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BOSTON UNIVERSITY

SCHOOL OF MEDICINE

Thesis

THE DEVELOPMENT OF AN ELISA FOR THE QUANTIFICATION OF ANTIBODIES AGAINST CD52G, A SPERM COATING GLYCOPROTEIN, IN THE SERA OF PATIENTS WITH INFERTILITY

by

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CLAIRE E. MARCUS

ABSTRACT

Antisperm antibodies (ASA) are thought to be a predominate cause of immune infertility by interfering with various aspects of sperm function in both the male and the female reproductive tracts. The precise mechanism by which these antibodies contribute to infertility, as well as their etiology, remains to be established. ASA are present in a variety of biological substrates, such as genital tract secretions, and the blood sera of both males and females. Although not all ASA underly infertility, a substantial body of research suggests that certain ASA, referred to as sperm immobilizing antibodies (SI-Abs) and sperm agglutinating antibodies, significantly impair sperm transportation in the female reproductive tract. High titers of sperm agglutinating or sperm immobilizing antibodies have been associated with reproductive failure. CD52g is a GPI anchored glycoprotein found on mature sperm and in seminal plasma (SP). Antibodies against a male reproductive tract-specific epitope of CD52g are known to readily agglutinate sperm. The current study sought to develop an ELISA to quantify the prevalence of CD52g antibodies in the sera of male and female patients with infertility, and to determine if there was a correlation between the prevalence of CD52g antibodies and the prevalence of sperm agglutinating antibodies in the sera of these patients. Ultimately,

CD52g antibodies were only detected in the sera of patients (21%) with sperm agglutinating antibodies. While detecting CD52g antibodies in sera via an ELISA proved challenging, the results of this study corroborate research demonstrating that CD52g antibodies have a remarkable capacity to agglutinate sperm. Elucidation of the mechanisms underlying this immune response would advance our understanding of immune modulation in human reproductive tracts, further the diagnosis of immune infertility, and are currently providing the basis for the development of a potent dual purpose immunocontraceptive, that both prevents unintended pregnancy, and prevents the transmission of sexually transmitted infections (STIs).

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LIST OF ABBREVIATIONS

ART..Assisted reproductive technologies

ASA..Antisperm antibodies

BSA..Bovine serum albumin

CASA..Computer assisted semen analysis

CM..Cervical mucus

ELISA..Enzyme-linked immunosorbent assay

FBS..Fetal bovine serum

FRT..Female reproductive tract

GTI..Genital tract infections

HCA..Human contraceptive antigen

HIV..Human immunodeficiency virus

HRP..Horseradish peroxidase

HSV..Herpes simplex virus

IF..Immunofluorescent assays

MRT..Male reproductive tract

NAA..Natural autoantibodies

NAb..Natural antibodies

PBS..Phosphate-buffered saline

PS..Polystyrene

RIA..Radioimmunoassay

SI-Abs..Sperm-immobilizing antibodies

SIT..Sperm immobilization test

SIV ....Sperm immobilization value

SP ....Seminal plasma

STI..Sexually transmitted infections

TMB .... 3,3′,5,5′ tetramethylbenzidine

UAB ....Universal assay buffer

UI ....Unexplained infertility

ZP..Zona pellucida

INTRODUCTION

The World Health Organization (WHO) defines infertility as "a disease of the reproductive tract characterized by the failure to achieve pregnancy after 12 months or more, of regular, unprotected, sexual intercourse" [1]. Infertility, a critical component of reproductive health, is a remarkably prevalent health condition. Worldwide, an estimated 8-12% of reproductive-age couples are infertile [2], with countries in Eastern Europe, North Africa/Middle East, Oceania, and Sub-Saharan Africa having the highest prevalence of infertility [3]. Males are found to be solely responsible for 20-30% of infertility cases [2]. Semen analysis, the evaluation of semen volume, sperm count, motility, and morphology, has been the chief means of diagnosing male factor infertility since the 1930s [4]. Infertility is attributed to ovulatory defects in 40% of infertile women and evaluation of such defects can involve the detection of hormonal indicators of ovulation, assessment of ovarian reserve, and radiographic evaluation of the uterine cavity and fallopian tubes [5]. If the results of the aforementioned tests fail to reveal marked abnormalities, a diagnosis of exclusion is reached, and the couple's infertility is described as "unexplained" [5]. Approximately 15-30% of infertile couples will be diagnosed with unexplained infertility (UI) [5]. The etiology of UI remains diffuse, with research suggesting that a complex interplay of aberrant endocrine, genetic, immunological and physiological mechanisms may be responsible [5].

The influence of immune mechanisms in the pathogenesis of unexplained infertility has been the subject of investigation since 1899, when "spermotoxic" antibodies in rabbits sensitized with bull semen were first found to immobilized sperm

cells [6]. In 1922, Dr. Samuel Meaker first documented the presence of antisperm antibodies (ASA) in women [7]. In humans, Rosenfled first surmised that immunizing women with human semen could lead to infertility [8]. Baskin subsequently turn these ideas into reality [9] and patented a "spermotoxic vaccine" in 1937 [10]. By 1954 Rumpke and Wilson independently demonstrated that antisperm antibodies were associated with male infertility [11,12]. Although research into ASA was eclipsed by the development of assisted reproductive technologies (ART) [13], fascinating questions concerning the dynamic role ASA play in reproductive biology remains especially relevant. Understanding the extent to which ASA impair not only sperm transport but directly interfere with sperm/oocyte interaction, could potentially help physicians choose the least aggressive, and most effective, intervention for patients seeking to achieve a pregnancy [14]. The identification of cognate antigens of ASA that impede fertilization could be used as targets for immune contraception [14]. The characterization of ASA and their cognate antigens provides a route by which to not only more thoroughly understand the molecular pathways underlying fertilization, but to also unravel the intricate immune mechanisms involved in establishing and maintaining pregnancy.

One sperm antigen potentially relevant to immune infertility is the glycoprotein CD52g. CD52g is found abundantly in the male reproductive tract, including mature sperm and seminal plasma (SP) [15]. Antibodies raised against CD52g have been shown to readily immobilize and agglutinate sperm [15]. Bronson et al. established a serum repository from patients with infertility and determined the subclass and incidence of ASA in each serum sample. The Anderson Lab obtained aliquots of the sera from the

repository and subsequently determined the degree to which each patients' serum agglutinated sperm. The objectives of the current study were to determine the prevalence of antibodies directed against CD52g in the sera and to determine if the prevalence of agglutinating antibodies in the sera correlated with that of CD52g antibodies. If there was a marked increase in the prevalence of CD52g antibodies in the serum of infertility patients, as compared to controls, and these serum samples exhibited a strong capacity to agglutinate sperm, this would corroborate decades of research suggesting that ASA, against antigens essential to sperm functioning, play a pivotal role in alloimmune challenges to human fertilization, and add further credence to the supposition that one function of CD52g may be to modulate immune responses to sperm.

CHAPTER 1. LITERATURE REVIEW OF ANTISPERM ANTIBODIES (ASA)

The development of ASA is a fascinating immunological phenomenon that has not been clearly elucidated. However, theories on the nature of ASA reactions have been posited, which seek to reconcile the many discrepancies and complexities that arise in the study of these curious proteins.

1.1 NATURAL AUTOANTIBODIES

Natural autoantibodies (NAA), or natural antibodies (NAb), are a collection of spontaneously secreted antibodies that are surprisingly difficult to define [16]. In its purest form, NAA are defined as polyreactive, germline-like (no N-additions and little somatic hypermutation), low affinity antibodies, generated in the absence of foreign antigen (though their production is influenced by them), and without helper T cells, that recognize both self-antigen and foreign pathogens [16,17]. In mice, NAA are predominantly generated by a subset of B-1 cells (B-1a or CD5+ B-1 cells). B-1a cells principally secrete IgM-NAA, but also IgA-NAA and IgG-NAA (mostly IgG3) [17]. Although there are significant challenges in discerning all the particular B cells types capable of secreting NAA in humans [16-18], the human equivalent of B-1a cells has been identified and represents 15-20% of circulating adult B cells [19]. Research concerning the physiological role of NAA, particularly IgM-NAA, has illuminated just how essential NAA are to modulating immune responses and protecting humans from infectious and autoimmune diseases. IgM-NAA combat invading pathogens until the sluggish B2 and T cells are deployed and hinders both the production, and machinations, of pathogenic IgG autoantibodies [17-19]. IgM-NAA can bind to a limited range of

highly conserved epitopes found in self-antigens and thereby hide them from pathogenic autoantibodies or induce the clearance of dead or senescent somatic cells [17,19]. Furthermore, IgM-NAA can bind to receptors on leukocytes including T cells, dendritic cells, and B-1 cells to modulate their activities and/or development [17].

The sperm surface is coated by a complex array of carbohydrate, lipid, and protein specificities that have been conserved over evolution [21], and as such, are prime targets for NAA. Essentially, sperm surface carbohydrates may serve to stimulate the production of NAA and thereby mask integral sperm antigens from immune surveillance [15]. Paradisi et al. reported on several interesting phenomena in this regard. Previous research has shown that antisperm antibodies are present in both fertile and infertile populations, although the prevalence of ASA in these populations varies depending on the assay utilized. ASA have been found in 10% of infertile men and 5% of infertile women [22]. However, they have also been detected in 1-2.5% of fertile men and 4% of fertile women [22]. Paradisi et al. theorized that sperm antigens should be classified into three groups or types: (1) antigens recognized by natural autoantibodies or cross-reactive antibodies (a product of molecular mimicry) that are functionally irrelevant and are present in both fertile and infertile populations, (2) antigens present only in infertile populations and pathogenic in nature, (3) antigens belonging to the first category that can elicit a heighted immune response, perhaps due to a breakdown in immune tolerance [23].

A strain of research that testifies to the significance of natural autoantibodies against sperm, concerns antisperm antibodies developed before puberty. Tung et al.

demonstrated that ASA reached their peak incidence in both boys and girls at ages 1-10 before puberty and thus before mechanisms of immunological tolerance are established [24]. They suspect that either molecular mimicry to microorganisms with similar epitopes, antigenic leak, and natural autoantibodies all may be potentially responsible for this phenomenon [24]. Furthermore, fascinating research by Flickinger et al. showed an increase in serum antisperm autoantibodies (including IgG autoantibodies) once Lewis rats reached puberty [25]. Flickinger et al. reasoned that as germ cells entered new stages of development and sperm neo-antigens arose, so too did an autoantibody response to sperm [25]. They remark that dynamic yet tightly regulated immune mechanisms to suppress orchitis must be operating. Paradisi et al.'s suggestion of a third category of sperm antigens composed of those that are initially recognized by NAA but subsequently evoke a more aggressive immune response is significant, as CD52g may belong in this third category of sperm antigens. Its core peptide, CD52, is found on a host of somatic cells while the glycosylated isoform is found on sperm cells [15]. It is possible that in the context of a particular inflammatory condition, the glycosylated isoform could induce an enhanced reactivity by natural autoantibodies [6,15,23]. The enhancement of a natural, physiological autoantibody response to a pathological level, in response to elevated levels of autoantigen, due to either a breakdown in tolerance and/or molecular mimicry following infection, could explain the existence of ASA in healthy pre-pubescent children and fertile individuals, as well as in men and women diagnosed with UI.

1.2 ANTISPERM ANTIBODIES AND MALE INFERILITY

At puberty, spermatogenesis coincides the formation of the blood testis barrier-a nearly impenetrable barricade, composed of various types of junctional proteins, that divides the seminiferous epithelium into two distinct compartments (basal and abluminal) [26]. The blood testis barrier was once thought to be solely responsible for preventing an immune response to self-antigens and alloantigens in the testis [26]. However, immune privilege in the testis is now a more complex and colorful picture. The blood testis barrier is still central to limiting immune activity in the abluminal compartment, but other mechanisms including the muted pro-inflammatory activity and the immunosuppressive character of testicular macrophages, immature testicular dendritic cells, and a propensity for the programmed death receptor-1/programmed death ligand-1 (PD-1/PD-L1) system to thwart any self-reactive T cells in the testis, all underly testicular immune privilege [27]. Furthermore, there is significant immune suppression by Sertoli cells and Leydig cells. For instance, Sertoli cells are efficient phagocytes of approximately half of the testicular germinal cells that undergo apoptosis, thereby helping to prevent an immune response to neo-antigens that arise as these germ cells further mature and/or die [28].

Seminal plasma (SP) provides not only transportation and nutrition to spermatozoa but also limits immune responses as spermatozoa mature in the testis and journey through the female reproductive tract [29]. Seminal plasma is a complex mixture of diverse biomolecules, derived from secretions from the testes, epididymides, prostate, seminal vesicles, and bulbourethral glands [30]. It is composed of an array of immunosuppressive substances that prevent autoimmune responses to sperm antigens.

Seminal plasma contains an abundance of prostaglandins, notably PGE2, which inhibits or restricts the activity of neutrophils, macrophages, natural killer cells, CD 8+ T cells, and T Helper type 1 responses [31]. Soluble Fc receptors, numerous regulators of the complement system, and lactoferrin-an invaluable weapon and modulator of immunity [32] are just a few of the many modulators of inflammation which constitute seminal plasma. The activities of all of these macromolecules, as well as a host of others, creates a milieu in the testis in which neoantigens are tolerated yet immune responses to potential pathogens are still effective [31].

Research suggests that any type of testicular pathology (testicular torsion, genital infections), and/or vasectomy, that induces the leakage of antigens beyond what can be contained by the aforementioned immunosuppressive mechanisms, would likely produce ASA [6]. Indeed, sperm agglutinating and sperm immobilizing antibodies have been detected in more than half of vasectomized men [6]. However, unlike in other species, male ASA do not induce systemic autoimmune disease [6]. Thus, despite the fact that sperm glycans contain epitopes shared by multiple antigens, and therefore would yield ASA that react with such common epitopes, among the ASA formed in men, there is a greater degree of sperm specific ASA compared to those developed in women [6]. Grygielska et al. investigated both the specificities and intensity of ASA produced in SCID mice (lacking adaptive immunity) and NOD/SCID mice (lacking both arms of immunity) after administering human peripheral blood lymphocytes (PBLs) from either ASA-positive or ASA-negative individuals [34]. The NOD/SCID mice allowed for unhindered PBLs sensitization and thus the NOD/SCID mice had higher levels of ASA

but the NOD/SCID mice also generated ASA that tended to be specifically reactive to sperm-membrane antigens. Such specific ASA subsequently showed a greater ability to agglutinate sperm. Thus, it may be hypothesized that in males, the breakdown of testicular tolerance, or inefficient active immunosuppressive mechanisms, can enhance the natural autoantibody response to sperm yielding more mature antibodies that have undergone somatic hypermutation and affinity maturation and thereby have a greater affinity to sperm antigens.

Beyond a breakdown in tolerance, there is research suggesting that molecular mimicry and cross-reactivity play important roles in the development of ASA. With regard to molecular mimicry, it may be that at least some ASA are developed against exogenous, pathological antigens and yet these antibodies also bind sperm, because a particular sperm antigen may be similar enough in conformation, structure, or amino acid sequence, to the exogenous antigen [35]. Kalaydjiev et al. found that the incidence of ASA increased in 47% of patients with shigellosis and 42% of patients with salmonellosis [36]. All patients in the study had been negative for ASA at the time of diagnosis and yet developed clinically relevant titers of ASA within a month after diagnosis. A similar finding was evidence by Dimitrova et al. in patients suffering from ulcerative colitis and in patients infected with *H.pylori* [37,38]. Tung et al. found that natural sperm autoantibodies cross react with *E.coli* [39] and Kurpisz and Alexander determined that the overwhelming majority of monoclonal antibodies produced against sperm cross-react with *E.coli* and *S.typhi* [40]. Thus, any disease or infection which can disrupt mucosal immune mechanisms and/or directly aid in the leak of bacterial antigens

from the bowel, could provoke an immune response in which sperm become an unwitting target.

Other research has emphasized that the inflammatory mediators involved in clearing the aforementioned infections, are likely culpable in the development of ASA. The unbridled activity of leukocytes, pro-inflammatory cytokines and reactive oxygen species (ROS), produced in response to acute, chronic, or latent infections, could irrevocably alter or damage sperm. Both exogenous and endogenous ROS production can induce an array of negative consequences including the degradation of the sperm membrane, sperm DNA fragmentation and chromatin damage, a premature acrosomal reaction, and disorders of capacitation [41-43]. Sanocka et al. studied markers of oxidative stress in semen samples from fertile and infertile patients with genital tract infections (GTI) [44]. In contrast to fertile semen samples with a GTI, infertile samples showed continued activation of xanthine oxidase, an enzyme which hampers the ability of catalase to remove hydrogen peroxide, long after the offending pathogen was eliminated. The ensuing preponderance of pro-oxidant and underwhelming anti-oxidants in infertile semen samples, points to the importance of oxidative stress underlying infertility. Bozhedomov et al. investigated ROS and its association with ASA [22]. In semen samples from infertile men, in which 50% or more of the sperm was coated with IgG, the ASA-positive sperm generated significantly elevated amount of ROS when compared to fertile, ASA-negative semen samples. Furthermore, the percentage of sperm cells with DNA fragmentation in ASA-positive, infertile men was significantly higher than infertile men without ASA and in fertile men. However, antioxidant therapy

diminished sperm DNA fragmentation, with a simultaneous decrease in ASA. Though the exact role of ROS in mediating an autoimmune response to sperm is undoubtably complex, it is possible that the accumulation of ROS could lead to the oxidation of lipids, proteins, and nucleic acids that overall damage sperm cells. Moreover, ROS-modified sperm macromolecules would be more immunogenic than native molecules, because epitopes previously hidden could be exposed upon oxidation. Thus, ROS-modified sperm antigens would be more readily recognized and internalized by antigen presenting cells (APC), which would present them as neo-antigens to lymphocytes. This may also promote B cell epitope spreading.

In B cell epitope spreading, the specificity of an antibody expands from the initial epitope that triggered the response, to include either other epitopes of an antigen (intramolecular epitope spreading) or another antigen altogether (intermolecular epitope spreading). Grygielska et al. immunized SCID and SCID/NOD mice with natively glycosylated or deglycosylated sperm antigens [34]. In both mice strains, the deglycosylated sperm antigen induced a significantly stronger ASA response, suggesting that the deglycosylated sperm antigens were a more powerful immunogen, as sperm epitopes were left uncovered. They argue that sperm glycans can be protective- masking the more restricted sperm antigens buried under the glycocalyx and preventing an immune response to such integral antigens. However, as Paradisi suggests, it is possible that these same glycans, which induce a low-affinity immune response (i.e. natural autoantibodies) could, in the presence of infection (and downstream oxidative stress), or due to a breakdown of immunosuppressive mechanisms, lead to epitope spreading and

subsequently can lead to a dangerous specific autoimmune response that hinder sperm functioning.

Essentially, sperm antigens may be more susceptible to becoming antigenic targets after being modified by ROS or molecular mimicry may stimulate B-lymphocytes to generate cross-reactive antibodies that bind sperm antigens. In any case, once self-tolerance to a particular sperm antigen is broken, epitope spreading can contribute to the development of IgG autoantibodies with exquisite specificity to a sperm-antigen that impairs sperm functioning. Bacterial infection could be one element that triggers such a cascade of events.

However, it is still unclear whether the impediment to sperm functioning is due to the binding of an antisperm antibody, in and of itself, to an altered sperm antigen, or if the impairment to sperm functioning is a consequence of the alteration itself- that a vital sperm protein required for fertilization simply no longer functions properly [42]. The degree to which ASA impair sperm functioning and fertilization depends on several factors; the proportion of ASA-positive sperm, the number and location of those ASA on the sperm (head, midpiece/neck, or tail), the affinity of ASA binding, the function of the antigen to which the ASA bind and the isotype of the ASA [22].

1.3 ANTISPERM ANTIBODIES AND FEMALE INFERTILITY

Upon entering the female reproductive tract (FRT) sperm and seminal plasma antigens face a relatively hostile environment. Host neutrophils, monocytes, and lymphocytes are present in the vaginal, cervical, and uterine epithelium, and could

potentially recognize sperm as foreign and mount an immune response [31]. It would therefore stand to reason that most women develop ASA upon coitus, however, most women, despite regular exposure to sperm and seminal plasma antigens, do not develop ASA [6]. Such a finding suggests that the FRT is an immune privilege site. Perhaps more so than in any other organ, the immune system's remarkable ability to adapt to a constantly changing environment is paramount. The immune system must concurrently manage infection and yet tolerate alloantigens to allow for successful reproduction [31,50]. Mechanisms fundamental to striking this delicate balance are numerous. For instance, the leukocyte response following the initial deposition of semen into the FRT is "silent" in that it occurs in the absence of a significant release of proinflammatory cytokines or ROS and serves to eliminate sperm of inferior quality [50]. Indeed, the FRT contains unique immune cells, mechanisms of T cell regulation, anti-inflammatory cytokine secretions, and reproductive hormones, that all promote alloantigen acceptance and protection from sperm isoimmunization [50].

Components of semen play an important role in this regard. Although sperm lack paternal MHC molecules preventing specific MHC-dependent adaptive immune responses, innate immune mechanisms must be modulated [50]. Seminal plasma (SP) has a host of immunosuppressive factors that nonspecifically dampen the immune response within the FRT. In addition, SP induces the expression of cytokines that recruit regulatory T cells (Tregs) populations into the FRT. Transforming growth factor (TGF) β and PGE2 in SP further augment the recruitment of Tregs, and establish an anti-inflammatory, Th2 dominate response, within the FRT, that supports alloantigen

acceptance [50]. SP components also recruit tolerogenic dendritic cells to endometrial tissues [31]. These dendritic cells secrete higher levels of anti-inflammatory cytokines and allow for the expansion of specific paternal Tregs that support pregnancy. Finally, soluble HLA-G, a molecule known to be found on the surface of extravillious cytotrophoblast cells, is found in SP [31,50]. HLA-G, both soluble and membrane bound forms, modulates a host of immune responses; inhibiting natural killer cells and CD8+ T cells, suppressing IFN-γ, and inducing Treg differentiation [31.50]. Thus, an array of diverse immune modulators in SP plays an important role in influencing the response of immune cells in the FRT to sperm.

Similar to the pathogenesis of ASA in males, there are several hypotheses regarding the development of ASA in females. Just as GTIs in men have been implicated in the pathogenesis of male ASA, the prevalence of ASA in women has correlated with gynecological infections. Forty-six percent of women with pelvic inflammatory disease (PID) were found to have sera and cervical mucus (CM) positive for ASA, compared with a prevalence rate of 20% in women who only had a lower genital tract infection [51]. In both men and women, ASA may be generated concurrent with infection with *chlamydia trachomatis* (*C.trachomatis*). Activated T cells secrete proinflammatory cytokines that can activate macrophages into phagocytosing both *C.trachomatis* and sperm [52]. This localized inflammatory process to eliminate *C.trachomatis* may also result in the development of antibodies to sperm [52]. Such an infection could induce an immune response that limits the expansion of Tregs specific for male antigens, and instead, promotes the release of aberrant cytokines that enhance, rather than modulate,

immune response to sperm. This could set the stage for sperm isoimmunization and infertility [52,53].

Molecular mimicry between sperm antigens and pathogens is also implicated in female isoimmunity to sperm. Immunoprecipitation and blotting experiments demonstrated shared epitopes between *C.trachomatis* and human sperm heat shock proteins (HSP) [54] and between Ureaplasma urealyticum (*U. urealyticum)* and human nuclear autoantigenic sperm protein (NASP), a protein required for DNA replication [55]. Interestingly, ASA incidence in women has been found to correlate with ASA incidence in their male partner. Twelve percent of men whose wives were positive for ASA, similarly tested positive for ASA. However only 6% of men whose wives were negative for ASA tested positive for ASA. Witkin argued that sperm already coated with male ASA and subsequently deposited within the FRT, could induce cytokines that would activate macrophages and thereby promote the presentation of sperm antigens to T helper cells [52,56]. In line with this, Kverka et al. demonstrated that PBMCs from infertile women with ASA, infertile women without ASA, and controls, produced markedly different cytokines upon encountering sperm cells [57]. Essentially, sensitized lymphocytes in the FRT could orchestrate an immune response against sperm, especially when sperm are coated with antibody, and this deviant cytokine signaling encourages a pro-inflammatory environment in the FRT that compromises sperm functioning.

Clarke argues that anti-idiotypic antibodies might explain the development of ASA in women [49]. The variable domain of an antibody contains the antigen binding site (paratope) and thus specifies an antibody's unique recognition of its antigen. This

part of an antibody is referred to as its idiotype or idiotypic determinate, and it is itself antigenic, as it can be recognized by anti-idiotypic antibodies. Because the antigen binding site or paratope of an antibody mirrors the epitope of a particular antigen, anti-idiotypic antibodies, which recognize the paratope, have specificities that similarly recognize the original antigen [49]. It is possible that if women were continuously exposed to semen that contained ASA, they could produce anti-idiotype antibodies against the paratope of the ASA, and thereby could initiate an antibody response against a sperm antigen because they are complementary to the specificity of the anti-idiotypes [49]. Clarke also argues that it is possible that all three mechanisms work in concert to induce ASA in women [49]. Either cross-reactive antibodies produced subsequent to infection or antibody-coated sperm can cause lymphocytes to secrete pro-inflammatory cytokines which can further potentiate and/or maintain an anti-idiotypic immune response to ASA in semen. Finally, as Paradisi suggested, natural autoantibodies to sperm may, due to a heighted immune response and epitope spreading, become harmful autoantibodies specific for sperm antigens that impair sperm functioning.

1.4 CD52G: A TARGET ANTIGEN FOR SPERM AGGLUTINATING ANTIBODIES

One of several assays to detect ASA is referred to as the complement-dependent sperm immobilization test (SIT) [58,59]. This assay involves using a mixture of the patient's sera, sperm, and complement, to detect impaired sperm motility [58,59]. Antibodies detected by these assays are referred to as sperm immobilizing antibodies (SI-Abs) [58,59]. The results of a SIT are represented as a sperm immobilizing value (SIV)

[58,59]. If the SIV is positive, then a quantitative SIT is performed, in which a patient's serum is serially diluted to determine the dilution that retains sperm motility up to 50% [58]. Assessment of this titer in infertile women is an important diagnostic tool and helps to determine which treatment method is most appropriate for a particular patient, depending on the degree to which SI-Abs may underly their infertility [58,59]. SI-Abs are most frequently found in the sera of women with unexplained infertility, with an incidence of 10%-15% [58]. Moreover, women with SI-Abs in their sera were found to have these antibodies at comparable levels in their cervical mucus (CM) and peritoneal and follicular fluids [60].

ASA can have both immobilizing and agglutinating activities. The term, agglutination, describes an ASA mediated phenomena, in which sperm stick to each other, usually in particular patterns, like head-to-tail. Assays measuring the extent to which ASA agglutinate sperm include the Sperm Escape Assay and the Agglutination Kinetic Assay. These tests infer the agglutinating ability of ASA by measuring either, the number of free, motile sperm that escape agglutination, or by measuring the time it takes to agglutinate a given concentration of sperm cells, upon exposure to ASA. Computer assisted semen analysis (CASA) software, that analyzes and assess sperm motility, as well as, manual microscopic methods are used to conduct these assays.

Elucidation of the epitopes recognized by SI-Abs is necessary for determining the mechanism by which these antibodies impair sperm motility. Such knowledge could provide a basis for the development of a treatment specific to ASA-mediated infertility [61]. To achieve this goal, given the heterogenous nature of ASA, Koyama et al.

established a human-mouse hybridoma that secreted high titers of a human monoclonal SI-Ab and designated it H6-3C4 [62]. The monoclonal antibody (mAb) H6-3C4 was of the IgM isotype and readily immobilized and agglutinated sperm. Immunofluorescent stainings of the male reproductive tract (MRT) with H6-3C4 revealed that the antibody reacted exclusively with the cauda epididymis but not the testis or other somatic tissues [63,64]. Western blotting and SDS-PAGE analysis of the immune complexes formed between H6-3C4 and sperm extracts, showed that the amino acid sequences of the proteins precipitated by H6-3C4 were found to be identical to those of human glycoprotein CD52 [63,64].

Human CD52 is expressed on the surface of a host of immune cells, including mature lymphocytes, monocytes/macrophages, and dendritic cells, among others. It was originally detected as an antigen recognized by the rat mAb Campath-1 [65]. Humanized Campath was first used in 1982 for the treatment of non-Hodgkin lymphoma [66]. Decades later, under its new name, Alemtuzumab, it has been used for the treatment of multiple sclerosis (MS), chronic lymphocytic leukemia (CLL) and in kidney transplantations for anti-rejection therapy [66]. The physiological role of CD52 in modulating immune activity remains to be fully explicated.

However, it is known that Campath is capable of inducing complement-dependent cytotoxicity (CDC), antibody-dependent cell-mediated cytotoxicity (ADCC), and caspase-dependent apoptosis in leukocytes [66-68]. CD52 is also believed to play a significant role in regulating the activities of T cells, capable of acting as both an activator and inhibitor of effector and regulatory T cells [68]. Campath was also found to

be reactive with human sperm, capable of immobilizing and agglutinating sperm, similar to that of H6-3C4 [64]. This was due to the fact that CD52 expressed in leukocytes and in MRT tissues share a common core protein. The core protein is composed of only 12 amino acids tethered to the plasma membrane via a glycosylphosphatidylinositol (GPI) anchor [6,68]. However, distinct structural modifications exclusive to mrt-CD52 (CD52g present on the surface of sperm, the epididymal epithelium, and in male reproductive tract fluids) but not expressed on leukocyte-specific CD52 were soon noted.

One such difference is that mrt-CD52 (CD52g) is more heavily glycosylated, having both O-linked and N-linked carbohydrate moieties that are almost completely sialylated and fucosylated [69]. Disparate glycosylation patterns explained the curious finding that H6-3C4 only reacted with sperm and not with lymphocytes. Western blot analysis of sperm extracts revealed that CD52g contained a unique glyosidic bond within its N-linked carbohydrate chain that H6-3C4 recognized [6,70]. Meanwhile, Campath was found to bind to the last three amino acids of CD52's core protein and components of the GPI anchor [69]. The structural differences between mrt-CD52 (CD52g) and leukocyte-CD52 implied that there are different physiological functions between the two proteins. The unraveling of the biology of both proteins is under investigation. However, research has provided the means for much conjecture regarding the function of CD52g.

As sperm embark on their developmental journey through the epididymis, the epididymal epithelium, via apocrine section, releases extracellular microvesicles called epididymosomes, into the intraluminal compartment of the epididymis [71]. CD9-positive epididymosomes preferentially fuse with sperm and are thought to transfer

CD52g into the maturing sperm membrane via its GPI anchor [30,71]. Thus, CD52g is found in abundance in epididymal sperm and in the ejaculate. Since sperm motility is one of the chief characteristics acquired by epididymal sperm, it is possible that CD52g's concomitant appearance functions to ensure sperm acquire this pivotal function [30,72]. CD52g's sialylated N-linked glycans, as well as, the nature of its O-linked glycans, confers a negative charge to CD52g that is thought to contribute to the net negative charge of the sperm surface [6]. This negative charge may prevent sperm from agglutinating with itself [6,15]. Flori et al. found that CD52g binds semenogelin I, one of the predominant structural proteins that constitutes a loose gel formed by semen after ejaculation [73]. Some of these CD52 molecules with bound semenogelin I are cleaved of their GPI anchor as this gel is degraded, thereby releasing sperm to penetrate cervical mucus [73]. Thus, CD52g is thought to play a central role in both the coagulation and liquefaction of semen [73].

Given that H6-3C4 and Campath were known to induce a complement-dependent cytotoxicity, Koyama et al. wanted to demonstrate the manner in which CD52g modulated the complement cascade. Koyama et al. used a hemolytic assay based on the Mayer method, in which sensitized sheep erythrocytes are exposed to titered complement components in sera [74]. Upon addition of CD52g, the titer at which 50% hemolysis occurred was significantly reduced [75]. In fact, the degree of hemolysis was reduced by CD52g in a dose dependent manner. However, the addition of antibody against CD52g reversed this trend, spurring the lysis of erythrocytes [75]. Thus, CD52g seemed to inhibit complement mediated cytotoxicity. Subsequent investigations determined that purified

CD52g reduced complement activity via the classical pathway [75]. Hardiyanto et al. conducted immunoprecipitation assays to determine at what stage of classical complement pathway CD52g functioned [76]. CD52g in epididymosomes and membrane-bound CD52g were both found to bind to complement component 1q (C1q), the initial protein that induces the classical pathway [76]. CD52g bound to C1q via its N-linked carbohydrate chain, a MRT-specific sperm modification [76]. Thus, this unique carbohydrate modification of CD52g may protect sperm in the FRT by inhibiting the function of C1q. If any immune complexes form on the surface of the sperm membrane, CD52g may regulate classical complement activation, such that bound ASA fail to exert any toxic effect [76]. However, if an antibody is raised against CD52g, it could negate its ability to protect sperm from complement-mediated lysis, when sperm enter the FRT [76]. Intriguingly, CD52 has been found in the female reproductive tract, perhaps indicative of a role protecting the developing oocyte as well as the embryo from complement activation [77].

1.5 MECHANISMS OF ANTISPERM ANTIBODY MEDIATED INFERTILITY

Another means by which ASA are thought to interfere with fertility is by compromising the ability of sperm to penetrate cervical mucus [78]. Under the influence of estrogen, the cervix secretes an extremely watery mucus (CM). CM represents an important barrier, through which sperm must pass, if they are to survive the stringent selection process they are subjected to as they traverse the FRT [79]. ASA impair the ability of sperm to penetrate this CM. The movement of sperm through CM is likely

influenced by an interaction between sperm and CM components (mucin glycoproteins and lipids). ASA coating the sperm surface could bind mucins and effectively trap sperm in CM [51]. The isotypes of ASA responsible for this impaired migration are predominantly IgA antibodies directed against the sperm head and IgG ASA directed against the neck or midpiece of sperm [51]. The inability of sperm to breech the CM is also a consequence of complement activation via either IgA or IgG ASA [51]. D'Cruz et al. used flow cytometry to demonstrate that exposing sperm to IgG ASA resulted in the deposition of complement components (C3d and C5b-C9) on the sperm surface [80]. This immobilizes sperm and induces the activation of granulocytes to the antibody and complement bound sperm [80]. Although researchers have not yet reached a consensuses on whether complement components are sufficiently present throughout the FRT to induce the aforementioned effects [51], it has been documented that complement components in CM were able to immobilize 50% of ASA-coated sperm after 1 hour and 70% after 3 hours [81]. ASA have also been found to hinder sperm migration from the uterus into the fallopian tubes [60].

The effects of ASA on capacitation, the acrosome reaction, and the ability of sperm to bind to the oocyte are thought to be determined by the antigenic specificity of ASA [51]. Sperm membrane proteins are dynamic- they form unique complexes, in specific locations, at specific times in the lifetime of an individual sperm and each complex has a specialized role in fertilization [82]. Thus, the characterization of immunogenic sperm proteins necessary for processes like capacitation, need to be elucidated, so that the role of cognate ASA can be determined. Given this, an interesting

study investigated the incidence of ASA-interference with zona pellucida (ZP) binding [83]. Fifty percent of patients whose ejaculated sperm was coated at the sperm head with IgG or IgA had impaired ZP binding [83]. However, the ASA coated sperm were able to bind to the ZP to some degree; zona pellucida binding was never entirely prevented by ASA [83]. The researchers concluded that when it comes to the role of ASA in impairing gamete interaction, what matters most is the relevance of the specific antigen [83]. Francavilla and Barbonetti argue that the degree to which ASA exerts a negative effect on fertilization, downstream of cervical mucus penetration, is hard to establish because it requires an assay that can quantify the amount of ASA on the sperm surface and knowledge of both the antigen of each ASA, and the role each antigen plays in mediating a successful sperm/oocyte interaction [84]. Research into these complexities is warranted, not only because it would further basic knowledge in reproductive biology but also because it would help physicians to choose the most effective and least invasive assisted reproductive treatment option for those with ASA-mediated infertility [84].

Much of the research into the etiology of ASA formation and the effects of ASA on the mechanisms underlying fertilization, was conducted over 20 years ago and needs to be repeated. The complex nature of ASA is testament to the complexity of the immune response in human genital tracts. Due to this complexity, questions regarding the very nature of ASA remained unanswered. The presence of ASA in prepubescents, and in both fertile and infertile adults, yet their rarity in the population as a whole, makes delineating whether they are an uncommon, or all-too-common, immunologic phenomena a challenge. The fact that the majority of ASA are not inherently sperm specific, but react

with pathogenic and endogenous epitopes, begs further investigation. Understanding the functional relevance of oligosaccharides that coat the sperm surface could elucidate why SI-Abs preferentially target carbohydrate moieties. Although hypothesis that seek to resolve these discrepancies were discussed, conclusive answers will only be brought about through continued research. ART are a backroad currently used to circumvent the molecular processes mediating fertilization that remain an enigma. Therefore, continued research into ASA, such as anti-CD52g, is of cardinal importance, for it has advanced our understanding of the immune mechanisms underlying fertilization, has contributed to the development of novel contraceptive methods, and has the potential to improve the diagnosis and treatment of immune infertility.

CHAPTER 2. LITERATURE REVIEW OF ENZYME-LINKED IMMUNOSORBENT ASSAYS (ELISA)

2.1 THE HISTORY OF ENZYME-LINKED IMMUNOSORBENT ASSAYS

The ELISA was pioneered by Peter Perlmann and Eva Engvall at Stockholm University in Sweden in 1971 [85]. Perlmann and Engvall combined aspects of immunofluorescent assays (IF) developed in the 1940s [86] and radioimmunoassay (RIA) introduced in the late 1950s [87]. RIA was a rather laborious process in which a specified concentration of the antigen of interest was radio-labeled and bound to an antibody, which was itself attached to a solid material [87]. The concentration of the antigen of interest in test sera was determined by the extent to which the sera derived antigen displaced the radio-labeled antigen [87]. In a desire to circumvent the use of radioactive labels, Avrameas et al. optimized the use of enzyme conjugated antibodies to detect the presence of immune complexes in the late 1960s [88]. Utilizing these techniques, Perlmann and Engvall developed a direct ELISA, in which an enzyme, alkaline phosphatase, rather than radioactive iodine, was used as an antibody label, to successfully quantify the level of IgG in rabbit serum [85,89]. The ELISA method provided to be easier, cheaper, and more adaptable than RIA or IF, and yet still retained the specificity and sensitivity required of a diagnostic tool [90]. The great versatility of the ELISA method is due to the fact that a researcher can vary the combinations of reagents used, depending on the objective of the assay [91]. The technique is simple, utilizing materials and equipment that are relatively inexpensive and require no extensive training or certification [91]. Furthermore, owing to enzyme amplification, ELISAs are sensitive

[92] and yield quantifiable data [91]. The ELISA would be modified over the next forty years to allow for the detection of a plethora of proteins found in human sera. Throughout the 1970s ELISAs were used to quantify the amount of antibodies in sera to strains of Salmonella [93], to detect cholera exotoxin [94] and infections caused by influenza and mumps [85]. Today, ELISAs are ubiquitous in diagnostic laboratories around the world [85]. They are employed for the detection of hepatitis A [95] and B [96], rheumatoid factor [97,98], HIV [99,100] and antinuclear antibodies [101]. ELISAs have still wider applications in various industries (food and pharmaceutical) as well as in both basic and applied scientific research [89]. The ELISA is thus a remarkable technique in that it's a straightforward assay, composed of simple, well-understood constituents, that has aided in the understanding of a diverse array of complex, unexplained biological phenomena.

2.2 ELISA: OVERVIEW OF THE PRINCIPLE AND PROCEDURE

ELISAs are conducted to detect and measure the concentration of biomolecules [89,91]. The general mechanism by which this is accomplished involves at least one antibody and its cognate antigen. The antigen of interest is immobilized, either by passive absorption to a 96-well plate, or by binding to an antibody, referred to as the capture antibody, that is itself absorbed to the plate [89,91]. The antigen or capture antibody will not bind to every conceivable site on the plastic well. Therefore, to prevent nonspecific absorption of proteins to these empty sites, a blocking agent is employed [89,91]. An enzyme-labeled antibody is subsequently added and binds to any immobilized antigen [89,91]. Upon addition of the proper substrate, the enzyme will catalyze a reaction that produces a colored product [89,91]. The intensity of the color generated from this

enzymatic reaction constitutes a signal that can be measured via colorimetry and correlates with the amount of antigen on the plate [89,91]. Substrate catalysis can be stopped via the addition of a strong acid that quickly denatures the enzyme [89,91]. Between the addition of each reagent in an ELISA, the plate is incubated and washed [89,91].

2.3 TYPES OF ELISAs

Depending on the objective of the assay, different strategies for conducting an ELISA have been established. They differ in how the antigen is immobilized and detected. There are three basic approaches to conducting an ELISA, each of which can be modified in the event that a competitive assay is required [91]. A direct ELISA involves adding the antigen of interest to the surface of the plate. Enzyme-labeled antibodies are subsequently added and bind directly to the immobilized antigen (Figure 1a). An indirect ELISA largely follows the same steps as a direct ELISA, except for the stage at which the enzyme-linked antibodies are added. After the antigen has been immobilized to the plate, cognate primary or detector antibodies are added. However primary antibodies are not enzyme-conjugated. The primary antibodies are themselves targeted by enzyme-conjugated antibodies (Figure 1b). Thus, the conjugated antibodies bind indirectly to the antigen, via binding to the primary antibody. The enzyme-linked antibodies are referred to as anti-species conjugates, as they are specific to the antibodies of the species in which the primary antibodies are generated [91]. For instance, if the primary antibodies were produced in rats, the enzyme-conjugated secondary antibody must be an anti-rat

antibody. Direct ELISA are constrained by the fact that the primary antibody must be enzyme conjugated [91]. Indirect ELISAs compensate for this, as there are thousands of commercially available secondary anti-species conjugates, of various isotypes, that can be used to detect particular primary antibodies in the assay [91]. Thus, indirect ELISAs are more flexible than direct ELISAs and are consequently more commonly used. Essentially, a single secondary antibody with the particular isotype of interest can be used to report the presence of many antibodies against a particular antigen. The sensitivity of the assay is also enhanced, as many secondary antibodies can bind to epitopes on the primary antibody, increasing the signal generated upon catalysis [91]. Yet one pitfall of indirect ELISAs is that they have varying degrees of non-specific binding [89,91]. Different species produce antibodies that share evolutionary conserved sequences. Therefore, it is possible that the paratope of an antibody generated in one species, could recognizing an epitope on an antibody from a different species [89,91]. This could result in a secondary antibody unintendedly binding to off-target, endogenous antibodies in the test sample and a falsely elevated signal would result.

To ensure greater specificity, a sandwich ELISA can be employed (Figure 1c). In this method, the antigen of interest is sandwiched between two antibodies. The first antibody, the capture antibody, is immobilized to the surface of the plate and selectively captures the antigen of interest. The second antibody bound to the captured antigen can be either enzyme conjugated (direct sandwich ELISA) or a detector antibody that is subsequently targeted by a labeled antibody (indirect sandwich ELISA). Sandwich ELISAs allow for a variety of different test samples to be tested against a single capture

antibody with a particular specificity, or for a variety of capture antibodies to be tested against various samples, on the condition that the enzyme-conjugated antibody does not react with the capture antibody [91].

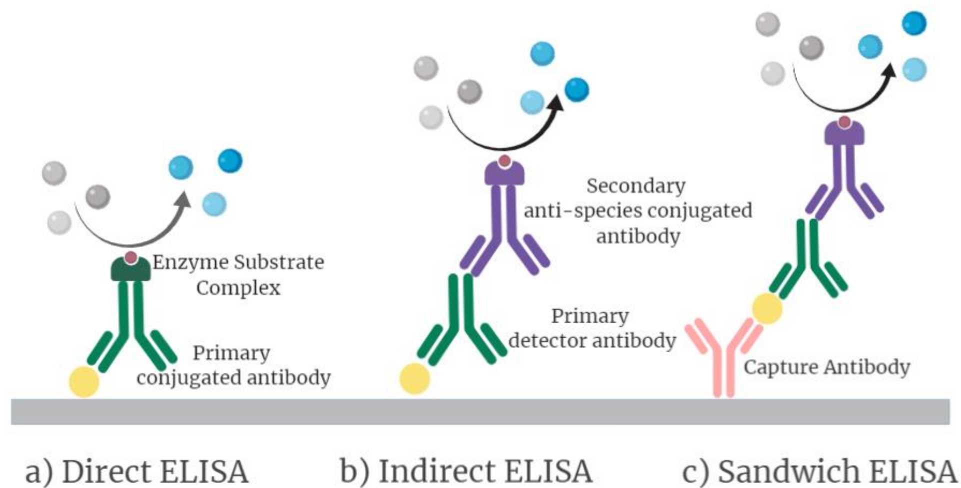


Figure 1. Types of ELISAs. a) Direct, b) indirect, and c) indirect sandwich ELISAs, are three approaches to detecting and quantify the concentration of an analyte using immune complexes.

2.4 STAGES IN ELISA

Each component required to conduct an ELISA has unique characteristics that account for the phenomena and influences the degree to which accurate, reliable, and reproduceable results are obtained. The first step in an ELISA involves coating a 96-well plate with either antigen or capture antibody. The 96-well plates typical of an ELISA are manufacture from polystyrene (PS), a hydrophilic polymer. Nonpolar protein components are thought to absorb to the PS plate via hydrophobic interactions [91]. In a sandwich ELISA, it is imperative to discern the appropriate concentration of capture antibody that must be added to the plate, as the manner in which capture antibodies absorb to the plate

can affect the results of the assay. If the concentration is too high, steric hindrance may prevent the antibodies from absorbing to the plate or may cause antibodies to stack on top of one another [91]. In both cases, the likelihood of a stable interaction between the capture antibodies and the antigen of interest decreases. Moreover, the orientation of the capture antibodies, and the degree to which the majority of the antibodies bind to the plate via their Fab portion, rather than their Fc portion, influences the amount of antigen captured [91]. Ideally, capture antibodies would absorb to the plate via their Fc and would be evenly spaced along the surface of the well, to allow for the maximum concentration of antigen to bind [91]. Another significant factor influencing the degree to which the capture antibodies or antigen absorb to the plate, is the length of time both are incubated and the temperature at which that incubation occurs. Higher temperatures increase the rate of protein absorption, but could potentially damage the protein components used to coat the plate [91]. The concentration of antigen or antibody used to coat the plate, also influences the incubation time required to ensure that a maximal number of hydrophilic interactions between the coating mixture and the plastic matrix occur [91].

After the first biomolecule has been immobilized on the plate, it is necessary to ensure that any extraneous proteins in the test sample, or any antibodies introduced in subsequent steps of the ELISA, do not absorb to the plate. If such proteins were to occupy empty spaces on a well, the anti-species conjugate could bind to these and give a falsely elevated signal [91]. To prevent such binding, one method involves incubation of a blocking agent to the plate before addition of the test sample. Common protein blockers

include bovine serum albumin (BSA) and non-fat dry milk [91]. These proteins attach permanently to any empty spaces on the surface of a well, preventing irrelevant proteins from doing so [91]. Another method to further prevent nonspecific binding is to add a low concentration of detergent to the dilution buffer of reagents used in the assay [91]. These detergents prevent and disrupt weak, nonspecific protein-protein interactions which could similarly give rise to a misleading, elevated signal.

Fundamental to ELISAs is the use of enzyme-conjugated antibodies to report the presence of a particular antigen. There are various enzymes that are readily amenable to conjugation with an antibody [91]. However, the most studied enzyme-antibody conjugate and one of the most commonly used, is horseradish peroxidase (HRP) [91]. Discovered in 1903, HRP is a heme containing protein extracted from the roots of the horseradish plant (*Armoracia rusticana*) [102]. Peroxidases are a large collection of enzymes that catalyze the oxidation of peroxides [102]. The reaction mechanism of HRP mediated catalysis is complex [102] but, essentially, HRP breaks down two molecules of hydrogen peroxide into water and oxygen. This is achieved by the reduction of hydrogen peroxide, as the heme group of HRP is simultaneously oxidized, to yield an unstable HRP intermediate. This intermediate has a lower affinity for the second molecule of hydrogen peroxide and one can select a hydrogen donor, such as 3,3′,5,5′ tetramethylbenzidine (TMB), to be oxidized by the HRP intermediate and thereby regenerate the native HRP. This redox reaction is exploited in ELISAs. When a hydrogen donor like TMB is oxidized, it yields a blue reaction product [91]. The colored product serves as a measure of the activity of the HRP enzyme and thus, in an ELISA, should indicate the presence of

immune complexes and by extension, allows for the detection and quantification of antigen of interest.

To stop the redox reaction, a strong acid such as sulfuric acid, is often utilized to denature HRP [91]. Stopping the reaction expedites the measurement of the colored product, as a single endpoint measurement can be obtained, rather than monitoring the rate of the reaction over time [91]. Therefore, once the stopping reagent has been added, the reaction product is measured by a colorimetric assay [91]. A beam of light is directed through each well of the plate. A plate reader derives the amount of light absorbed in each well based on the transmittance. Each well is given an absorbance value in optical density. The intensity of the color of the solution in each well represents the amount of protein of interest present in the test sample. Adding the stopping reagent before reading the plate alters the color of the test solution from blue to yellow, with a maximum absorbance at 450nm [91]. Thus, in an ELISA, the plate reader transmits light (at 450nm) through each well and determines how much light of that wavelength is absorbed by each test sample.

After the addition of each reagent in an ELISA, the plate must be incubated and subsequently washed. The incubation period provides time for specific antigen-antibody interactions to occur [91]. As the antigen and antibody must to come into close enough contact to allow for their characteristic non-covalent bonds to be established, the incubation period is essential. However, numerous factors affect this interaction including the concentration of the antibodies and antigen, and the time and temperature of the incubation [91]. The washing process involves emptying the plate wells and flooding

them with washing buffer three times. Flooding the wells with the wash buffer is undertaken to remove any unbound (irrelevant) reagents from the wells and thereby increase the accuracy of the ELISA, as only those reagents that react with the antigen or antibodies introduced in the different phases of the ELISA remain in the well [91]. A common washing buffer is phosphate-buffered saline (PBS) mixed with a small concentration of the detergent Tween-20 [91]. The detergent serves to further break up any nonspecific protein-protein interactions such that extraneous proteins can be removed from the assay [91]. If such extraneous proteins (such as endogenous sera antibodies) were to remain in the well, they could be detected in the following phases of the test, giving a high background signal. Knowledge of the systems, stages, and components of an ELISA will all be considered in order to optimize the performance of the assay to detect CD52g antibodies in human sera.

2.5 ELISA: A TOOL FOR THE QUANTIFICAITON OF CD52G ANTIBODIES IN HUMAN SERA

An overview of the initial indirect and the sandwich ELISA protocols used to detect CD52g antibodies will be described below, accompanied by a pictorial presentation (Figure 2) of these steps. The experimentally derived specifics of each step, as well as modifications to these protocols, will be described in Chapter 3.

When developing an ELISA for CD52g Abs in serum, two ELISA techniques were utilized, a typical indirect and sandwich ELISA. In the indirect ELISA, CD52g is bound inside the wells of the plate via coating the wells with seminal plasma (SP). Human sera from patients with infertility are subsequently introduced. If CD52g

antibodies are present in the sera, binding to CD52g will occur. The addition of anti-human IgG that has been enzyme (HRP) linked indicates the degree to which anti-CD52g antibodies are present in each serum sample. In addition, controls, as well as, solutions containing known concentrations of CD52g antibodies are tested. A standard curve is constructed using optical density values. The standard curve is used to calculate the relative concentration of CD52g antibodies in each patients' sera. In a sandwich ELISA, rather than coating the wells with CD52g/SP, the wells are coated with Campath and CD52g/SP is added in a subsequent step. Between each step in either type of ELISA, there is an incubation period, followed by washing with 0.05% Tween-20 in PBS. What follows is an outline of the indirect and sandwich ELISA protocols for detecting and quantifying CD52g antibodies.

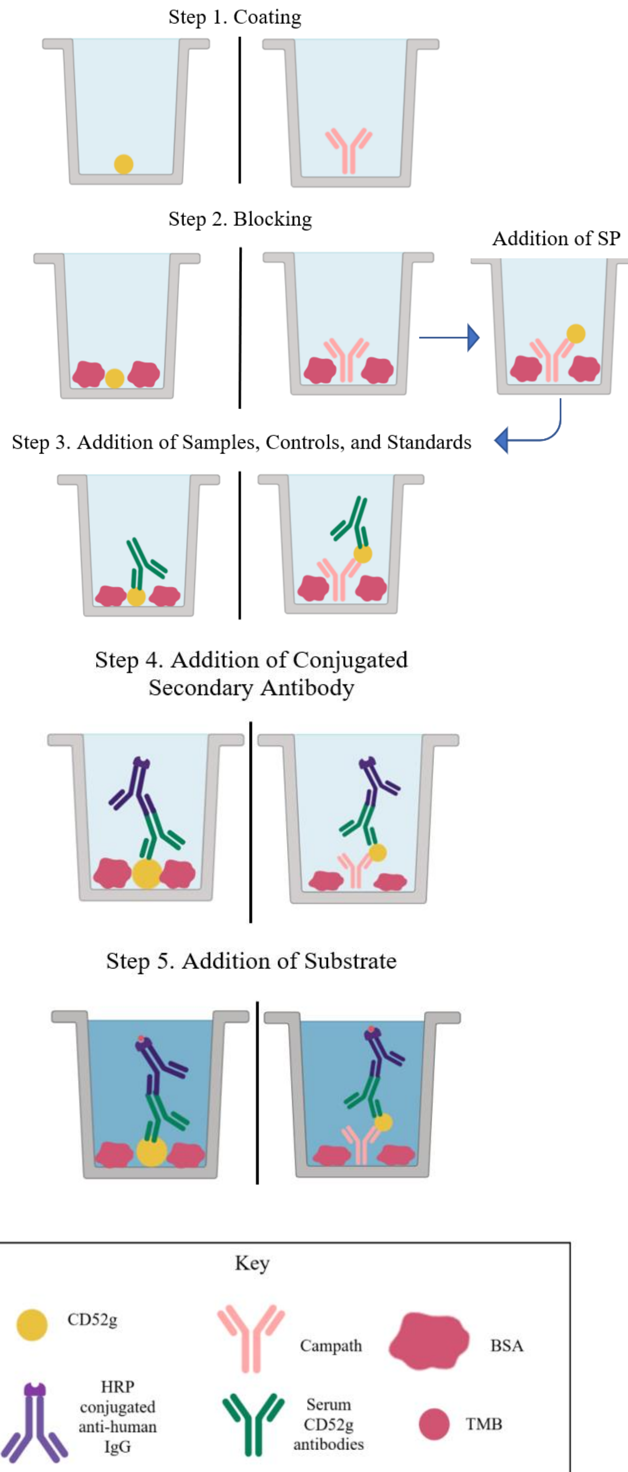


Figure 2. Schematic of Indirect and Sandwich CD52g ELISA

Step 1. Coating: The first step in both an indirect and sandwich ELISA requires a 96 well plate. In an indirect ELISA, seminal plasma, containing CD52g, is diluted in an appropriate buffer and coats the bottom of each well. In a sandwich ELISA, the plate is coated with diluted Campath, a monoclonal antibody against the protein core of CD52g. Campath is used as the capture antibody in this assay. Campath concentrations are calculated in µg/ml, indicating the amount of the antibodies that are present in solution.

Step 2. Blocking: The major component of fetal bovine serum (FBS) is bovine serum albumin (BSA). In the blocking step, diluted FBS is added to the plate, such that BSA can absorb to regions of the well surface that are not covered by the antigen or by the capture antibody. BSA will thereby prevent non-specific antibody binding to the plate.

Step 3. Addition of sera test samples, antibody standards, and controls: Test samples of human sera are diluted into a buffer. Solutions with known amounts of the CD52g antibody HCA-4, are diluted in a range of concentration for use as a set of antibody standards. HCA-4 (Human Contraceptive Antigen-4) a humanized monoclonal antibody raised against the carbohydrate moiety of CD52g. Each serum sample is added to individual wells on the plate in duplicate or triplicate. The HCA-4 standard concentrations are calculated in µg/ml. Positive and negative controls were added as a measure of the assay's validity.

Step 4. Addition of Conjugated Antibody: The conjugated antibody added is an anti-human Immunoglobulin G (IgG). The IgG has been conjugated with HRP. If the serum sample added to the plate is positive for CD52g antibodies, the conjugated IgG antibodies will bind to any CD52g antibodies that have bound to CD52g inside the well. If, however, there are no CD52g antibodies in the test sample, the conjugated antibody will be removed during the washing step. Thus, theoretically, only the wells containing sera that are positive for CD52g antibodies will contain the conjugated antibody.

Step 5. Addition of HRP substrate: The substrate that is added is 3,3',5,5'-tetramethylbenzidine (TMB). The oxidation of TMB causes the solution in each well to become a blue color. The intensity of this color is indicative of the amount of CD52g antibodies. The reaction is then quenched using sulfuric acid.

Step 6. Reading: The ELISA plate is inserted into a plate reader which reads each well by colorimetry at 450nm. The intensity of the color in each well is given in optical density, which is directly proportional to the amount of CD52g/CD52g antibody binding that occurred within the well. The optical density values for each of the antibody standards is used to plot a standard curve.

Note: In a sandwich ELISA, the protocol is slightly different in that diluted SP, containing CD52g, is added after blocking. The sequence of steps then continues unchanged.

2.6 OBJECTIVES OF THE CURRENT STUDY

The chief objective of this study was to develop and optimize an ELISA to determine the prevalence of CD52g antibodies in the sera of patients with infertility as compared to controls. The second objective was to determine if there was a correlation between patient sera that was shown to agglutinate sperm and the prevalence of CD52g antibodies in these samples.

CHAPTER 3. DEVELOPMENT OF AN ELISA FOR THE DETECTION OF CD52G ANTIBODIES IN HUMAN SERA

The initial methods used to produce an ELISA to measure the concentration of CD52g antibodies will be described first. A description of the modifications made to this procedure as a consequence of experimentation conducted for this study will subsequently be detailed.

3.1 MATERIALS AND METHODS

Sandwich ELISA: Method for Coating ELISA Plates with Capture Antibody

Campath (1000 µg/mL), a commercially available monoclonal antibody (IgG2b) against the protein core of CD52g is diluted in PBS. This solution constitutes the coating buffer. The coating buffer (50µL/well) is added the ELISA plate. The plate is sealed with an adhesive, clear, plastic cover to prevent the reagents from evaporating or being contaminated. The plate is subsequently incubated overnight at 37°C. Following

incubation, the plate cover is removed and the coating buffer is discarded. The plate is washed three times with PBS (200µL/well).

Method for Blocking ELISA Plates

The blocking buffer consists of PBS with 5% Fetal Bovine Serum (FBS). The blocking buffer (200µL/well) is added to the ELISA plate. The plate is sealed and is incubated at 37°C for 1 hour. Following incubation, the blocking buffer is discarded and the plate is washed three times.

Method for Seminal Plasma (SP) Addition

Indirect ELISA: SP Coating

Frozen aliquots of seminal plasma (SP) are brought to room temperature. The SP is diluted in PBS. The SP solution (200µL/well) is added to each individual well of the plate, such that it absorbs to the well surface. The plate is sealed and is incubated overnight at 37°C.

Sandwich ELISA

Frozen aliquots of SP are brought to room temperature. SP is diluted in sample diluent. The sample diluent is composed of PBS with 10% FBS and 0.5% Triton X-100. The SP solution (200µL/well) is added to each well of the ELISA plate, such that molecules of CD52g contained within it can be captured by Campath antibodies coating the plate. The plate is sealed and is incubated at 37°C for 2 hours. Following incubation, the SP is discarded and the plate is washed three times with wash buffer.

Method for Preparation of Serum Samples, Controls, Antibody Standards and Conjugate Antibody

As the SP is incubating, the antibody standards are produced. The humanized monoclonal antibody (IgG1) HCA, raised against the carbohydrate epitope of CD52g is used to prepare standard solutions. Concentrated HCA is serially diluted in sample diluent to known concentrations and added to the plate (50µL/well). Aliquots of each serum sample are brought to room temperature, vortexed, and subsequently diluted in sample diluent and vortexed again before addition to the plate (50µL/well). Horseradish peroxidase (HRP) conjugated anti-human rabbit Immunoglobin G (IgG) is added to PBS to create a 1:5000 dilution and added to the plate (100µL/well).

Method for Conducting an ELISA Assay

After the plate was blocked and CD52g has been immobilized to the plate, the HCA standards and the serum samples were added in duplicate or triplicate to the microtiter plate. The loaded assay plate was sealed and incubated at 37℃ for 1 hour. The plate was washed with wash buffer three times to remove any unbound material from the well. The conjugated antibody was added to each well and the plate incubated for 1 hour at 37℃. The plate was subsequently washed three times with wash buffer to remove any unbound conjugate antibody. The HRP substrate, 3,3',5,5'-tetramethylbenzidine (TMB, 100µl/well), was added to each well. The liquid in the wells that contained CD52g antibodies should turn a blue color. The plate was incubated at room temperature to allow time for the blue color to develop. Theoretically, the intensity of this color should correlate to the concentration of anti-CD52g antibodies in the original serum sample.

Sulfuric acid (2M, 100µL/well) was added to each well to quench the HRP-catalyzed reaction. Sulfuric acid was added when the lowest standard turned blue, approximately 15-20 minutes after the addition of TMB. Upon addition of sulfuric acid, the liquid in the plate turn a yellow color. The plate is then inserted into a plate reader which assigned an optical density value to each well via absorbance colorimeter at 450nm. The standard curve is generated from the known concentrations of the HCA standards. The concentration of anti-CD52g antibodies can be derived from this curve.

Method for Washing Plates

After the addition of each reagent, the ELISA plate is washed three times with wash buffer. The wash buffer is composed of PBS with 0.05% Tween-20 (200µL/well). After flooding the plate with wash buffer, the plate is inverted and the buffer is discard into the sink. The inverted plate is tapped against paper towels to remove any residual buffer.

Statistical Analysis of ELISA Data

A Repeated Measures ANOVA (analysis of variance) procedure was used to compare the mean absorbance values derived from antigen coated and non-antigen coated (background) wells for all serum samples. If the results of the ANOVA were significant ($p < 0.05$), post-hoc analysis was conducted via the Tukey Test. GraphPad Prism, a commercial graphing and statistics software was used to perform these analyses and graph OD values.

RESULTS

3.2 SANDWICH ELISA: OPTIMIZATION OF CAPTURE ANTIBODY AND PRIMARY ANTIBODY CONCENTRATIONS

Optimization of Campath Concentration

The considerable specificity of sandwich ELISAs engendered them as the best strategy for detecting CD52g antibodies in the complex milieu of human serum. Campath is a rat monoclonal antibody specific to the protein core of CD52g. When used as a capture antibody, Campath immobilizes CD52g from seminal plasma via an antigen/antibody interaction. The first stage of this study involved determining the proper concentration of Campath that would constitute the coating buffer. A microtiter plate was coated with two different concentrations of Campath (10µg/mL or 20µg/mL) to test its affect on assay performance.

Optimization of Primary Antibody (HCA) Concentration

The humanized monoclonal antibody HCA-4 (470µg/mL) was used the primary antibody. HCA is raised against the carbohydrate moiety unique to CD52g. As Campath would immobilize CD52g via binding to CD52g's protein core, HCA would bind to CD52g specific glycans. HCA would serve as a positive control and/or standard in subsequent experiments to detect CD52g antibodies in human sera. Therefore, it was necessary to determine the appropriate concentration of HCA-4 to detect CD52g immobilized by Campath (at 10µg/mL or 20µg/mL). This was accomplished by serially diluting HCA-4 and examining the resulting trend in the signal generated.

Production of Assay Components

A typical capture antibody concentration of 10µg/mL was tested against a concentration of 20µg/mL. An amount of concentrated Campath was added to PBS to produce the two desired concentrations. Each coating buffer, with a concentration of Campath at either 10µg/mL or 20µg/mL, coated 18 wells on one plate. Seminal Plasma (SP) was brought to room temperature. A 1:10 dilution of SP (200µL/well) was used for this assay. A 2-fold dilution series of HCA-4 starting at a concentration of 100µg/mL was assayed. Since 100µg/mL of HCA was previously determined to be the optimal concentration at which HCA agglutinated sperm, this was the highest HCA concentration to be tested for its ability to detect CD52g in ELISA. The recommended dilution factor for the conjugate antibody, rabbit anti-human IgG, ranged between 1:1000-1:10,000. Therefore a dilution factor of 1:5,000 was selected.

Assay

Two concentrations of Campath and six different concentrations of HCA-4 were assayed on a single plate using the sandwich ELISA procedure described in section 3.1.

Assay Results and Discussion

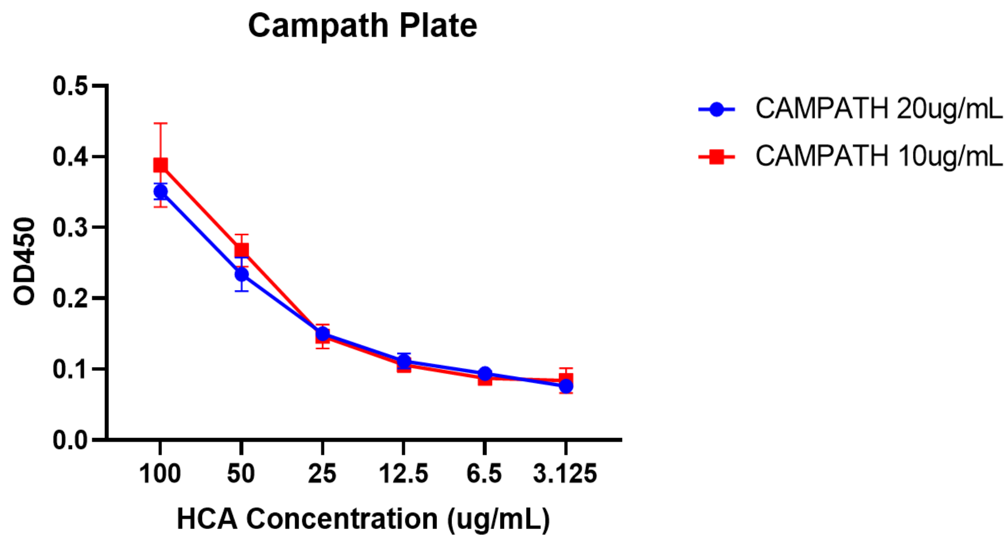


Figure 3. Sandwich ELISA optimization of Campath and HCA-4 concentrations. Concentrations of Campath at 10µg/mL or 20µg/mL plotted against concentrations of HCA-4 ranging from 100µg/mL to 3.125µg/mL. Error bars represent standard deviation.

The first experiment conducted sought to determine the optimal concentrations of both the capture antibody, Campath, and the primary "detector" antibody HCA-4. As shown in Figure 3, the strongest signal was obtained with Campath at 10µg/mL and HCA-4 at 100µg/mL. The higher concentration of Campath (20µg/mL) may have exceed the binding capacity of the surface of the plate, such that the ability of Campath antibodies to distribute evenly on the well surface, via their Fc, was impaired. This would have resulted in Campath antibodies either failing to trap CD52g or, conversely, capturing CD52g, but due to failed absorption, being removed upon washing. In either case, the depressed signal at the higher concentrations of Campath (Figure 3) would result. The concentration of HCA-4 at 50 µg/mL or higher, exceeds the concentration of CD52g

bound to Campath at 20µg/mL, and a less intense signal compared to Campath at 10µg/mL is generated as a result. Thus, adding a higher concentration of Campath is a waste of the reagent, as the higher concentration did not increase the amount of CD52g immobilized to the plate. Regardless of the Campath concentration, decreasing the concentration of primary antibody, decreased the signal generated. This was expected, as decreasing the concentration of primary antibody would result in fewer available epitopes to which the conjugate antibody could bind to. The optimal concentration of Campath used to coat all subsequent plates was 10µg/mL. The concentration of HCA-4 that could optimally detect CD52g immobilized by Campath (at 10µg/mL) was 100µg/mL.

3.3 INDIRECT ELISA: OPTIMIZATION OF SEMINAL PLASMA DILUTION AND PRIMARY ANTIBODY CONCENTRATION

Optimization of SP Concentration

While the enhanced specificity of a sandwich ELISA made the format an excellent choice for detecting CD52g antibodies in sera, the technique required a 2 hour incubation of SP and varying quantities of Campath, which is a costly reagent. It was worth investigating whether CD52g could be detected via a less expensive and more expedient strategy, such as an indirect ELISA, without compromising the strength of the signal produced. Again, seminal plasma was used as the source of CD52g. To determine the optimal dilution of SP required to coat the microtiter plate of an indirect ELISA, three dilutions of SP were produced and assayed.

Optimization of Primary Antibody (HCA) Concentration

HCA was serially diluted to determine the concentration that would allow for optimal detection of CD52g that was absorbed to the plate.

Production of Assay Components

SP was serially diluted in PBS (1:10, 1:20. 1:30) to produce three different coating solutions, each of which used to coat individuals wells in triplicate (200µL/well) on a single microtiter plate. The primary and conjugate antibody conditions were kept the same as in the previous experiment. Specifically, a 2-fold dilution series of HCA-4 was assayed and the conjugate antibody, rabbit anti-human IgG, was diluted 1:5000 in PBS. VRC01 (IgG1), a broadly neutralizing antibody against HIV-1, was used as an isotype control. VRC01 is of the same subclass as HCA-4, but is specific to the CD4 binding site of the glycoprotein, gp120, an HIV envelope protein. As seminal plasma is a complex, heterogenous mixture, it is possible that HCA-4 could bind to endogenous components of SP, other than CD52g. To gauge the degree to which this nonspecific binding occurred, VRC01 was assayed.

Assay

Three different dilutions of SP (1:10, 1:20. 1:30) and six different concentrations of HCA-4 were assayed on a single plate using the indirect ELISA procedure described in section 3.1

Assay Results and Discussion

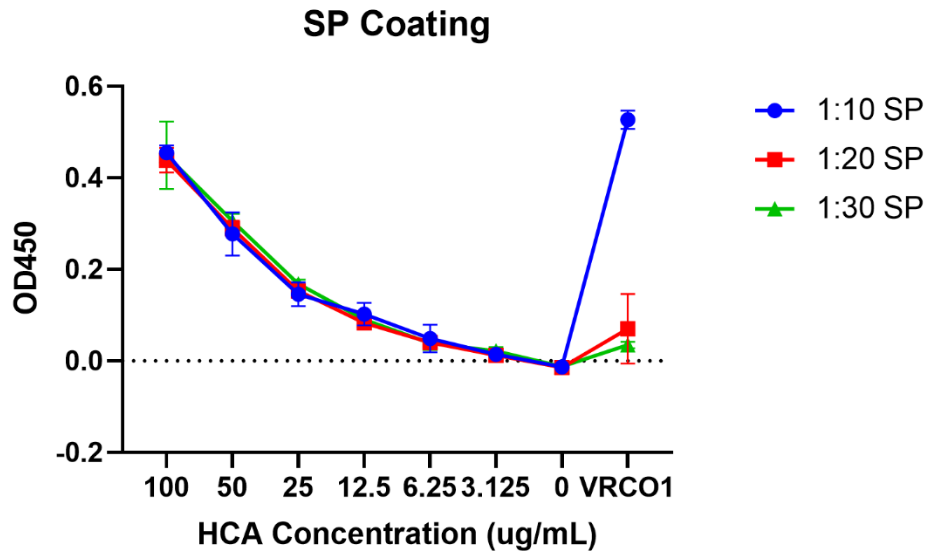


Figure 4. Indirect ELISA optimization of SP coating dilution and HCA-4 concentration. Dilutions of SP were coated to the plate and were plotted against concentrations of HCA-4 ranging from 100µg/mL to 0 µg/mL. Error bars represent standard deviation

All three SP dilutions yielded signals with nearly the same intensity at each of the different concentrations of HCA. In other words, there was no decrease in signal intensity as the concentration of CD52g was decreased on the plate. Thus, the plate wells adsorbed a similar amount of CD52g regardless of the extent to which the SP was diluted. This implies that the capacity of the plastic microtiter plate to bind CD52g is finite and thus even if higher levels of CD52g (smaller dilutions) were assayed, no more antigen could possibly attach to the well surface. The isotype control, VRC01, generated a considerable signal when the SP coating was at a 1:10 dilution. In fact, the mean OD of the isotype control at a 1:10 dilution of SP was slightly higher than the signal produced from HCA-4 at 100µg/mL, suggesting that the HCA-4 signal generated was unlikely to accurately

represent an interaction between HCA-4 and CD52g. However, it is possible that the intense signal generated by VRCO1 is simply an artifact and repeating the experiment would have elucidated the degree to which it was a false positive result. Nonetheless, the great variety of carbohydrates and proteins present in SP could allow for HCA-4 to bind non-specifically these molecules, if they contained similar epitopes to that recognized by HCA-4. The difference in the intensity of the signal generated by VRC01 versus HCA-4 when SP was further diluted (1:20 and 1:30) suggested that diluting the SP minimized such background noise reactions, perhaps by simply decreasing the number of available biomolecules to which the primary antibody (HCA-4) and/or the isotype control could interact with. The SP dilution at 1:20 yielded a slightly higher signal than that of the SP dilution at 1:30, with only a negligible difference in the isotype control (VRC01) signal. Therefore, to optimally detect CD52g via an indirect ELISA, a SP dilution of 1:20 and HCA at 100µg/mL was deemed optimal.

3.4 INDIRECT ELISA: OPTIMZATION OF SERA DILUTION

Titration of Individual Positive and Negative Serum Samples

Sera collected from 88 patients diagnosed with infertility and positive for ASA, as well as, sera from 11 healthy controls, negative for ASA, were available for assay development. Aliquots (100µL-200µL) of these samples were frozen at -80°C. Bronson et al. previously determined the incidence of antisperm antibodies (ASA) in each serum sample and the localization of bound, sera-derived ASA, on the sperm surface, via an immunobead test (IBT). As both the proper dilution of SP and optimal concentration of

the primary antibody, HCA-4, to detect CD52g, via an indirect ELISA, had been deduced (Figure 4), the assay protocol could be further modified to determine the concentration of CD52g antibodies present in each of the 88 serum samples positive for ASA. The dilution of sera required for such an assay was now determined by titrating the serum samples against a constant dilution of CD52g. There was a concern that activated complement components in sera could alter the measurement of CD52g antibodies in sera. For instance, active C1q could be retained on the plate and enable endogenous IgG antibodies, not specific to CD52g, to be immobilized in to the plate. C1q could also bind immune complexes in sera and potentially trap them within the ELISA system. The subsequent addition of the conjugate antibody would produce a falsely elevated OD value. In order to ascertain the degree of such interference, heat-inactivated human serum (HIHS) was assayed.

Production of Assay Components

A 1:20 dilution of SP was erroneously produced using sample diluent (which contains the surfactant Triton X-100) and served as the coating buffer. A two-fold dilution series (50µL/well) of each serum sample in sample diluent (1:20 to 1:640) was added to the ELISA plate after the blocking step. A single serum sample positive for ASA and a single serum sample negative for ASA were assayed. A set of antibody (HCA-4) standards were assayed for use as a positive control.

Assay

An indirect ELISA was performed following the procedure described in section 3.1

Assay Results and Discussion

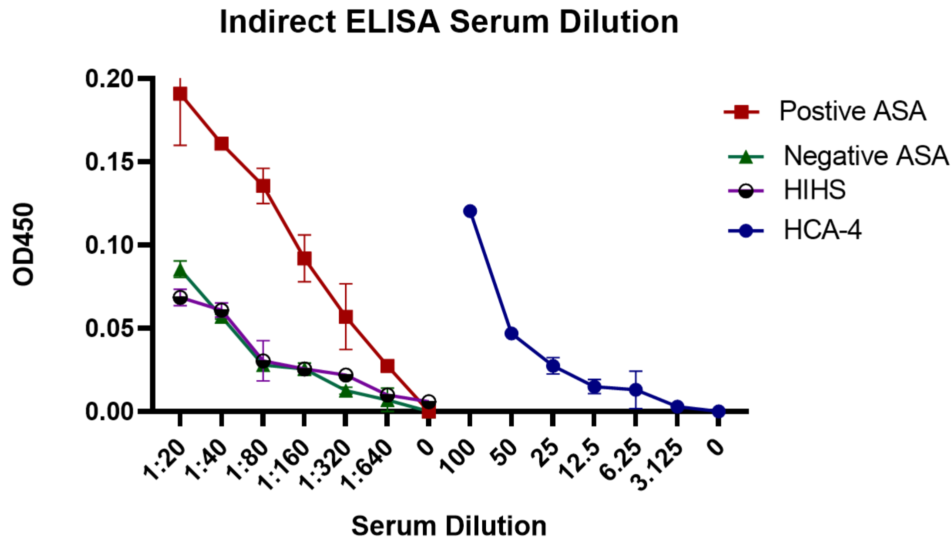


Figure 5. Indirect ELISA optimization of serum dilution. A serum sample positive for ASA and a serum sample negative for ASA were serially diluted and tested against a constant 1:20 dilution of SP. Heat-inactivated human serum (HIHS) was assayed as an indication of the degree of complement interference in the assay. HCA-4 concentrations ranging from 100µg/mL to 0 µg/mL were used as a positive control. Error bars represent standard deviation.

The depressed HCA-4 signal, as compared to that generated in the previous ELISAs (Figs. 3&4) was due to the erroneous dilution of SP into sample diluent (0.5% Triton X-100) rather than PBS. The presence of Triton X-100 disrupted the hydrophobic interactions necessary for an optimal amount of CD52g to adsorb to the plate. Thus, in this assay, CD52g may only be present in minute quantities on the plate. The challenge in this study was to detect CD52g specific antibodies present in human sera. Titration of the serum sample negative for ASA fell within the range of the standard HCA-4 curve,

casting doubt on the negativity of the sample and the specificity of the assay. Though there was discrimination between the positive and negative ASA samples, it was entirely unclear to what degree CD52g-specfic antibodies were actually being detected. Titration of the negative serum sample and the heat-inactivated sample yielded absorbance values with nearly the same intensity. Thus, if CD52g antibodies were actually being detected in the assay, their detection in the negative serum sample was likely not mediated by active complement. Given that the HIHS was derived from an individual not diagnosed with infertility, it is possible that a baseline level of CD52g antibodies were measured in the HIHS and the negative serum samples. This would be analogous to previous research that found ASAs in the sera of both fertile and infertile individuals. This hypothesis is discussed further in section 3.16. However, it is also very possible that the measured absorbance of both the negative and positive sera was entirely a consequence of non-specific binding of sera immunoglobins to the diverse array of SP components. With regard to the positive ASA serum sample, it is possible that the signal generated represents a genuine interaction between CD52g and CD52g antibodies. However, the physiochemical properties of sera, which can contribute to falsely elevated signals has not been accounted for. The preponderance of non-CD52g antibody binding substances, in human sera, and in seminal plasma, underscores the fact that both seminal plasma and human sera are diverse matrixes. Both are composed of immunoglobulins and other unsuspecting endogenous substances that could lead to an inaccurate measurement of CD52g antibodies. Therefore, this assay elucidated it would be necessary to determine the degree of background noise reactions in each individual sample, as such nonspecific

reactions could exceed the number of true CD52g-CD52Ab reactions. Though it was not possible to truly differentiate between positive and negative samples in this assay, as background noise reactions were not measured, a dilution of sera between 1:80 and 1:640 was within the range of the HCA-4 standard curve and demonstrated a difference in signal intensity between the negative and positive samples. Therefore, a 1:100 dilution of sera was chosen as an appropriate starting dilution for subsequent assays.

3.5 MODIFICATIONS TO THE ELISA PROTOCOL

In a preemptive attempt to reduce the incidence of non-specific binding and enhance the sensitivity and strength of the signal generated, several aspects of the assay protocol were altered.

Modification: Purifying IgG from Seminal Plasma

Given that seminal plasma contains IgG antibodies, it was possible that these antibodies could absorb to the plate and upon addition of the conjugate antibody, (rabbit anti-human IgG) would bind the conjugate and generate a falsely elevated measure of CD52g antibodies (high background). To mitigate this, SP (1:20) was put into a Protein G Agarose column and the flow-through was collected and assayed. Protein G Agarose is composed of recombinant Protein G bound to agarose beads. Protein G binds preferentially to the Fc region of IgG thereby removing IgG from SP. The Thermo Scientific™ Protein G Agarose protocol was followed in this regard.

Modification: Addition and Incubation of Serum, Controls, and Standards

The serum samples, HCA-4 standard and any controls were produced as described in section 3.1 but were added to the wells of a non-tissue cultured treated, V-bottom, 96 well microtiter plate, and subsequently transferred, by multichannel pipette, to the coated and blocked ELISA plate. This method would minimize the time difference between addition of the first and last sample, and thereby ensure that the results of the assay were not affected by differences in incubation times. This method would also prevent the wells from drying out between the addition of each sample. The activity of assay reagents, particularly the HRP-conjugate, could be compromised if the wells dry out, weakening the signal generated. To account for variation in the antibody titer of each patient, it was important to have a wide enough range of dilutions to capture most of the patient titers. Therefore, highly concentrated HCA-4 (400µg/mL) was added to the assay standard. To strengthen the intensity of the signal, the plates were incubated overnight at 4℃ (rather than for two hours at 37℃) after addition of the test samples, the HCA-4 standard and any controls. The protocol then proceeded as described in section 3.1.

3.6 INDIRECT VS SANDWICH ELISA: BACKGROUND NOISE REACTIONS AND PLATE VARIABILITY

Background Noise Determination

The same serum samples assayed in the previous ELISA (Figure 5) were again assayed using two different ELISA formats, a sandwich ELISA and an indirect ELISA. In this assay, the degree of background noise inherent in each serum sample was determined by

adding serum positive for ASA to wells that were blocked but that did not contain CD52g (PBS and not SP was added to these wells). The OD of the wells with and without antigen could then be compared.

Negative Controls

Blank wells, coated with either SP or capture antibody and blocked, but lacking serum and antibody conjugate were assayed to account for any variation in the OD due to the plate itself. Wells with immobilized CD52g and blocked but containing only the conjugate antibody and not serum were also assayed. High OD values measured in these wells would demonstrate nonspecific binding of conjugate antibodies, which should be prevented by the blocking buffer. Thus, these wells essentially measure the effectiveness of the blocking buffer in preventing binding of the secondary antibody.

Indirect ELISA: Assessment of Well-to-Well Variation

Two sets of HCA-4 standards were plated (in triplicate) to assess variation in the measured OD between wells across the plate; variation could occur as a consequence of poor technique (inaccurate pipetting, bubbles in wells, inconsistent washing), variable incubation times between wells, or inconsistences with reagents (insufficient vortexing, and/or inconsistent freezing and thawing of different serum samples)

Sandwich ELISA: Confirmation of Campath Concentration and Assessment of Well-to-Well Variation

Two sets of HCA-4 standards were again plated (in triplicate) to assess any source of variation across the plate. The concentration of Campath (10µg/mL or 20µg/mL) used to coat the plates was repeated to confirm the results of previous experimentation (Figure 3).

Production of Assay Components

Serum was diluted 1:100 in sample diluent. All other reagents were diluted according to the protocol in section 3.1.

Assay

The sandwich and indirect ELISAs were performed following the protocol described in section 3.1 along with the aforementioned modifications outlined in section 3.5.

Assay Results and Discussion

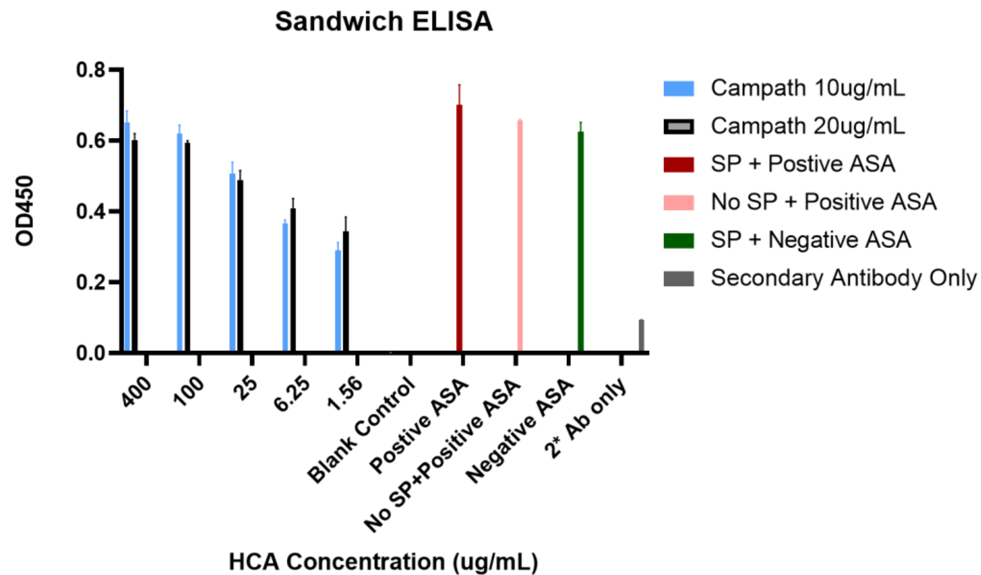


Figure 6. Background noise and plate variability in sandwich ELISA detection of CD52g antibodies in serum samples (1:100). Error bars represent standard deviation.

The intensity of the signals generated with Campath at 10µg/mL, upon addition of higher concentrations of HCA-4, was slightly stronger, though not significantly so, from that generated when Campath was at 20 µg/mL. Thus, the lower concentration of Campath maximized signal intensity, while minimizing the amount of reagent required per assay. At lower concentrations of HCA-4 (6.25 µg/mL and 1.56 µg/mL) a depressed signal was generated in wells with Campath (10µg/mL) compared to those coated with the higher concentration of Campath (20µg/mL). This is likely the result of CD52g being bound in excess of HCA-4 when the wells were saturated with 10µg/mL of Campath. The more limited amount of CD52g bound to Campath at 20µg/mL was optimally detected by the smaller concentration of HCA-4. Therefore, the optimal concentration of Campath (10µg/mL) was confirmed. The HCA-4 standard demonstrated that there was little

variation across the plate. As the concentration of HCA-4 decreased, so did the signal generated upon addition of the conjugate, in a consistent manner across the plate. If great disparities in the measured OD values existed between the replicates then accuracy of the data would have been negated. Thus, well to well variation was low. The background noise OD values obtained in the wells that lacked CD52g (i.e. lacked SP) were nearly as high as those obtained in wells with CD52g. Therefore, it was impossible to distinguish antigen-antibody reactions from non-specific reactions. The fact that the serum seemingly negative for ASA generated a signal with an intensity nearly equal to those of the highest concentration of HCA-4, and the wells measuring positive serum antibody reactions, further challenged the degree to which the ELISA was accurately measuring CD52g antibodies in the presence of other serum components. The lack of a signal in the blank control wells confirmed that the plastic of the ELISA plate and/or buffers (sample diluent or wash buffer) were not contributing to the measured absorbance values. However, the presence of a signal in the wells in which only the conjugate antibody was added suggest that blocking and/or washing was insufficient. The secondary antibody was binding to regions of the plate that were not adequately coated with BSA or could have bound to SP or sera components that were not effectivity removed from the system during the washing process. This assay evidenced the importance of measuring background noise in both the positive and negative serum samples when performing all subsequent ELISAs.

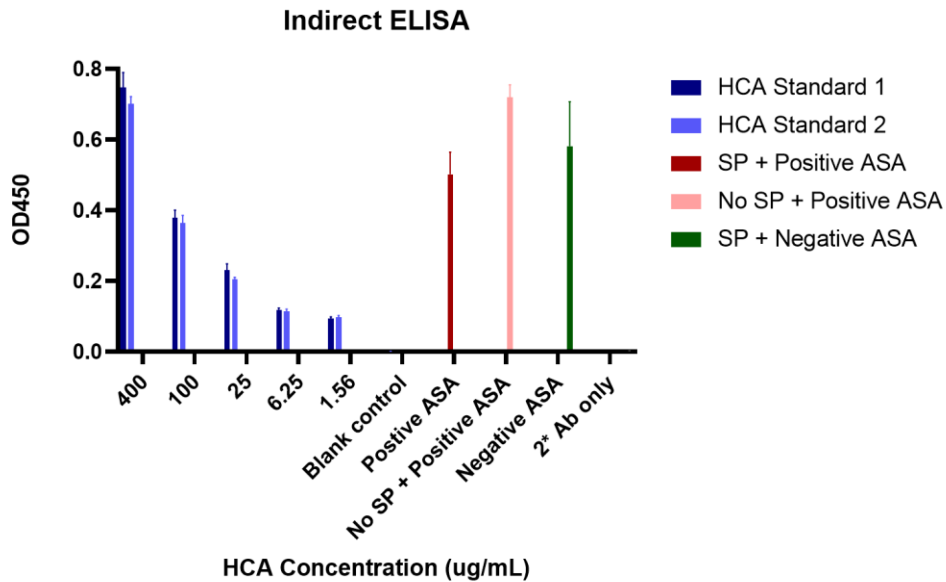


Figure 7. Background noise and plate variability in indirect ELISA detection of CD52g antibodies in serum samples (1:100). Error bars represent standard deviation.

Similar to results of the sandwich ELISA method (Figure 6), the indirect ELISA demonstrated that variability across the plate was negligible, but background noise reactions were not. The replicates of the HCA-4 standards were consistent across the plate. In this assay, the background noise reactions actually exceeded the antibody-antigen reactions measured in both the negative and positive ASA serum samples. Therefore, as in the sandwich ELISA, the false positive results are likely a consequence of non-specific reactions, the incidence of which needed to be reduced, in order for the assay's data to be interpreted correctly. In the indirect ELISA, the most intense signal was generated from the background wells, in contrast to the sandwich ELISA, in which the OD value measured for the positive ASA serum was slightly greater than that of the background wells (Figure 6). The specificity of the sandwich ELISA, the fact that Campath captures CD52g and other seminal plasma components should be washed away,

may have slightly minimized the background noise derived from seminal plasma components. However, interference caused by sera immunoglobulins remained a distinct concern. Since both negative controls failed to generate a signal, it suggested that blocking was sufficient and that plate components and buffers used in the assay were not contributing to absorbance values. Methods to reduce the contribution of serum components to background noise reactions needed to be considered along with determination of background noise reactions in all serum samples both negative and positive. The sandwich ELISA remained the best strategy for detecting CD52g antibodies, as it allowed for more confidence in assessing the assay's ability to detect CD52g specific antibodies as opposed to endogenous sera immunoglobulins binding non-specifically to SP antigens.

3.7 CONTINUED OPTIMZATION OF SERA DILUTION AND DILUENT

Optimization of Sera Dilution and Diluent

A simple method to attempt to reduce non-specific binding of antibodies in sera was to further dilute the sera. In doing this, the concentration of antibodies in sera would be reduced and would thereby limit the capacity of low affinity non-specific interactions to be maintained while favoring specific, higher affinity antigen-antibody interactions. Universal Assay Buffer (UAB), a commercial buffer developed for diluting serum for use in Luminex assays, was compared with sample diluent (PBS with 10% FBS and 0.5% Triton X-100) for its ability to contribute to the reduction of background noise reactions.

Production of Assay Components

Campath (10µg/mL) diluted in PBS was used to coat two microtiter plates. A 1:20 dilution of SP was produced in sample diluent. A ten-fold dilution series (1:100 to 1:1,000,000) of serum samples (50µL/well) diluted in sample diluent or UAB was assayed. A single sample positive for ASA and a single sample negative for ASA were assayed.

Assay

Two sandwich ELISAs were conducted according to the protocol outlined in sections 3.1 and 3.5

Assay Results and Discussion

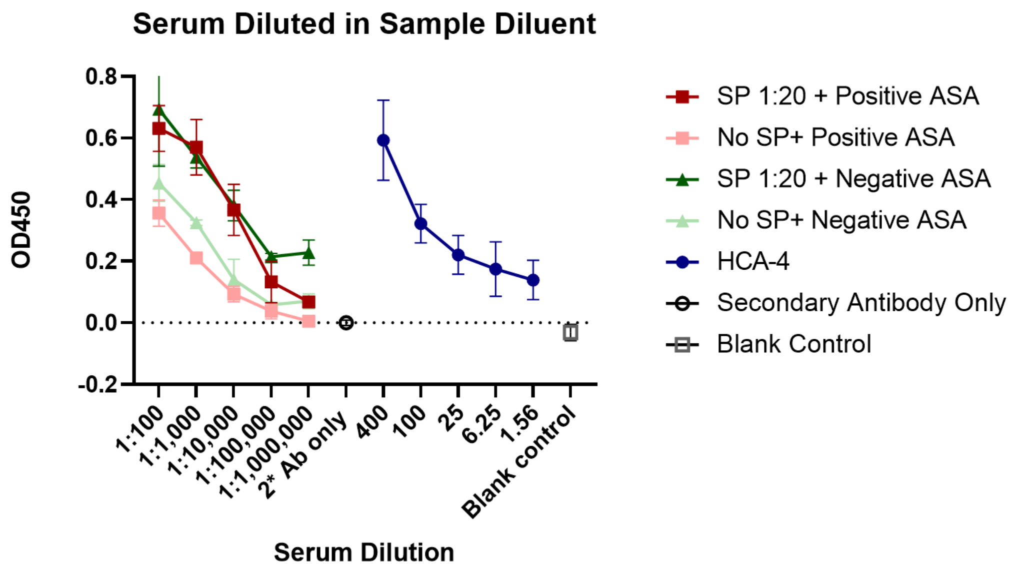


Figure 8. Sandwich ELISA: series dilution of sera in sample diluent (PBS with 10% FBS and 0.5% Triton X-100 and background noise determination in the detection of CD52g antibodies in serum samples. Error bars represent standard deviation.

Analysis of the measured absorbance values via a one-way ANOVA and post-hoc analysis with Tukey's test, determined that there was no significant difference in absorbance between the positive and negative serum samples in the presence of CD52g ($p=0.406$). Moreover, there was no significant difference in the mean OD of positive sera wells with and without CD52g ($p=0.067$). Such findings reinforced the supposition that non-specific antibody reactions and other undesirable phenomena were interfering with the measurement of CD52g specific antibodies. Thus, methods to ensure that the assay can differentiate between background noise reactions and true CD52g antibody reactions were paramount. There was a significant difference in absorbance between the negative serum sample and background noise ($p=0.002$, $p< 0.05$). It may be that further diluting the negative serum sample was more effective at reducing background as compared to the positive sample, due to inherent differences in the amount of endogenous antibody in each serum sample.

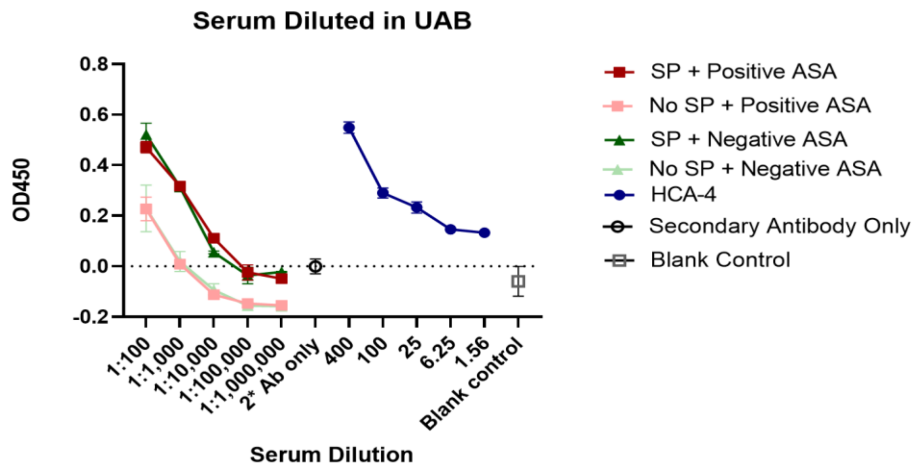


Figure 9. Sandwich ELISA: series dilution of sera in universal assay buffer (UAB) and background noise determination in the detection of CD52g antibodies in serum samples. Error bars represent standard deviation.

In this sandwich ELISA, the positive and negative serum samples were diluted with UAB rather than sample diluent. Although there no significant difference in absorbance between the negative and positive serum samples, there was significant differences in the absorbance between positive and negative serum samples and background noise reactions. The higher OD values generated when the sera were diluted in sample diluent (Figure 8) are likely the result of more non-specific reactions, and not an indication that using sample diluent produced a more sensitive assay. In contrast, with the use of UAB, a 1:10,000 dilution of sera still yielded a signal that that was significantly different from background noise reactions. Antibody-antigen reactions in both the positive and negative serum samples could be discriminated from background noise reactions for the first time. This suggested that the UAB was more effective than the combination of BSA and Triton X-100, in the sample diluent, at reducing non-specific binding of sera derived antibodies. It is possible that the UAB is composed of a more effective detergent, or a detergent at a higher concentration such that weak, non-specific antibody reactions could not withstand its effects. The UAB could contain other antibody binding substances that compete with endogenous human antibodies for binding to CD52g and thereby facilitate the binding of high affinity antibodies to CD52g. At a sera dilution between 1:1,000 and 1:10,000 the assay still produced a signal and background noise reactions were below the limit of detection, suggesting that a dilution in this range could sufficiently eliminated background noise. However, differentiating between samples positive and negative for ASA remained a challenge. It was possible that the serum sample positive for ASA and

the ELISA was accurately assessing the relative degree of CD52g antibodies between two patients.

3.8 SANDWICH ELISA: ASSESSMENT OF BLOCKING AGENTS AND SERA DILUTIONS

Optimization of Sera Dilution

All of the previous ELISAs had been conducted using the same single positive and single negative serum sample. Since there was bound to be great variation in the degree of CD52g antibodies (and interfering substances) in sera from patient to patient, utilizing pooled samples would increase the yield of reactive CD52g antibodies assayed and could potentially help to discriminate between positive and negative serum samples. Highly dilute serum (1:10,000) was thought to be optimal, as higher dilutions would decrease the concentration of IgG in the serum and perhaps, thereby, decrease any non-specific reactions or deposition of sera IgG to the plate. Yet the high dilutions of serum would inevitably reduce the intensity of the signal generated by the assay. Previous experimentation, albeit with one patient's serum sample (Figure 9), did show minimal background noise with an intense signal at a serum dilution of 1:1,000. Therefore, it was worth investigating whether a lower dilution of serum along with another blocking method, would enhance the intensity of the signal generated by the ELISA, while reducing background noise reactions. The only concern was that at a lower dilution it was very likely that the background noise reactions would exceed the real antigen-antibody reactions. This would then result in a misinformed conclusion. Nevertheless, to see if the

intensity of the measured OD values could be enhanced with a lower dilution of serum, without a corresponding increase in background noise, pooled serum samples were diluted either 1:1,000 or 1:10,000.

Optimization of Blocking Agent

Two common blocking agents, BSA, and non-fat dry milk, were assayed to determine if one was more effective at reducing background noise reactions.

Production of Assay Components

Pooled serum samples (50µL/well) were diluted 1:1,000 or 1:10,000 in UAB and assayed. The blocking buffer called for in the ELISA protocol consisted of PBS with 5% Fetal Bovine Serum (FBS). In this assay, the blocking agent previously used (BSA) was compared to that of PBS with 5% non-fat dry milk. All other components were produced according to the protocol in section 3.1.

Assay

A sandwich ELISA was performed as outlined in sections 3.1 and 3.5

Assay Results and Discussion

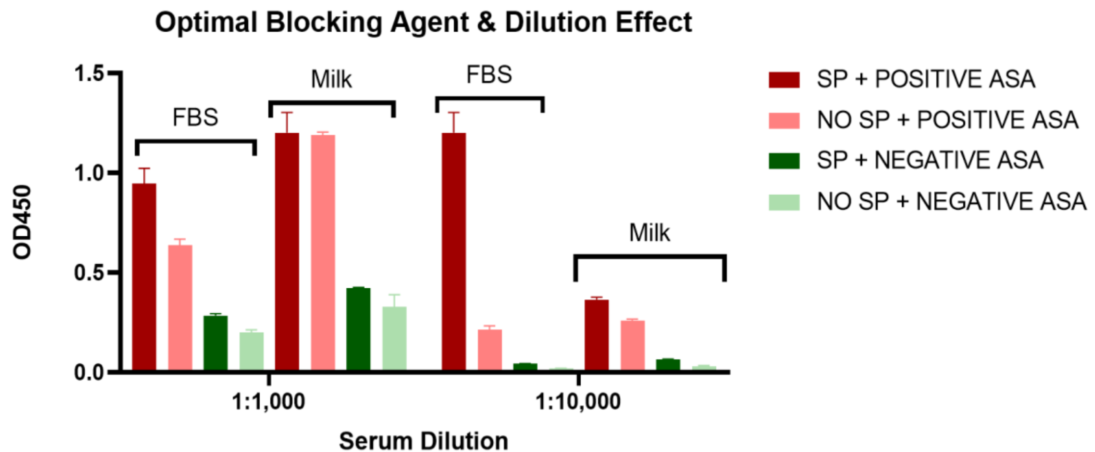


Figure 10. Sandwich ELISA: Comparison of the effects of blocking agents and sera dilutions on background noise reduction. Error bars represent standard deviation.

The combination of blocking agent and serum dilution that generated that highest measured OD value, with the least background noise for the pooled positive serum samples was the FBS/BSA block when the sera was diluted 1:10,000. This same combination of reagents was optimal for assaying the pooled negative serum samples as, it yielded the smallest signal with minimal background. Regardless of dilution, the FBS block was more effective at reducing background noise reactions from the serum samples. In fact, background noise was eliminated from the pooled negative serum diluted at 1:10,000. It is possible that the serum antibodies specific to milk proteins bound and generated an intense background noise signal. However, neither blocking agent was totally effective at eliminating background in the pooled positive sera. Both FBS and milk proteins were equally effective at preventing non-specific reactions of the conjugate antibody in blank wells-wells that contained no sera, as the measured OD

values in such wells were below the limit of detection. Repeating the assay to validate this result would be warranted. This corroborated the previous ELISAs (Figs. 8,9,11) in which there was no non-specific binding of the conjugate antibody to the wells of the plate, suggesting that the plate is optimally blocked, in terms of the ability of the conjugate to empty regions of the plate. However, the background noise is this assay is likely not a consequence of non-specific binding of the secondary antibody to the plate, but that of antibodies in the serum. The conjugate antibody might not be able to bind to the surface of the plate, but it could bind to sera IgG that is potentially bound to the plate. High dilutions of the sera that greatly reduce the total concentration of sera antibodies are probably more responsible for the reduction in background noise than the FBS. In other words, only at the proper dilution of serum will the blocking agent be effective at reducing the non-specific binding of serum to the plate. However, it is probable that even if the plate is optimally blocked, there is bound to still be interference or background noise derived from serum antibodies. With the ponderance of IgG antibodies in serum, it is inevitable that there will be some degree of hydrophobic binding of serum IgG to the plate. It is also possible strong protein-protein interactions between serum antibodies and antigens either derived from the serum or from assay reagents could interfere with the binding of CD52g antibodies to CD52g and give rise to false positive results. This is discussed further in section 3.16.

3.9 SANDWICH ELISA: ESTABLISHMENT OF NEGATIVE CONTROL SERA

Screening Negative Sera for anti-CD52g Antibodies

As there had been no clear, reproducible distinction in the level of CD52g antibodies between populations positive or negative for ASA (Figs 6-9), it was decided that a limited number of negative sera, which showed minimal background noise reactions and relatively low OD values, would act as negative controls in future sandwich ELISA assays.

Production of Assay Components

All eleven serum samples negative for ASA (50µl/well) were diluted in UAB (1:10,000) and assayed via a sandwich ELISA. A pooled serum sample positive for ASA was similarly diluted and assayed. All other components were produced as previously described in section 3.1

Assay

A sandwich ELISA was conducted according to the protocol in section 3.1 and section 3.5

Assay Results and Discussion

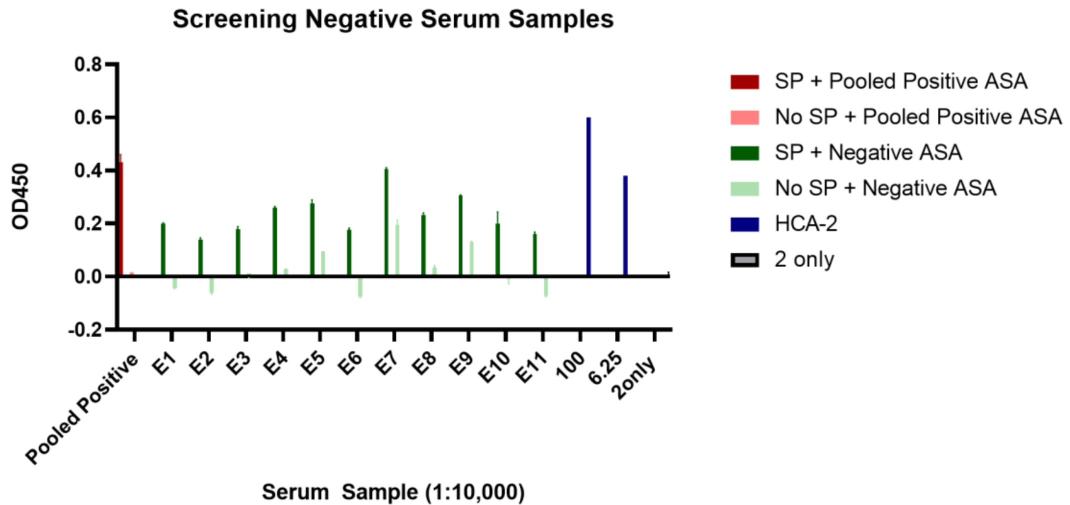


Figure 11. Sandwich ELISA: Screening of CD52g antibodies in serum samples determined to be negative for ASA via an immunobead assay. Error bars represent standard deviation.

In all the negative serum samples, CD52g antibodies were potentially detected to varying degrees. However, there was a distinct difference in the absorbance values between negative and positive sera. Moreover, there were four serum samples in which background noise reactions were below the limit of detection (E1,E2,E6,E11). These samples were pooled and would act as negative controls in the remaining assays.

3.10 SCREENING OF SERUM SAMPLES POSTIVE FOR ANTI-SPERM ANTIBODIES

Assaying Positive ASA Sera For CD52g Antibodies

Eighty-eight serum samples positive for anti-sperm antibodies were diluted 1:10,000 in UAB and assayed (50µL/well) against the pooled negative control serum (50µL/well).

Production of Assay Components

All other components were produced as previously described in section 3.1

Assay

A sandwich ELISA was conducted according to the protocol in section 3.1 and section 3.5

Assay Results and Discussion

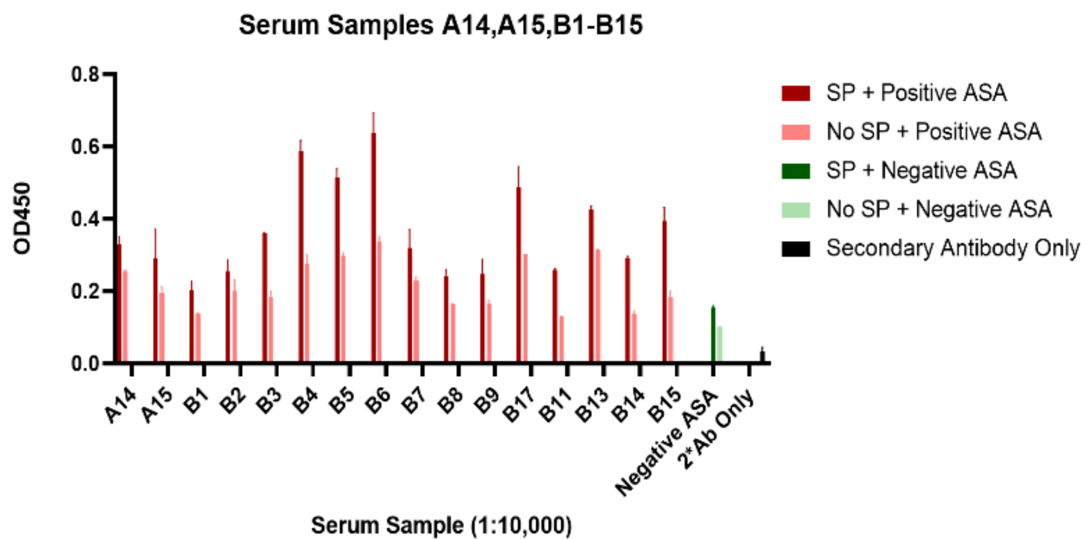


Figure 12. Screening sera for CD52g antibodies. A total of 88 samples were screened. All serum samples were diluted in UAB at 1/10,000 and assayed via a sandwich ELISA. Error bars represent standard deviation.

There was no significant difference in the level of CD52g antibodies in the serum samples positive for ASA as compared to negative controls. Background noise reactions continued to remain as just as high or exceed the measured OD values of the majority of the serum samples assayed and prevented the detection of CD52g antibodies via the

current ELISA. The degree to which diluting the serum in UAB reduced interference varied significantly depending on the serum sample.

3.11 FUTHER OPTIMIZATION OF SAMPLE DILUENT

Optimization of Sample Diluent

As the serum samples assayed still demonstrated significant background noise reactions, likely due to low affinity antibodies in sera, it was thought that perhaps increasing the concentration of detergent in the sample diluent could have an inhibitory effect on non-specific binding of sera immunoglobulins, perhaps to a greater extent than that of the UAB.

Production of Assay Components

Increasing concentrations of Triton X-100 were added to PBS with 10% FBS. Pooled positive and negative sera samples were diluted 1:10,000 in the sample diluent.

Assay

The sandwich ELISA protocol outlined in sections 3.1 and 3.5 was followed.

Assay Results and Discussion

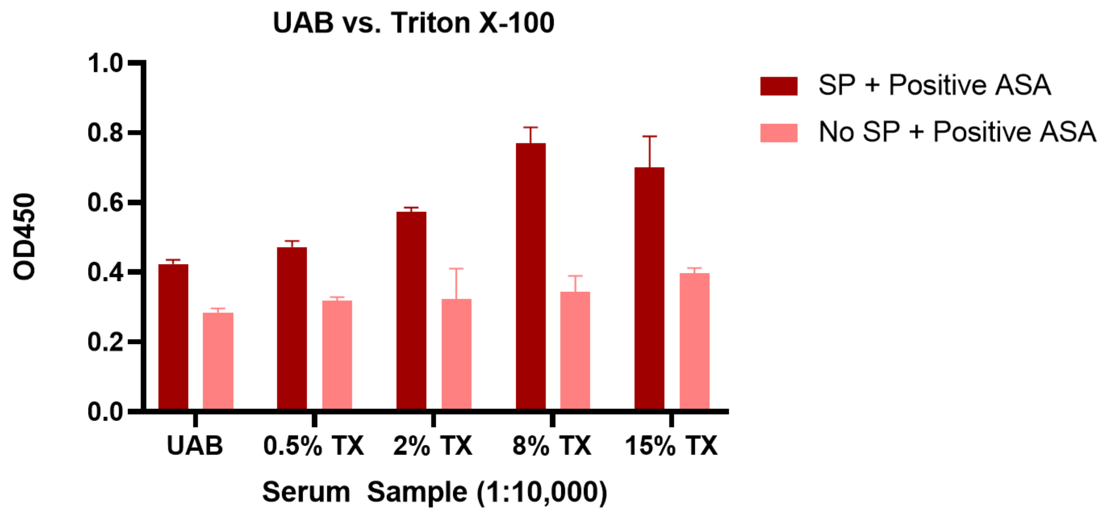


Figure 13. Sandwich ELISA detection of CD52g antibody levels and background noise reactions as a function of the sample diluent. Serum samples were diluted in UAB or sample diluent with increasing concentrations of the detergent Triton X-100 at 1/10,000. Error bars represent standard deviation.

Serum positive for ASA diluent in 8% Triton X-100 and 10% FBS in PBS was just as effective as the UAB in reducing background noise and allowed for an increase in the intensity of the measured OD value. Perhaps, increasing concentration of surfactant inhibited the rather weak interactions between sera immunoglobins and extraneous serum components or components of the ELISA, allowing for more numerous, high affinity interaction between CD52g specific antibodies to be maintained, and thereby generate a more intense signal. Any CD52g antibodies binding non-specifically to serum components, the ELISA plate, or Campath, would not be able to resist the activity of the more concentrated surfactant. Increasing the concentration TritonX-100 beyond 8% was excessive and the activity of the detergent may have competed with the ability of specific

CD52g antibodies to bind to CD52g. This experiment was repeated to confirm the concentration of Triton X-100 that would increase signal intensity, while minimizing background as much as possible. The results are shown below (Figure 14).

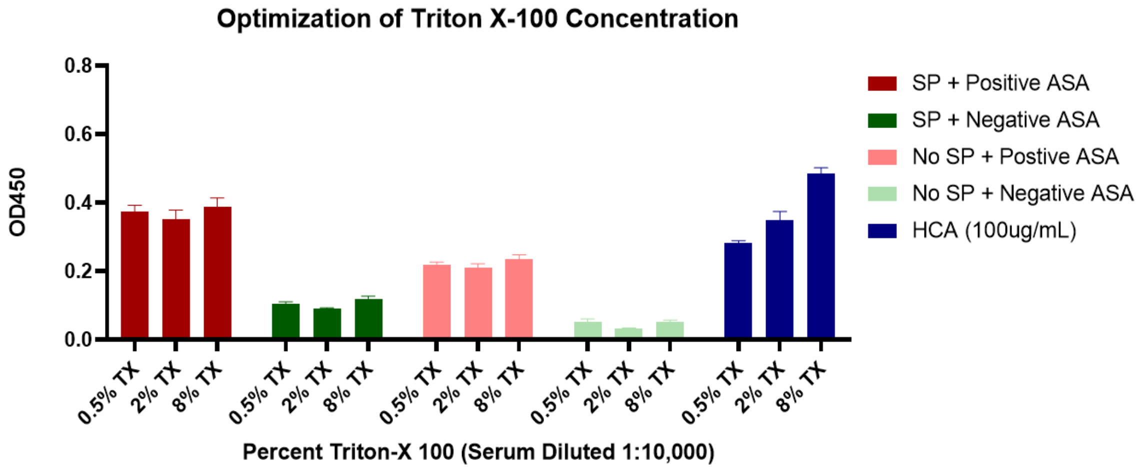


Figure 14. Sandwich ELISA detection of CD52g antibody levels and background noise reactions as a function of the concentration of the detergent Triton X-100 in the sample diluent(PBS with 10% FBS). Serum samples were diluted in sample diluent at 1/10,000. Error bars represent standard deviation.

Although there was no difference in the measured OD of the positive serum as a function of increasing concentration of Triton X-100, background noise reactions were significantly reduced when the positive sera was diluted in 2% Triton X-100 compared to 8% Triton X-100. However, there was no statistically significant difference between the 0.5% Triton X-100 and 2% Triton X-100 sera dilutions, in terms of signal intensity or background noise reduction for the positive serum. Diluting the negative serum in 2% Triton X-100 was no better than the 0.5% Triton X-100 at reducing background, but both concentrations generated a weaker signal than the 8% Triton X-100 solution. In terms of background noise derived from the negative sera, the measured OD values were

significantly reduced with the 2% Triton X-100 solution compared to both the 0.5% Triton X-100 and the 8% Triton X-100 solutions. Therefore, sample diluent with 2% Triton X-100 was used to dilute all subsequent serum.

3.12 SCREENING AGGLUTINATING SERA FOR CD52G ANTIBODIES

Agglutinating Samples for CD52g Antibodies

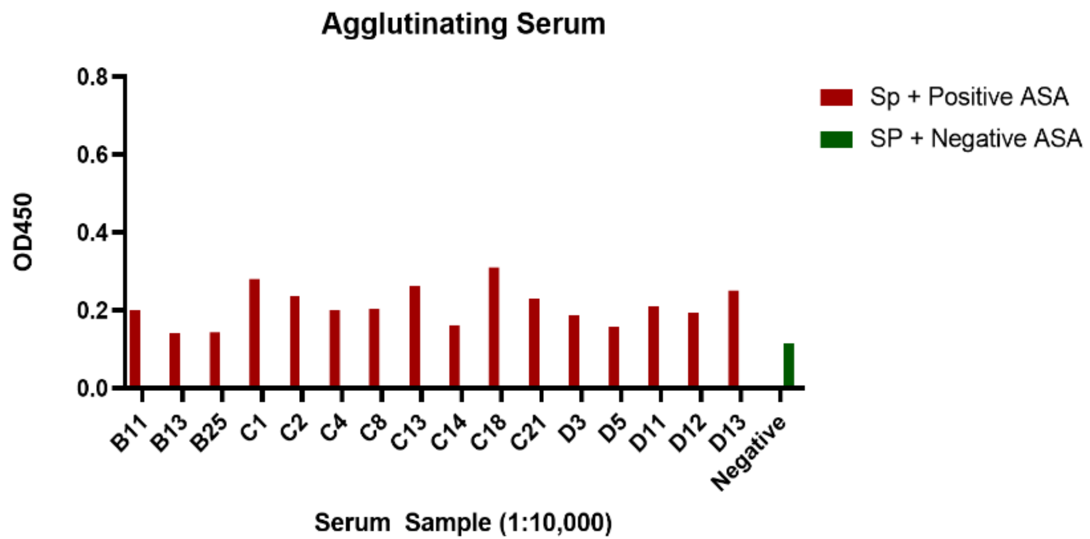
Since anti-CD52g antibodies agglutinate sperm, the ASA sera were assayed for agglutination activity. Sera that were found to agglutinate sperm were retested for CD52g antibodies, as the 2% Triton X-100 was hypothesized to increase the intensity of the measured OD values while minimizing background noise reactions.

Production of Assay Components

Twenty-four serum samples that were found to agglutinate sperm, as well as negative controls, were diluted in PBS with 10% FBS and 2% Triton X-100. All other components were produced according to section 3.1

Assay

The sandwich ELISA protocol was utilized as described in sections 3.1 and 3.5.



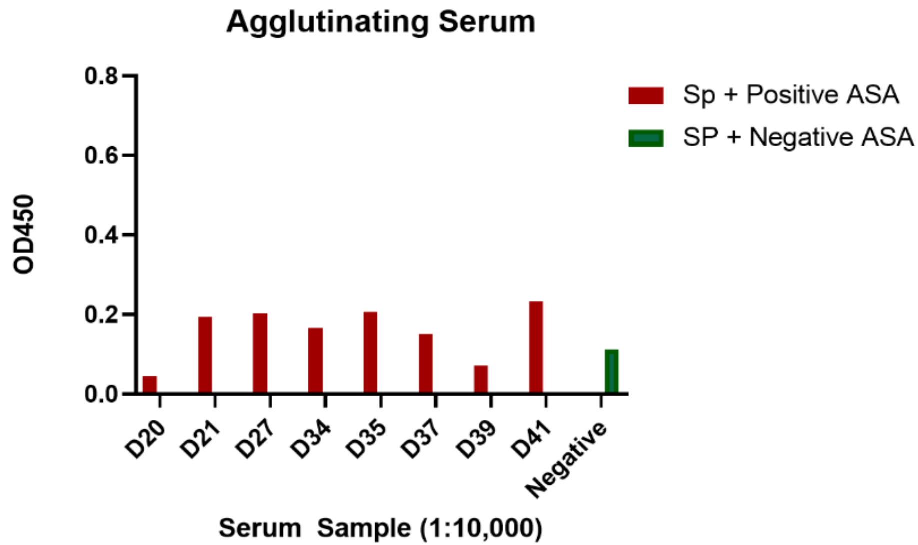


Figure 15. Sandwich ELISA detection of CD52g antibody levels in sera found to agglutinate sperm. Graphed is the level of CD52g antibody upon subtracting the background OD values in antigen-uncoated wells from the OD values in antigen-coated wells. Serum samples were diluted in sample diluent (PBS with 10% FBS and 2% TritonX-100) at 1/10,000. Error bars represent standard deviation.

Twenty-four serum samples that were positive for ASA and found to agglutinate sperm were retested after being diluted in 2% Triton X-100. Increasing the concentration of detergent in the sample diluent, decreased background depending on the serum assayed. An ANOVA revealed that of the 24 samples, 5 samples had measured OD values significantly different than that of the negative control ($p > 0.05$). CD52g antibodies were detected in the sera (21%) of infertility patients whose serum was found to agglutinate sperm.

CHAPTER 4. DISCUSSION

The current study suggests that CD52g antibodies are not readily detectable by ELISA in serum from patients with infertility. After extensive assay optimization, convincing anti-CD52g activity was detected in only 21% of patients with sperm agglutinating antibodies. This suggests that antisperm antibodies, in a majority of cases were not directed at CD52g sperm antigen. Although the data from this study are preliminary and need to be repeated, they support previous research which has demonstrated that CD52g antibodies agglutinate sperm and may thereby contribute to the pathogenesis of immune infertility.

A principle concern was natural autoantibodies (natural antibodies) and anti-idiotypic antibodies in sera. Previous research has suggested that at least some ASA are natural autoantibodies-polyreactive antibodies against diverse, poorly defined antigens which have a relatively weak affinity for any particular antigen. Other research has emphasized the role that anti-idiotypic antibodies may play in binding to sperm antigens

and thereby mediating immune infertility. A challenge in trying to measure the concentration of CD52g antibodies, some of which may be natural autoantibodies or anti-idiotypic antibodies, is that by their very nature, these antibodies react to a variety of antigens, not limited to sperm antigens, that are present in the sera of both diseased and control populations. Control populations negative for ASA via an immunobead test may be positive for CD52g antibodies in sera as measured by ELISA. Immunobead testing and sera ELISAs are detecting different types of ASAs. ASAs present in serum are directed against a variety of sperm or seminal plasma antigens, and using ELISA the specificity of such ASA can be deduced. ASA detected by immunobead assays are direct against antigens found on the surface of sperm. As CD52g is both soluble (in epididymosomes) and membrane bound, it is reasonable that sera negative for membrane bound sperm antibodies could still be positive for C52g antibodies as a consequence of a natural or idiotypic antibody response. Natural autoantibodies to CD52g may bind to immobilized CD52g in ELISA but such binding may be relatively weak. Such weakly reacting sera may have been negative in the immunobead testing because the binding was not sufficient to bind live motile sperm and therefore was not subsequently detected by beads coated with anti-human immunoglobulins. Thus, the positive signal from negative control sera, may not necessarily be a consequence of antibody inference or a defect in the assay.

As discussed by previous researchers, it is unlikely that antibodies against one sperm antigen are responsible for all sperm agglutination or immobilization activity. Indeed, researchers have emphasized that antibody localization, concentration, and

isotype as well as the intrinsic functions of the target antigens altogether determine the degree to which ASA impair sperm functioning. As such, the ELISA developed in this study does not indicate the functionality of the antibodies that are being measured. CD52g antibodies may be one of several ASAs, each of which has its own unique biological function and which acts in affiliation with each other, and complement proteins, to impair sperm motility in vivo.

Complicating matters is that natural autoantibodies and idiotypic antibodies to antigens other than CD52g could have interfered with the ability of the assay to accurately measure CD52g antibodies in sera. These antibodies could react with a variety of antigens either derived from the sera itself, or from assay reagents, and generate false positive reactions in both ASA positive and negative populations. Natural autoantibodies and idiotypic antibodies could both potentially bind to the capture antibody, Campath. Anti-idiotypic antibodies may actually mimic the binding of CD52g to Campath due to its mirror-image structure. By linking the capture antibody and the conjugate antibody, these antibodies would falsely elevate the level of CD52g antibodies detected in sera. Thus, by competing with CD52g specific antibodies, natural autoantibodies and idiotypic antibodies are likely one type of background noise reaction that confounded the results of the assay and, if not measured, could have precipitated the misinterpretation of those results. It is also possible that natural autoantibodies and idiotypic antibodies may interfere with the ELISA in a non-competitive way, such as absorbing to the plate. Approximately 80% of serum is composed of IgG, with normal adult serum levels of IgG in the range of 7-16 g/L [103]. However, the concentration of CD52g specific IgG is

serum is considerably lower, in the order of μg/mL. It is, perhaps, unavoidable that a degree of sera derived IgG will absorb to the plate and potentially bind the conjugate antibody or its enzyme (HRP). Antibodies in serum against complement components or circulating immune complexes could also potentially remain in the ELISA system if activated complement managed to absorb to the plate. Methods to mitigate interference caused by natural autoantibodies and idiotypic antibodies included diluting the serum and increasing the concentration of detergent the sera was diluted in. The success of these two strategies depended largely on the characteristics of the individual serum sample. Even after diluting 1:10,000 and/or diluting sera samples in 2% Triton X-100, intense background noise reactions remained just as high as measured antigen-antibody reactions, in the overwhelming majority of sera samples. Blocking the plate with BSA or milk proteins was also considered to reduce the absorption of serum antibodies and immune complexes to the plate. However, natural autoantibodies, or antibodies specific to BSA or milk proteins, present in the sera could have reacted with their target antigen and been a source of considerable background noise. Indeed, in wells devoid of CD52g, the addition of FBS and sera did yield a signal regardless of the sera dilution (Figure 10). A blocking agent devoid of such animal derivates may be required in order to more effectively reduce non-specific binding of sera components to the ELISA plate. Indeed, human anti-animal antibodies are reported to occur in 30-40% of patients [104]. These high affinity antibodies, raised against a specific animal immunoglobulin class, are most commonly anti-mouse antibodies but can also include rats, rabbits, and cattle [104]. As assay reagents were produced in, or derived, from these animals, anti-animal antibody in

sera could have potentially reacted not only with BSA in the sample diluent and blocking buffer but potentially with the capture antibody or the conjugate antibody. ASA are also known to cross-react with a variety of bacterial antigens including *E.Coli*, *S.typhi*, *H.pylori*, *C.trachomatis*, and *U.urealyticum*. If these bacterial antigens were present in sera, immune complexes could form, deposit on the plate, and be detected by the conjugate antibody, resulting in an over-estimation of the concentration of CD52g antibodies. On the other hand, bacterial antigens could compete with CD52g for antibody, and thereby result in the underestimation of the concentration of CD52g antibodies.

A challenge in developing this assay was that there was a need to reduce the concentration of natural autoantibodies and idiotypic antibodies that inferenced with the assay-that bound non-specificity to ELISA reagents or components. Yet some of these natural autoantibodies and idiotypic antibodies may have actually bound CD52g and deciphering the exact target of natural autoantibodies and idiotypic antibodies in the ELISA was not possible. Therefore, in trying to reduce the extent of natural autoantibodies and idiotypic antibodies in sera the goal was that high affinity CD52g specific antibodies would be detected. However the concentration of natural autoantibodies and idiotypic antibodies is unique to each individual patient. In one individual, the concentration of these antibodies could be low, but have a relatively high affinity for an array of epitopes. Meanwhile, in another patient, the concentration of natural autoantibodies and idiotypic antibodies could be high but bear a relatively low affinity for target antigens [104]. This variability complicates determining the

concentration of blocking agent or detergent that should constitute the sample diluent or blocking buffer, in order to effectively reduce non-specific antibody binding in all serum samples. This fact, coupled with ineffective blocking, may explain why the strategies employed in this study were insufficient to overcome background noise reactions in the majority of the sera assayed. Future experimentation could involve assaying serum samples with a high degree of background and control serum on BSA or skimmed milk coated plates for IgG deposition. Efforts could also be undertaken to compare the ability of detergents like Triton X-100, purified BSA, or milk protein as buffer additives to reduce background noise reactions.

In light of these confounding factors, to assay CD52g antibodies in human sera it was necessary to include wells not coated with Campath (i.e. containing no antigen) to determine the background noise reactions of each sample. Although there was no significant difference in anti CD52g levels in patients positive for ASA as opposed to controls, 21% of sera that agglutinated sperm was positive for CD52g antibodies in the sandwich ELISAs. In order to further prove the CD52g specificity of the antibodies, competition assays need to be performed using purified CD52g. Only a subset of the ASA positive sera from infertility patients demonstrated the capacity to agglutinate sperm suggesting that the presence of agglutinating antibodies is suggestive of, but not a definitive marker of immune infertility. Moreover, serum CD52g antibody levels may not accurately reflect the immunological situation in genital tract secretions. If the antisperm immune response originated in the genital tract mucosa, it is possible that antibody titers would be higher in genital tract secretions than in sera. Sera derived ASA may be largely

natural auto-antibodies and/or anti-idiotypic antibodies and thus have a variable affinity for CD52g. These antibodies may react to sperm antigens like CD52g but not necessarily mediate infertility. Direct evaluation of the relative abundance and titer of CD52g antibodies in the secretory fluid of reproductive tissues in men and women would more accurately reveal the role in which CD52g antibodies play in agglutinating sperm in vivo. Yet, given that a fraction of agglutinating sera was positive for CD52g, it does support the hypothesis that the effectiveness of sperm movement is influenced by antibodies bound sperm surface antigens like CD52g.

Even though the ability of the ELISA in this study to detect CD52g antibodies was limited, the importance of ASAs in mediating immune infertility should not be underestimated. Among the varied conditions that might underly the pathophysiology of unexplained infertility, alterations in sperm motility due to immunological reactions are significant. Treatment of ASA mediated infertility relies on ARTs, of which intracytoplasmic sperm injection (ICSI) has been the most successful [51]. Progress in understanding and treating infertility that arises as a consequence of auto or iso-immunity has been feasible, largely through studying individual candidate antigens like CD52g. Such research has enabled the discovery of new mechanisms and paradigms of immune modulation that are indispensable to the proper functioning and health of the human reproductive tract.

Moreover, highly specific ASAs, raised against antigens that are involved in sperm functioning, may be effective immunocontraceptive agents. Development of a contraceptive device (a vaginal film or intravaginal ring) that delivers HCA, as well as

monoclonal antibodies to HIV and HSV, into the cervicovaginal mucosa is currently in progress by researchers in the Anderson Lab. This novel contraceptive relies on passive immunization with cost-sensitive, humanized monoclonal antibodies, that are engineered in the *Nicotiana* plant and its development is an exciting proposition. Major global issues, such as migration, climate change, population growth and ageing, gender equality, child care, and medical ethics, are indelibly linked to reproduction, specifically, when and how reproduction should happen. In this milieu, an effective dual purpose immunocontraceptive, that both prevents unintended pregnancy and STI transmission, would be invaluable. Such a novel technology has the potential to become a highly effective contraceptive method and, simultaneously, could improve the acceptability of, and adherence to, STI prevention and control efforts, and furthermore, to reduce the stigma surrounding such endeavors. These benefits elucidate that understanding the basic biology of both fertilization and individual sexually transmitted pathogens, in the broader context of the immune environment in which they exist, is paramount to developing preventative healthcare measures that would improve the reproductive health and emotional well-being of women worldwide.

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