominates, IL-2 and IFN-γ production increases, resulting in a cytotoxic response to the pregnancy.

Another presently accepted pathway leading to immune tolerance of the feto-placental unit is through the production of transforming growth factor beta type 2 (TGF-β2), which is produced by natural suppresor cells in the decidua[41]. Decidual-derived TGF-β2 is a potent local suppressor of cytotoxic cells. Using the murine model, stress has been shown to decrease production of TGF-β2, while allogeneic mononuclear cell immunization has been shown to increase TGF-β2 production.

A third mechanism involves the upregulation of progesterone receptors on CD8+ T cells and subsequent production of a "progesterone-induced blocking factor" which suppresses cytotoxic activity of natural killer cells[45]. Low levels of "activated" CD8+ T cells have been associated with a history of habitual abortion.

At the present time our understanding of the immune response to pregnancy at the cellular and molecular level is significantly beyond our ability to assess clinically couples with unexplained, possible alloimmune habitual abortion. Comparing HLA tissue types between partners is of questionable importance. The mixed lymphocyte culture has been abandoned recently because of control data which showed that the presence of the "blocking factor" correlated well with the total weeks of pregnancy in a woman life, and not with habitual abortion. Maternal allosensitization was previously assessed using the

complement-dependent cytotoxicity (CDCC) assay[55,56,57]. In 1989, Gilman-Sachs et al. proposed that the FCXM has advantages over the standard CDCC assay because, in addition to the complement-dependent cytotoxic antibodies, other complement-independent antipaternal antibodies were detected. These other antibodies were thought to be relevant to the maintenance of pregnancy[78]. Subsequently, Gilman-Sachs et al. also proposed that the FCXM was a more sensitive technique than the CDCC assay; 69% of women receiving paternal or third party mononuclear cell immunotherapy became FCXM positive while only 36% became CDCC positive[79]. Thereafter, Gilman-Sachs et al. used the FCXM exclusively to evaluate maternal allosensitization following paternal mononuclear cell immunization.

The FCXM methodology used by Gilman-Sachs et al. was briefly described in their papers published to date[78,79]. In their methodology, the maternal serum was heat-inactivated, as was done in the CDCC assay. This step does not appear to be necessary, according to our unpublished data comparing heat treated and not treated serum: the heat-treating the maternal serum resulted in erroneously lower channel shifts[80]. As well, in the Gilman-Sachs et al. methodology, no preincubation step with either fetal calf serum or goat immunoglobulin was mentioned. This step is important to inhibit nonspecific Fc binding of human IgG to paternal cells.

In the papers by Gilman-Sachs et al., positive control sera were not used[78,79]. Without positive control sera, a negative result could be truly negative or could be due to technical

problems. In flow cytometry it is imperative to run negative and positive control sera with each test serum, to ensure that there are no technical difficulties at the time of the test.

In 1993, Maruyama et al. concluded that dual-colour FCXM could be used to predict subsequent pregnancy outcome in women who received paternal mononuclear cell immunization[81]. Unfortunately, little detail was given in regard to their FCXM methodology. The paper stated, "The flow cytometric crossmatch was performed with a modification of the technique proposed by Gilman-Sachs et al.," unfortunately, the modification was not described. Maruyama et al. also used heat-inactivated maternal serum and no positive control sera were used.

The FCXM has been used more extensively in renal transplantation to detect alloantibodies in potential renal allograft recipients[67,68]. A positive FCXM prior to transplantation is predictive of early graft failure or rejection, despite having a negative result using a standard complement-dependent cytotoxicity assay[67,68,69]. After assessing several parameters of the FCXM, Bray et al. presented a standardized FCXM methodology for assessment of potential transplant recipients, which they proposed should be used widely so that direct comparisons of results could be made between different laboratories[70]. In the renal transplantation literature, a positive FCXM is most commonly defined in terms of channel shift above the linear mean channel fluorescence of the negative control serum, as in this Master's thesis. Bray et al. estimated a channel shift

of 10 corresponded with a 20% increase in the percentage of positive cells[70].

The dual-colour FCXM methodology presented is similar to the methodology used in renal transplantation. Since the definition of a positive FCXM is in terms of channel shift above the negative control serum, the reliability of the negative control serum is crucial. Therefore, with a new pool of commercially prepared negative control serum, the channel shift would need to be re-evaluated. As well, the use of positive control sera is essential to check the validity of the test. Our source of positive control sera was from renal allograft recipients. Sera from seven recipients was consistently positive for IgG, but we found only a single recipient who was consistently positive for IgM alloantibodies. Various parameters, including reagents and commercially prepared fluorochrome-conjugated antibodies, were evaluated in regard to cost and sensitivity prior to their use in this project[80].

Although the flow cytometric crossmatch has been used clinically in a few North American recurrent pregnancy loss centres over the past five years, the definition of what constitutes a positive FCXM was not published prior to the completion of this reference range study[77]. A standardized FCXM methodology is vital in order to ensure reproducibility of results between laboratories and to encourage collaborative research between academic centres. With further development of the surface marker technology and refinements in the identification of antibodies or target cells involved in the maintenance of pregnancy, the FCXM should become an even more useful tool in the

assessment of unexplained habitual abortion.

This thesis provides an overview of the current knowledge about recurrent pregnancy loss, in particular, about potential mechanisms by which recurrent pregnancy loss may occur on an immunological basis. As well, current immunological investigations and treatment options were discussed. The basic principles of flow cytometry and its use in reproductive immunology were reviewed. Methods to isolate mononuclear cells as well as methods to preincubate mononuclear cells were compared. A reference range study was performed to determine what constitutes a positive FCXM to be used in the assessment of couples with a history of unexplained habitual abortion. Finally, the FCXM was assessed as a clinical tool to detect maternal allosensitization following paternal mononuclear cell immunization in couples with a history of primary unexplained habitual abortion. The subsequent pregnancy outcomes were very encouraging. Based on this pilot study, a prospective, randomized, double blinded, placebo-controlled trial has been proposed, to assess further this modified method of paternal mononuclear cell immunization, with boosting until the FCXM is positive, for couples with primary unexplained habitual abortion.

There are many intriguing questions in regard to the normal and abnormal immune response to pregnancy. It is hoped that this research project may help to bring us a little closer to some of the answers. With the standardization of the FCXM methodology, as presented in this thesis, the clinical significance of the maternal alloantibodies and

maternal allosensitization following immunotherapy can now be addressed in a collaborative manner.

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