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Development and Characterization of Monoclonal Antisperm Antibodies: Potential for Contraception

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by

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ABSTRACT

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May, 1991

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There is an expressed need to develop a greater variety of safe contraceptive methods which would find acceptance worldwide, particularly in developing countries. Immunization against spermatozoa might be such a method as judged by the accumulated evidence from studies on human females and in a number of female animal species.

Two extraction techniques were used for sperm membrane antigen isolation. The first technique involved NP-40 detergent for antigen extraction from human motile sperm and the second technique employed homogenized human testis for antigen extraction. Using these immunogens, sperm membrane-specific monoclonal antibodies (MAbs) were developed. When these antisperm MAbs were subjected to evaluation against an extensive panel of human tissues, no cross-reactivity to somatic tissues was observed, but staining was seen on sperm cells in testis, caput- and cauda epididymis, and vas deferens. Four of these antisperm MAbs were against epididymal antigens, three were against seminal vesicle factors, and seven were against testis specific antigens. These

evaluations indicated sperm specificity of these antibodies, fulfilling one of the important criteria for contraceptive vaccine development set forth in a World Health Organization (WHO) monoclonal antibody workshop report. The results of immunofluorescence (IF) and immunochemical staining (ICS) studies on methanol-fixed sperm indicated that these antibodies recognized antigens on the plasma membrane overlaying different regions of the sperm, i.e., acrosome, post-acrosome, equatorial, midpiece, and tail. When these antisperm MAbs were tested on fresh, capacitated, and acrosome reacted spermatozoa, a differential reactivity with fresh sperm as compared to acrosome reacted sperm was observed. The wide species cross-reactivity of these antisperm antibodies indicated shared antigens in these species, raising the possibility of employing experimental animal models to test contraceptive vaccines.

These antisperm MAbs demonstrated the multiplicity of antigens having a role in the process of fertilization. Eight of these antisperm MAbs were able to inhibit at least one sperm functional test in vitro, which satisfied another criterion for immunocontraceptive vaccine development. Since one MAb (DH22) was capable of binding to the acrosomal cap region of acrosome reacted sperm, this MAb could be used as a marker to identify acrosomal reacted sperm among the different matured stages found in a sperm population. Purification and biochemical characterization of these sperm antigens to which these antisperm MAbs were directed, would

be of interest to better understand their immunocontraceptive potential.

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I DEDICATE THIS WORK TO MY PARENTS

Ali Akbar Khan Lodi
and
Altafun Nahar Khan Lodi

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CHAPTER 1

INTRODUCTION

The world population is increasing at an alarming rate. As we enter the 1990s, excessive population growth continues worldwide. The world population will increase to about ten billion in the 21st century and ninety-five percent of this global population growth is concentrated in developing countries (U.N., 1984). This continued growth means a severe scarcity of the available meager resources with a subsequent lowering of the standard and quality of living in these already poor countries. In light of the above, a continued search for safe, acceptable and reliable means of fertility regulation must be maintained and accelerated.

The following is a list of some contraceptive methods currently in use for fertility regulation:

Steroidal contraception (oral or long acting)
Intrauterine devices (IUD's)
Barrier methods (condoms, diaphragms etc.)
Spermicidal products
Vasectomy and female sterilization

To a large extent, the methods of birth control available at this time still do not satisfy all of the varied cultural and service demands of different populations. There is an expressed need to develop a greater variety of

methods which would find wide acceptance, particularly in developing countries.

At present, vaccines are the greatest practical benefit that immunology has given humanity. After all these years, what is still essentially Jenner's vaccine against smallpox appears to have eliminated the disease worldwide. To date, most vaccines for human and animal health have been composed of whole organisms, viruses, or bacteria capable of inducing high levels of immunity over long periods of time. A birth control vaccine eliciting a specific, safe, low-cost, and effective antifertility immune response lasting for 1-2 years would have a major impact in family planning programs.

The basis for a vaccine lies in the induction of a specific immune response to gamete or pre-embryo antigens. In order to be effective, an antifertility vaccine requires that the antigen be unique to the reproductive tract and that the antigen have a fertility-related function. Thus, the antigen must be absent from other tissues, and it must have an action that can be blocked by an antibody or be located on a cell that can be lysed by complement. Efficacy and reversibility are two important criteria for an immuno-contraceptive vaccine. To be used for fertility regulation, a vaccine antigen must be expressed on the sperm surface (either an integral membrane protein or epididymal or seminal plasma-derived coating protein) and be accessible to immune attack. It must be immunogenic so that either alone

or in concert with conjugated proteins or adjuvant, a sustained and effective immunologic contraceptive response can be elicited. Furthermore, the antigen must be sperm specific so that no tissue damage occurs from cross-reaction (Anderson and Alexander 1983).

The possibility of inducing antibodies against reproductive tract antigens by deliberate immunization was suggested as early as 1899 by the pioneering work of Landsteiner (1899) and Metchinikoff (1899), and immunocontraception research has in recent years taken on new importance and such a great increase in activity. Experimental immunocontraception approaches are directed at inactivation of either one or more hormones, gametes, or preembryo antigens.

The development of an effective contraceptive vaccine appears attractive in the male. Currently three possible immunogens are being considered. Two (gonadotropin-releasing hormone and follicle stimulating hormone) are polypeptide hormones and the third (lactic dehydrogenase-C4) is a sperm-specific protein. Although sperm-specific antigens have been shown to be effective immunogens (Goldberg et al. 1986), their contraceptive efficacy has only been demonstrated by immunizing females, and, as such, they do not qualify as a vaccine for the male.

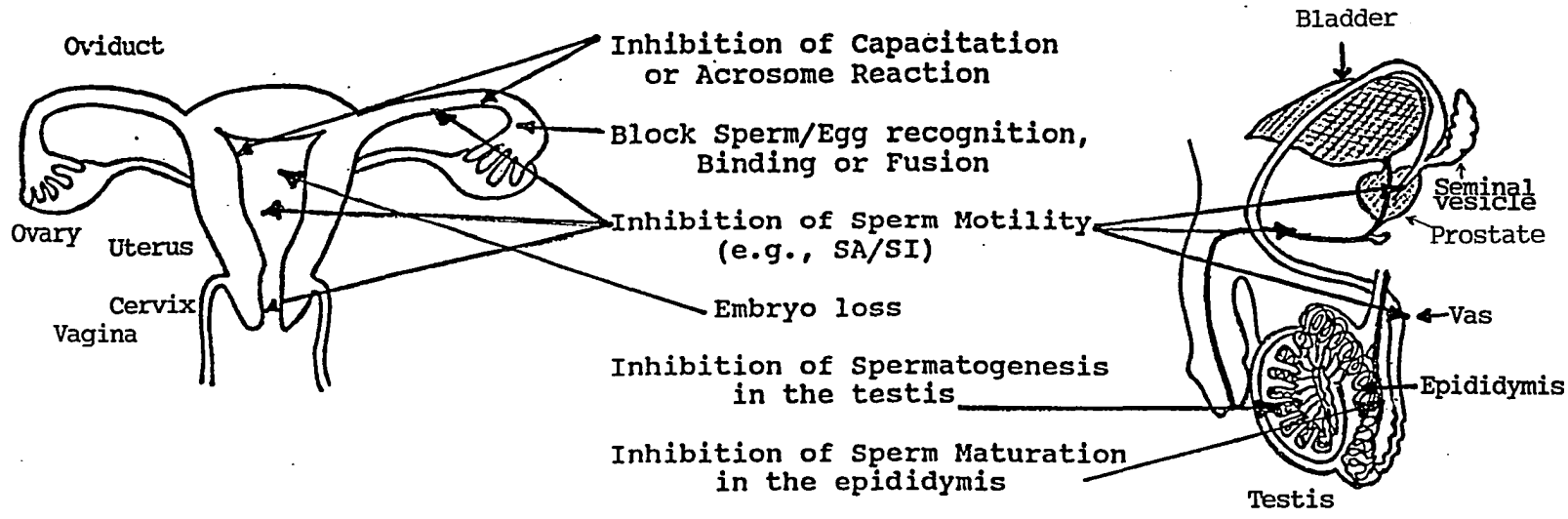
A number of other immunogenic sperm specific antigens [acrosin, hyaluronidase, and rabbit sperm autoantigens

(RSA-1)] have been identified and characterized. Such an approach would involve immunization of females. The feasibility of this approach is based on two lines of evidence. The first line of evidence comes from numerous studies of human involuntary infertility which demonstrated that anti-sperm antibody-mediated immunoinfertility reduces fertility in men and women (Menge, 1980). The second line of evidence comes from immunization studies of male and female animals of various species with sperm specific antigens. Immunization with extracts of sperm or mature testis has been shown to result in a significant reduction of fertility, embryo failure, or pre-implantation embryo mortality (Edwards, 1964; Menge, 1970; Kiddy and Rollings, 1973).

Immunological Aspects of Contraception By Immunization

Infertility can be induced in experimental animals when they have been immunized against spermatozoa, seminal plasma, testicular, oocyte, and trophoblast antigens. Theoretically, immunological factors would operate at any stage in the reproductive process (Fig. 1) since the gametes and the fertilized eggs, as well the hormones, tissues and other secretions in their environment, are all potentially antigenic and capable of eliciting an immune response. Fertility can be impaired in mammals by employing active or passive immunization against testis antigens, spermatozoa or seminal plasma in females (Mancini 1971; Tung 1980). The resulting

FIG. 1 Some Immunological Antifertility Effector Mechanisms in the Male and Female Reproductive Systems



Female Reproductive System

Male Reproductive System

antibodies seem to prevent spermatozoa from achieving fertilization. Similar studies have also been carried out on males in attempts to destroy the seminiferous epithelium or to suppress the fertilizing ability of the spermatozoa in the male reproductive tract (for review see Shulman, 1971).

Spermatozoa, although immunologically foreign to women, usually do not stimulate humoral immune responses with coitus. Deliberate immunization of females with sperm can impair their fertility due to a combination of antisperm antibody effects. Intrinsic sperm antigens are produced during germ cell maturation in the testis (O'Rand et al., 1981) or epididymis. Sperm coating antigens are added during sperm passage through the epididymis (Blaquier et al., 1986) and upon exposure to seminal plasma at ejaculation. Antibodies to these intrinsic antigens and coating antigens are associated with unexplained infertility (Isojima et al., 1968). Clinical syndromes have been identified where male and female infertility almost certainly has arisen due to antibodies against spermatozoa (Ansbacher, 1973; Alexander, 1983). Serum and cervical mucus from the female partner of infertile couples with poor postcoital test reports were tested (Dondero et al., 1978) against sperm from the normal male partner as well as sperm from a donor to see whether the infertility arises due to antibodies against spermatozoa. Also vasovasostomized men frequently have had antisperm antibodies in their seminal

plasma. Antisperm antibodies identified on the male partner's sperm using both the immunobead assay and the radiolabeled antiglobulin assay have been associated with an inhibition of cervical mucus penetration (Fuchs and Alexander, 1983).

Various cellular responses and immunoglobulin classes or subclasses could be involved in the reactions leading to infertility. Investigations of infertile women have revealed the presence of plasma IgG and IgM reactive with spermatozoa (Bronson, 1990). Antisperm antibodies of the IgM, IgG, and IgA classes may agglutinate sperm so that these sperm are unavailable to complete any further reproductive functions (Franklin and Dukes, 1964). For example, this agglutination may prevent normal progress through the cervical mucus during transit in the female reproductive tract.

Immunity to sperm can compromise fertility by: (1) a reduction in the number or quality of maturing sperm cells (Mancini et al., 1965); (2) interference with sperm motility (Mathur et al., 1984, 1986); (3) capacitation or the acrosome reaction (Saling, 1986); (4) decreased penetration of cervical mucus (Fjallbrant, 1969; Kremer and Jager, 1976); (5) enhanced phagocytic clearing of sperm from the female genital tract (Muscato et al., 1982); (6) sperm cytotoxicity given adequate levels of complement (Mathur, 1988); (7) inhibition of sperm enzyme (Dunbar, 1976);

(8) impairment of sperm-egg interaction (Saling et al., 1985); (9) embryonic loss (Menge et al., 1982; Jones, 1976).

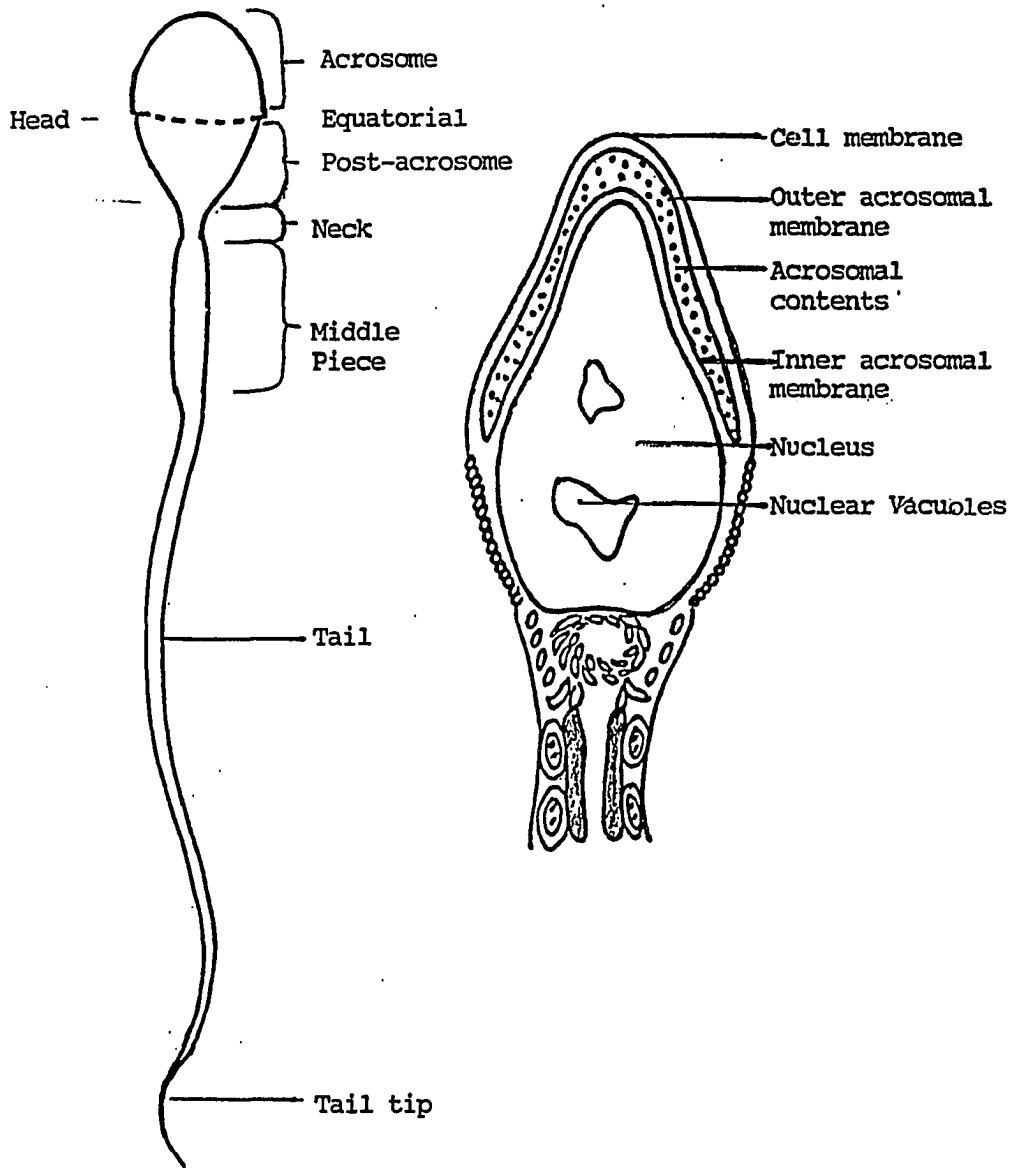
Since many of the vital events of sperm/egg interaction are associated with the sperm membrane, it is likely that antibodies to the membrane antigens may make ideal candidates for immunocontraceptives.

Sperm Antigens

The sperm plasma membrane is a mosaic of antigenic domains. Many of them are only produced in male reproductive tract. Antibodies produced to these antigens may be responsible for reducing fertility in immunized animals. Normal fertile ejaculates contain spermatozoa and a fluid called seminal plasma. Upon ejaculation, the sperm surface is coated with the seminal plasma components, some of which are so tightly bound that they cannot be readily eluted by simple washing with physiological saline or organic solvents (Scacciati and Mancini, 1975).

Human spermatozoa can be divided into three parts: head, neck, and tail. The sperm head is composed of the nucleus and the surrounding membrane structures (Fig. 2). The nucleus contains a haploid number of chromosomes and nucleoproteins, and it often has small, irregular shaped of clear areas referred to as nuclear vacuoles. The membrane structure of the sperm head consists of four parts: (1) the plasma membrane that covers the entire surface of the sperm

FIG. 2 Schematic Diagram of Human Spermatozoa
a. Normal spermatozoa showing 7 different regions
b. Cross section of human sperm head



head; (2) the acrosome, a bag-like structure that surrounds the anterior portion of the nucleus; (3) the post nuclear cap, a cytoplasmic sheath that covers the posterior part of the nucleus; and (4) the equatorial segment.

Antigens of human sperm have been mapped and many researchers report that naturally occurring human antisperm antibodies bind to antigens located on the acrosome, post acrosome, equatorial region, midpiece, and tail. Three types of semen specific antigens have been defined: (1) antigens in seminal plasma; (2) sperm coating antigens (adsorbed epididymal and seminal plasma components on the surface of spermatozoa); and (3) genuine or intrinsic spermatozoal antigens. The various groups of intrinsic spermatozoal auto- and iso-antigens are listed (Hjrot et al., 1982) in Appendix, Table A.

The most potent sites for interfering with the fertilization capacity of spermatozoa are likely to be on the outer and inner acrosomal membrane. Attachment of antibodies to such sites would be expected to interfere with several crucial aspects of sperm function which include the vesiculation of the plasma membrane and subsequent release of soluble enzymes, as well as the binding of spermatozoa to receptor sites on the zona pellucida and oolemma.

Specific cell-cell contacts that occur during mammalian fertilization are probably initiated through non-covalent protein-protein and protein-carbohydrate interactions

between surface membrane components (Fig. 3). Any comprehensive understanding of fertilization therefore requires a definition of these surface components and their chemical nature, localization on the cell surface and expression during gamete maturation and fertilization.

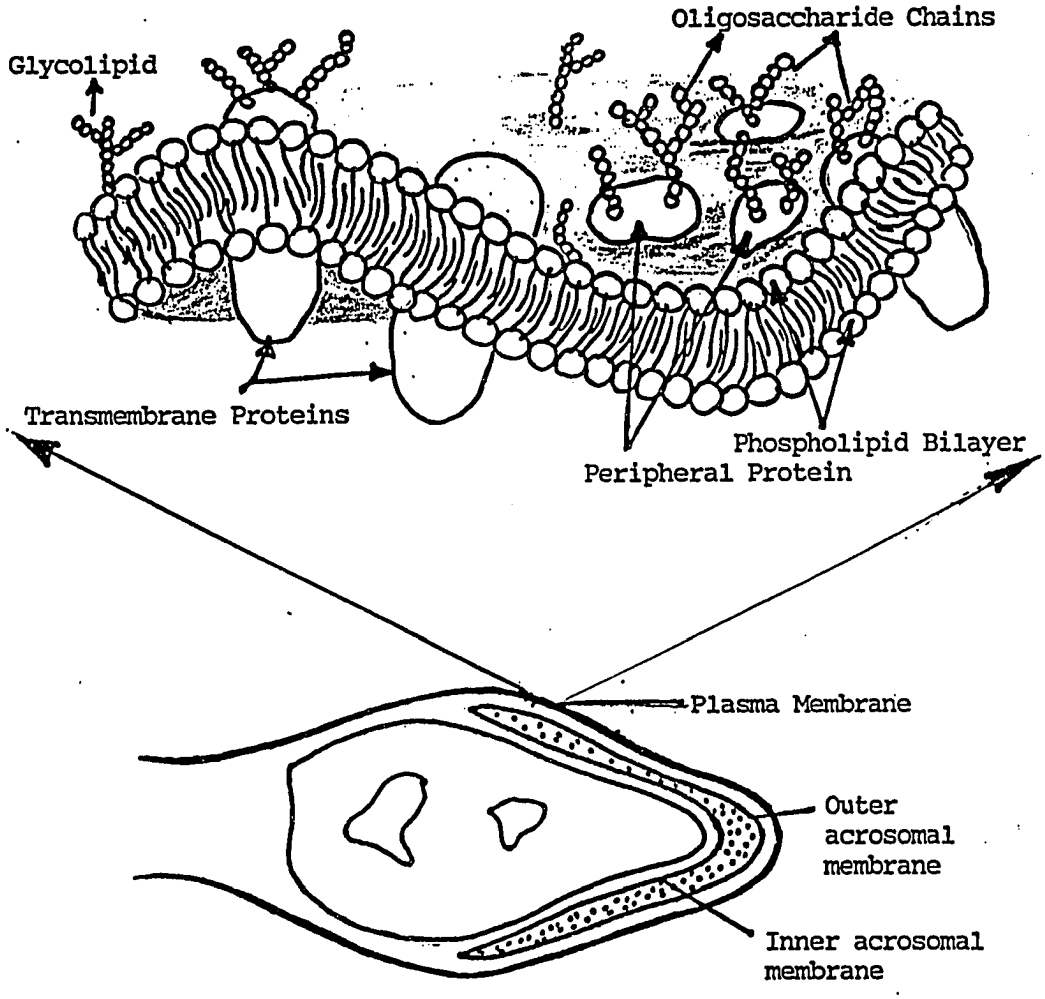
The Development And Changes Of Sperm Membrane Antigens During Spermatogenesis And Epididymal Maturation

Human spermatogenesis occurs in the seminiferous tubules at an average age of 13 years as the result of stimulation by anterior pituitary gonadotropic hormones. Figure 4 illustrates the various portions of the male reproductive system associated with spermatogenesis and sperm maturation. During spermatogenesis, dynamic cellular interactions take place. These are governed partly by initial recognition events involving plasma membranes of the cell within the seminiferous tubules. The expression of many sperm surface macromolecules occurs at a precise time in spermatogenesis.

1. Testicular Spermatozoa

In all mammals, including man, testicular spermatozoa are not capable of forward movement and are infertile. They are, however, similar in biochemical composition and structure to spermatozoa in other regions of the reproductive tract. Compared to ejaculated spermatozoa, testicular

FIG. 3 Schematic Diagram of Sperm Plasma Membrane Components

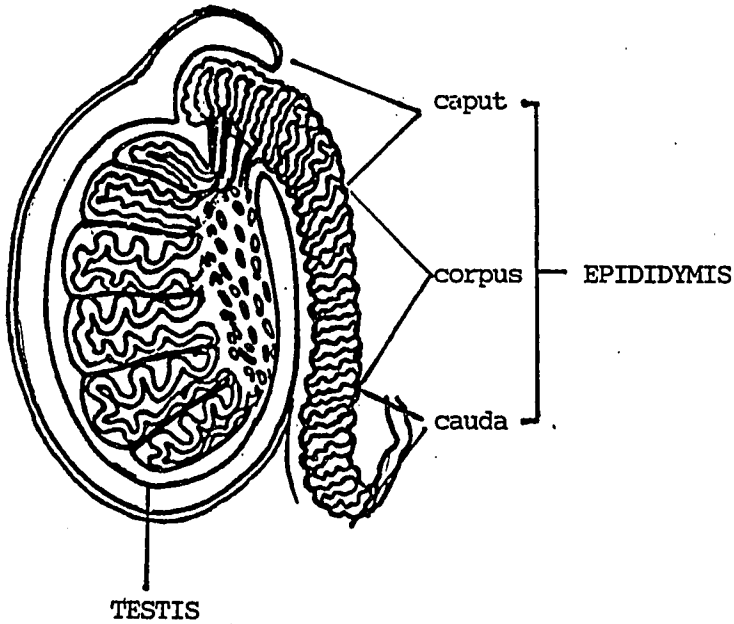


SPERM HEAD

FIG. 4 Schematic Diagram of Portions of the Male Reproductive System Associated with

Spermatogenesis (TESTIS) and

Sperm Maturation (EPIDIDYMIS)



spermatozoa contain more proteins, sodium, calcium, and have a high rate of glucose oxidation and lactic acid production, high levels of neutral lipids and phospholipids and low levels of fatty acids and ATPase. Differences in the amount and composition of lipids in the plasma membrane of testicular spermatozoa may account for their higher charge density at the phospholipid-water interface of these spermatozoa, as well as for their higher membrane fluidity compared with that of epididymal and ejaculated spermatozoa.

Studies in animals reveal that testis-specific autoantigens appear on the late pachytene spermatocytes and persist through spermatogenesis (Millette et al., 1977). However, some antigens, which appear first on B spermatogonia, are found at all stages of spermatogenesis, on spermatids, and even on residual bodies, but not on mature sperm (Romrell and O'Rand, 1978). Additional antigens appear on the plasma membrane after the midspermatid stage of spermatogenesis (Romwell et al., 1978). Phelps and Myles 1987 have reported that three monoclonal antibodies, PH20, PH21, and PH22, bind to the PH20 protein of guinea pig sperm. They all bind to the entire surface of testicular sperm, and their binding sites are restricted to the posterior head of the cauda epididymal sperm. On the other hand, PH30 represents an antibody that does not bind to testicular sperm even though PH30 protein is present on testicular sperm. In this case the epitope recognized by the PH30 monoclonal

antibody is not available on testicular sperm. Binding of this monoclonal antibody is first observed on cauda sperm where the antigen is restricted to the posterior head region (Primakoff et al., 1987). Similar human germ cell- and sperm-specific antigens, as revealed by mapping studies with monoclonal antibodies, develop during spermatogenesis (Anderson et al., 1987) and on the sperm surface during epididymal transit (Feuchter et al., 1981). The testicular sperm surface membrane is characterized by the presence of major species-specific high-molecular-weight proteins (105-115KD in the boar, Dacheux et al., 1989; 95-119KD in the ram, Dacheux and Voglmayr, 1983; 110-130KD in the rat, Jones et al., 1981; 110KD in man, Dacheux et al., 1987). Most of these testicular surface proteins gradually disappear in the first part of the epididymis. Some other compounds are removed very soon, such as 75-97KD compounds in the boar (Dacheux et al., 1989) and 78-88KD compounds in the ram (Dacheux and Voglmayr, 1983).

2. Epididymal Spermatozoa

The testicular spermatozoa and fluids pass to the caput region of the epididymis, then through the corpus portion into the cauda portion. Transport of the spermatozoa from the corpus to the cauda requires from one to three weeks. Spermatozoa, as they transit the epididymis, mature and gain fertilizing ability (Asch and DeCherney, 1987).

Sperm undergo changes in surface charge, surface carbohydrate composition, intramembranous particle distribution, plasma membrane fluidity, plasma membrane lipid composition, and surface protein and glycoprotein makeup during epididymal maturation (Eddy et al., 1985; 1988). Changes in surface charge can be observed by an increased binding of cationic colloidal iron particles, cationic ferritin, and positively charged beads (Olson and Danzo 1981). Spin resonance studies have detected a decreased charge density at the phospholipid-water interface. These changes in net surface charge probably result from a variety of additions, modifications and deletions of sperm surface moieties during epididymal maturation (Rodriguez-Rigau et al., 1982).

Changes in sperm surface carbohydrate composition have been observed during maturation and include decreases in the total bound sialic acid, and increases and decreases in the binding of various lectins to the sperm surface. These changes appear to result from the addition and removal of saccharide moieties (Eddy 1988). For example, galactosyltransferase activity is present on the surface of the sperm and in epididymal fluid. This enzyme is required for mouse sperm binding to zona pellucida (Lopez et al., 1985).

Major changes in sperm surface protein and glycoprotein composition occur during maturation through (1) the addition of new components, (2) the unmasking or modification of preexisting moieties, or (3) the loss of surface

components. Changes in individual surface proteins or glycoproteins often occur within discrete regions of the epididymis, probably as the result of regionally specialized activities of the epididymal epithelium. The disappearance of sperm surface proteins is sometimes associated with the appearance of new proteins. Among the new membrane surface compounds of the sperm appearing during epididymal transit, most are characterized by a low molecular weight (range, 14-36K). Such events have been described in the rat (Brown et al., 1983; Brooks and Tiver, 1984), the ram (Voglmayr et al., 1982, 1985), the hamster (Moore and Hartman, 1986), the chimpanzee (Gould et al., 1984), and the human (Dacheux et al., 1987) sperm.

During maturation, the sperm plasma membrane undergoes changes in lipid content. There is a decrease in cholesterol, and there are significant decreases and increases in a variety of plasma membrane lipids, but there are no significant changes in the cholesterol/phospholipid ratio (Nikoloupoulou et al., 1985). However, changes in amount and composition may not occur uniformly in all regions of the sperm plasma membrane during maturation. Freeze-fracture studies have demonstrated changes of intramembranous particles within the plasma membrane during maturation, specifically in the acrosomal and post acrosomal region (Friend and Fawcett 1974). Expression of this maturation phenomenon are manifested in the changes in antigenic

properties of spermatozoa during epididymal transit as a result of the addition, substraction and modification of antigens on the cell surface (Tezon et al., 1985b; Eddy et al., 1988). Substantial progress in this area was achieved in recent years with the demonstration that androgen-induced glycoproteins secreted by the epididymis became associated with the surface of spermatozoa during maturation (Tezon et al. 1985a). Synchronous with this interaction, the cells developed the fertilizing ability which characterizes mature sperm. Major advances in characterizing the changes that occur at the sperm surface during epididymal maturation have come from the use of immunological tools to study this process. These approaches have been useful for demonstrating quantitative changes in the sperm surface during epididymal transit, for identifying new surface components that arise during epididymal maturation, and for determining where such components originate in the epididymis. Supporting evidence of a biological role of epididymal glycoproteins in maturation was obtained by using the alternative approach of blocking the biological activity of these antigens with specific antibodies (Fournier et al. 1985).

Tezon et al. (1985) have shown that the antigens secreted by the proximal segments of the epididymis tend to accumulate in the epididymal plasma and on the spermatozoal surface as they pass through the organ. This finding suggests a gradual incorporation of antigens from the

surrounding milieu into the cells. Based on this observation, they extracted these antigens from the surface of ejaculated spermatozoa. A fraction corresponding to the proteins of epididymal origin was used to raise a polyclonal antiserum in rabbits. When incubated with sperm, the antiserum localized the antigens mainly on the acrosomal cap region of the ejaculated spermatozoon with minor localization on the midpiece and flagellum. These researchers also showed that these epididymal antigens have a role on sperm-oocyte interaction. Blanquier et al. (1986) also suggested a significant relationship between infertility and defective antigen content or localization.

The Prefertilization Stages of Spermatozoa During Transit Through Female Reproductive System

The interaction and fusion of male and female gametes in the fertilization process is a multistep phenomenon that is still not well understood at the molecular and biochemical level. Immediately following coitus in primates and ruminants, spermatozoa encounter the cervical mucus, a fluid with complex physical properties. The physical properties of mucus contribute to several important cervical functions in these species, including exclusion of seminal plasma, the selective exclusion of certain sperm cells with morphological and, possibly, functional abnormalities; and the

retention and conservation of sperm for later migration to the upper tract.

During transit in the female reproductive tract, spermatozoa undergo physiological and biochemical changes during a process called capacitation. This process involves loss of sperm-surface molecules, including lectin and antibody-binding sites (Koehler, 1978; Schwarz and Koehler, 1979). Capacitation prepares the spermatozoa to undergo the acrosome reaction and the hyperactivated motility that may enhance the ability to penetrate the zona pellucida. Capacitation has been described as a reversible process involving shifting and shedding of surface components of the plasma membrane acquired during epididymal transit and at ejaculation (Weinman & Williams, 1964). During capacitation, there is a decrease in membrane cholesterol and a change in the phospholipid composition in the plasma membrane, thus altering the cholesterol/phospholipid ratio (Langlias & Roberts, 1985). These alterations modulate membrane fluidity. The net negative charge of the membrane is reduced due to the removal of sialic acid residues (Farooqui, 1983) and sulfate residues (Langla et al., 1981) by hydrolases present in the female reproductive tract. Antisperm antibody H316 appears to bind to the human sperm acrosome only after capacitation and may be useful as a probe identifying capacitated sperm (Anderson et al., 1986). Antibodies directed against the plasma membrane of sperm can affect the mobility of membrane

particles; thus impeding or possibly hastening capacitation. Support of this hypothesis comes from the finding that some antisperm antibodies exposed to sperm prior to capacitation prevent sperm fusion with hamster eggs (Alexander, 1984).

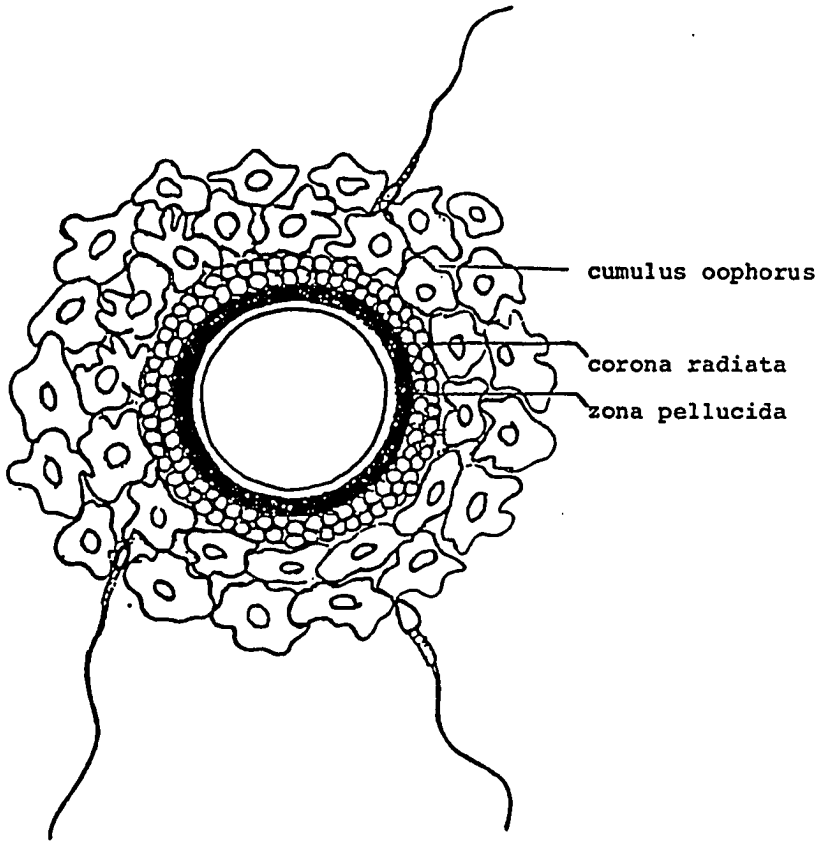
One end point of capacitation may be the hyperactivation of sperm. This vigorous pattern of movement observed in capacitated sperm was first reported in the hamster in 1970 (Yanagimachi, 1970). Later it was reported in other species, including man (Burkman, 1984). These movements may enable sperm to overcome the physical resistance of the vestments which surround the oocyte. When sperm antibodies directed against the plasma membrane affect sperm capacitation, they may inhibit sperm hyperactivation too. If sperm do not become hyperactivated, no penetration of zona pellucida or fertilization can occur (Fraser, 1981).

The end point of capacitation is the acrosome reaction, an exocytotic event made up of a series of steps. The anterior region of the head swells and the outer acrosomal membrane fuses with the plasma membrane. The release of most of the acrosomal contents occurs next. Finally there is a loss of the shroud of the fenestrated membrane so that the inner acrosomal membrane becomes the external face of the sperm. Although it is still debatable whether the zona pellucida initiates the acrosome reaction, this oocyte vestment does provide the primary sperm contact during fertilization (Wassarman, 1988). Antisperm antibodies may

mask the sites of induction of the acrosome reaction or reduce membrane mobility, and thus prevent its occurrence. Saling et al. (1986) reported that a monoclonal antibody directed against a sperm antigen prevents zona penetration by inhibition of the acrosome reaction.

During penetration, the spermatozoa must pass through the outer oocyte layers and arrive at the vitelline membrane in a state that is appropriate for fusion with this membrane. A mature ovulated egg (Fig. 5) has three layers of outer vestments through which the spermatozoa must pass: (1) the cluster of surrounding follicle cells, the cumulus oophorus, (2) the dense cellular layer present between the cumulus and the zona, the corona radiata, (3) the noncellular coating known as zona pellucida. The major constituent of zona is glycoprotein. Antibodies can also impede sperm-egg fusion (Saling et al., 1985). Antibody directed against the zona can prevent sperm binding (Henderson et al., 1987; Sacco, 1987). In the rabbit and guinea pig, antisera to sperm membrane antigens interfere with zona binding and penetration (Primakoff et al., 1988; O'Rand, 1981). Anti-sperm antibody in patient sera may also interfere with zona interaction (Bronson et al., 1982; Tsuku, 1986; Mahony et al., 1991). Several studies have demonstrated that anti-sperm antibodies can reduce hamster-egg penetration by human sperm (Alexander, 1984; Haas et al., 1980). Sperm penetration through the zona pellucida requires an initial period

FIG. 5 A Mature Ovulated Human Egg With 3 Vestments Through Which The Spermatozoa Must Pass



of time during which the spermatozoa attach and bind to the zona surface. This binding of spermatozoa to the zona is a tight, species-specific interaction and suggests that the zona pellucida may have sperm receptors on its surface. The putative presence of sperm receptors on the zona pellucida makes it likely that there are complementary egg-binding proteins. The murine zona pellucida is composed of three sulfated glycoproteins designated ZP1, ZP2, ZP3 (Bleil and Wassarman, 1980). ZP3, with a molecular weight of 83,000 daltons, has been proposed as a species specific sperm receptor. Antibodies directed against these receptor sites could interfere with zona attachment and subsequent penetration. Recently researchers from our institute (Coddington et al., 1991) evaluated some monoclonal sperm antibodies to see if any antisperm antibodies could impede sperm-zona binding. Two monoclonals significantly decreased zona binding after exposure to MA-14 and MA-27. Another study from our laboratory (Mahony et al., 1991) evaluated 26 antisperm monoclonal antibodies from a WHO (World Health Organization) panel with the hemizona assay. Among these, six monoclonals (S04, S19, S34, S58, S61, and S65) showed inhibition of zona binding (HZ index <50). Little is known about the mechanism of the binding process. It may bind to an intact plasma membrane, or a vesiculated surface, or an inner acrosomal membrane, or through a combined sequential reaction involving all three.

Monoclonal Antibodies and Their Significance to Studies of Immunoreproduction

Antibodies produced from a cell line that has arisen from the growth and division of a single cell to form a clone are called monoclonal. Ever since the beginning of the experimental immunology at the end of nineteenth century, scientists have exploited the specificity of antibodies to detect, isolate, and analyze biological material. The power of antibodies as probes for biological structure underwent a quantum increase in 1975, when Kohler and Milstein discovered a method for generating monoclonal antibodies. A number of hybridomas producing antibodies reactive against sperm have been developed. Monoclonal antibodies (MAbs) have proven to be highly useful probes for studying sperm surface components and for dissecting the structure and function of the male gamete. They have been used to map the domains of the sperm surface, to determine the time of appearance of future sperm surface components during spermatogenesis, to examine the species specificity of antigenic determinants on the sperm surface, and to follow the fate of sperm components after fertilization (Eddy, 1988). MAbs have also been used to detect auto-antigens and to test the role of surface components in the binding of the sperm to the zona pellucida and in fertilization (O'Rand, 1981; Saling, 1986). Because these monoclonal antibodies permit the study of single sperm antigens,

researchers have produced monoclonal antibodies that recognize components of sperm from a variety of species including man (Lee et al., 1982; Isahakia and Alexander, 1984; Naz et al., 1984; Wolf et al., 1983). Human sperm antigens are being studied through application of sperm specific monoclonal antibodies and evaluation of their biochemical and functional characterization. These antibodies will provide insight into the mechanisms of immunological infertility as well as contributing to the development of contraceptive vaccines. The WHO Monoclonal Antibody Workshop identified a number of antisperm monoclonal antibodies that recognized functionally significant sperm antigens (Anderson et al., 1987). The production of monoclonal antibodies directed against sperm provides a powerful tool for definition of new antigens that may be involved in fertilization.

CHAPTER 2

LITERATURE REVIEW

Ever since Landsteiner (1899) discovered the antigenic properties of human spermatozoa, various investigators have studied the phenomenon of immunologic infertility. Early attempts have been made to induce infertility in females by immunization with whole spermatozoa and with seminal plasma.

In the last ten years, research has centered on using various antigens in purified forms to induce infertility in mice (Edwards, 1964), guinea pigs (D'Almeida and Voisin, 1979; Primakoff et al., 1988), rabbits (Munoz and Metz, 1978), and primates (Goldberg, 1981). To be used for fertility regulation, a vaccine antigen must be expressed on the sperm surface (either an integral or coating protein). It is therefore logical to develop antisperm antibodies to sperm surface antigens. Definition of mammalian sperm surface domains at the molecular level has been achieved using lectins as probes (Schwartz et al., 1979), covalent protein labels such as [^{125}I]-diiodofluorescein isothiocyanate (Herr and Eddy, 1980), polyclonal antibodies raised against sperm antigens (Herr and Eddy, 1980), and monoclonal antisperm antibodies (Myles, 1981; Schmell, 1982; Wolf, 1983). Additionally, discrete patterns of intramembranous

particle distribution in the plasma membrane have been defined by freeze-fracture replica techniques (Friend et al., 1977; Yanagamachi, 1990). At present, however, relatively little is known about the association of such restricted domains to cellular function. Characterization of the human sperm surface is in its infancy.

Studies of membrane antigens have been facilitated greatly by the availability of monoclonal antibodies to individual determinants of antigens in complex mixtures. Clones of antibody-producing cells can provide banks of antibodies for use in typing cells according to the antigens present on their surfaces, and sufficient quantities of antibodies can be produced readily to support antigen isolation. The method for generating monoclonal antibodies has been used to develop a number of hybridomas producing antibodies reactive against sperm from mouse (Saling, 1985a, 1985b), rabbit (O'Rand et al., 1984) and guinea pig (Primakoff et al., 1988). Monoclonal antisperm antibodies are being used to study antigen expression during development and differentiation in spermatogenesis and epididymal maturation in the mouse (Bechtol, 1979). A WHO-sponsored workshop, mentioned in the last chapter, was held to review the current status of monoclonal antibodies regarding their effects on molecular events underlying reproduction and the feasibility of using this approach to identify trophoblast and sperm specific antigens that might represent suitable

candidates for contraceptive vaccine development. A list of 66 mouse monoclonal antibodies reacting with human sperm submitted by 29 laboratories is shown in Appendix A (Anderson et al., 1987). The immunizing antigen in most cases was washed human ejaculated sperm or human sperm antigen extract; exceptions included S23-29 (purified mouse LDH-C4), S22 (baboon epididymal sperm), S12 and S57 (syngeneic mouse sperm), S49 (rabbit sperm) and S60 (human chorionic villous surface membrane). S51 and S50 were two controls reactive against sheep red blood cells respectively, and NS.1 is a Ig-deficient ascites fluid produced by an unfused murine myeloma cell line. These antibodies were evaluated in coded form by 42 laboratories with the appropriate expertise in biochemistry, immunohistology and the testing of reproductive cell function. The majority of antisperm monoclonal antibodies cross-reacted with cellular elements in non-reproductive tissues. However, three antisperm MAbs appeared to demonstrate sufficient specificity to warrant further investigation as reagents for the identification of antifertility vaccine candidates. These three sperm monoclonal antibodies, S20, S37, and S61, were identified as primary vaccine candidates based on three criteria: (1) reactivity with human testicular germ cells and to the abundant surface antigens on mature spermatozoa; (2) minimal cross reactivity with somatic cells; and (3) inhibition of at least one sperm function assay (Lee et al., 1984; Moore

et al., 1985; Herr et al., 1986). Although a panel of these monoclonal antibodies has been made from several animal species, (Feutcher et al., 1981; Schmell et al., 1982; Isahakia and Alexander, 1984) only a few have been examined as to their effects on fertilization (Naz et al., 1984a; 1984b). Numerous human testicular germ cells and placental cDNA libraries are now available and provide a resource for cloning these vaccine candidates. To probe the library, monoclonal antibodies that can specifically affect fertilization without cross-reacting with normal somatic cells are required.

The Characterization of Sperm Surface Antibodies

A. Sperm Functional Analysis

Two tests are commonly used to detect free antisperm antibodies, the sperm agglutination test (Kibrick et al., 1952) and the sperm immobilization test (Isojima et al., 1968). Sperm-agglutinating antibodies are most commonly found in vasectomized men (65%); sperm-immobilizing antibodies are found in 40% of vasectomized men (Ansbacher, 1971). Agglutinating antibodies are found in less than 10% of the infertile women, whereas sperm-immobilizing antibodies are found in about 8%. This agglutination and immobilization may prevent normal progress through the cervical mucus during transit in the female reproductive tract. Complement dependent immobilization may result in damage to both the

acrosome membrane and the entire plasma membrane (LeBouteiller, 1975). Antisperm antibodies of the IgM and IgG classes may agglutinate sperm (Ingerslev et al., 1979) but only IgM and some subclasses of the isotype IgG are able to immobilize sperm by initiating the classical complement cascade (Brown et al., 1984). Antibodies of isotype IgA do not fix complement so they cannot immobilize sperm.

Recently an immunobead test involving micrometer-sized polyacrylamide beads coated with covalently bound anti-immunoglobulin antibody was developed for the detection of antisperm antibodies to sperm surface membrane antigens (Bronson et al., 1984). This assay can provide information on the location of the antibodies on the sperm surface (for example, on the head, tail, midpiece or tail-tip) and immunoglobulin class G, M or A (Bronson et al., 1981; 1982).

Many monoclonal antisperm antibodies exhibit actions and specificities similar to those of naturally occurring antisperm antibodies in human beings; for example, one has been reported that causes complement-dependent immobilization but not agglutination of human sperm (Isahakia & Alexander, 1984). A monoclonal antisperm antibody may cause neither sperm agglutination nor sperm immobilization but prevent gamete interaction. There are some in vitro and in vivo tests which can be used to detect any inhibition of sperm egg interaction due to antisperm antibodies. The first interaction of the spermatozoon and egg involves tight

binding of spermatozoa to the zona pellucida. This highly species specific binding involves complementary sites on the receptors of the surface of the gametes (Ahuja, 1985). The irreversible binding of sperm and zona necessary for fertilization and some types of male infertility have been demonstrated to be due to failure of spermatozoa to complete this adhesion (Overstreet et al., 1980). Overstreet et al. (1976) first suggested the use of zona intact non-living oocytes to examine the sperm-zona pellucida binding. Burkman et al. (1988) at our Institute have further modified this hemizona assay (HZA) technique. The HZA is being used as a clinical tool to evaluate patients and is predictive for fertilization potential (Franken et al., 1989b). The hemizona assay can be used to evaluate whether antisperm antibodies impede binding. When sperm were coincubated with antibody for one hour, some monoclonal antisperm antibodies markedly inhibited binding, thus providing evidence that such antibodies could potentially reduce fertility (Mahony et al., 1991).

The zona-free hamster penetration test (SPA) has been used extensively in many laboratories to examine the role of antisperm antibodies in fertilization. Our laboratory (Alexander et al., 1983) and others (Haas et al., 1980; Dor et al., 1981) have observed that antisperm antibodies, especially of the IgG isotype, interfere with human sperm penetration of hamster egg membrane. When sera from patients

with immunologically-mediated infertility were exposed to normal sperm, the ability of their sperm to penetrate hamster eggs was much reduced or eliminated. A penetration rate of 59% was observed for the control sera as compared to only 15% for the test sera. Fully 90% of the sera from patients with antibodies to sperm caused a reduction in egg penetration rates.

Antisperm antibodies can significantly inhibit in vitro fertilization of mouse eggs by murine sperm (Naz et al., 1984a). In vivo (by passive immunization) fertilization can be used (Naz et al., 1984a; Saling and Waibel, 1985) on animal models for primary evaluation of fertility effects due to antisperm antibodies. Immunization with an anti-idiotypic antibody which mimics sperm antigen structure could be completed (Carron et al., 1988). The mouse monoclonal antisperm antibodies M42.14 and M29.6 recognized distinct mouse sperm antigens which prevented mouse fertilization in vitro and in vivo (Saling, 1986). There are several advantages associated with the application of human antibodies for passive immunization using animal models to test their effect on fertilization: (1) They are a cheap source of unlimited amount of well characterized and serologically very specific reagents; (2) The degree of immunization is directly proportional to the amount of the antibody applied, and does not depend on individual variation response; (3) The action is shortlived and is fully

reversible; (4) As they are human proteins, severe immunization against other cells do not seem to be probable even if they are applied several times sequentially.

B. Biochemical Characterization

Western blot techniques have revealed that, even though an antibody reacts to what appears to be the entire acrosome, antigens of differing molecular weights may be involved. Primakoff and Myles (1983) have found guinea pig sperm head antigens ranging from 18 to 70 Kd, and our laboratory has observed human sperm acrosomal antigens varying from 34 to 240 Kd. In elegant studies, Reynolds and Oliphant (1984) have used monoclonal antibodies to characterize an acrosome-stabilizing factor, a 360 Kd dimer synthesized in the corpus epididymides and found in seminal plasma and caudal epididymal fluid. This substance maintains the acrosome during epididymal storage and prevents the acrosome reaction.

As was mentioned, during the fertilization process, the sperm cell must successfully interact with two primary surface constituents of the egg, the zona pellucida (an extracellular glycoprotein layer that surrounds the egg) and the egg plasma membrane. Sperm-zona interaction involves the binding of the sperm to species-specific receptor molecules present within the zona (O'Rand, 1988). A number of studies have indicated that carbohydrate moieties present

within the zona pellucida or on the surface regulate this interaction. Competitive inhibition studies using various monosaccharides, polysaccharides or glycoproteins have indicated a role for specific carbohydrate moieties in mediating sperm-zona interaction in hamsters (Ahuja, 1982) and mice (Wasserman and Bleil, 1982). Treatment of either the zona pellucida or sperm with glycosidic enzymes also inhibits sperm binding to the zona in a variety of species (Ahuja and Gilbert, 1985; Shur and Hall, 1982). The possibility that sperm-egg fusion involves carbohydrate components of the sperm and/or egg plasma membrane is suggested by observations in other membrane fusion systems where glycoproteins play a critical role in the fusion process. Once these antigens have been elucidated, they can be used both in infertility assessment and production of a contraceptive vaccine.

Lectins, such as concanavalin A and wheat germ agglutinin, are plant proteins that have multiple binding sites for specific sugars. Wheat germ agglutinin, for example, binds to glucose and mannose. Most plasma membrane glycoproteins have multiple exposed sugar groups; thus, lectins cross-link glycoproteins that have multiple appropriate sugar substituents. Monoclonal antibodies against sperm membrane glycoproteins often react with antigenic determinants common to more than one cell-type, and such

cross-reactivity is mainly due to glycoconjugates on the cell surface (Anderson et al., 1987).

Production and characterization of antisperm monoclonal antibodies will aid in understanding fertilization and be a step toward contraceptive vaccine development.

CHAPTER 3

SPECIFIC AIMS

Specific aims of this project are to

1. Develop murine monoclonal antibodies directed against human sperm surface antigens.
   a. Prepare sperm membrane (both coating and integral) antigen extraction from human ejaculated sperm and from human testicular sperm.
   b. Immunize Balb/c mice
      i. by conventional immunization technique and
      ii. by first tolerizing mice with common antigenic sugar moieties to human sperm and somatic cells and then immunize mice with human sperm extracted antigens.
   c. Perform the classical hybridoma technique to generate Mabs against extracted sperm surface antigens.
   d. Determine the class (isotype) and quantitate antibodies.

2. Localize antigens reacting with a particular MAb restricted to the sperm surface on human by

immunofluorescence (IF) and immunochemical staining (ICS).

3. Determine the molecular weight of antigens from extracted sperm antigens that are recognized by these MAbs by the immunoprecipitation technique and SDS-PAGE (sodium-dodecyl sulfate polyacrylamide electrophoresis).

4. Determine the reactivity of MAbs with fresh, capacitated, and acrosome reacted human sperm by enzyme linked immunosorbant assay (ELISA) and ICS.

5. Determine whether these MAbs are against human sperm surface antigens by the live sperm ELISA and the immunobead test (IBT).

6. Perform cross-reactivity studies evaluating generated panel of MAbs using ELISA /or CB-RIA (cell-binding radioimmunoassay) to sperm and other cell types (human lymphocytes, erythrocytes) or do IF and ICS against a panel of human tissues.

7. Localize the origination of sperm antigens in the reproductive system reacting with a particular MAb by IF and ICS on frozen human reproductive tissue section.

8. Determine the cross reactivity of MAbs with other species spermatozoa by IF and ICS.

9. Determine whether these antisperm antibodies have functional significance.
   a. Sperm agglutination assay (SA)
   b. Sperm immobilization assay (SI)
   c. Cervical mucus penetration assay (CM)
   d. Hamster egg penetration assay (SPA)
   e. Hemizona assay (HZA)

10. Determine whether a carbohydrate moiety is involved on the sperm surface antigen activity by means of inhibition of carbohydrate moiety by sodium metaperiodate.

11. Use passive immunization of any particular promising antibody that also cross-reacts with mouse sperm to determine whether a reduction in fertility occurs.

CHAPTER 4

PURPOSE & SIGNIFICANCE

At present, no group of sperm antigens useful for human immunocontraception has been defined. The current literature shows that the majority of extant monoclonal antibodies cross-react with cellular elements in nonreproductive tissues.

For vaccine development, the reactivity to the abundant surface antigens on mature spermatozoa and the minimal cross reaction with somatic cells are important criteria (Anderson et al., 1987). Therefore, the aim of our study is to develop antisperm monoclonal antibodies to sperm membrane surface antigens.

One approach is to use human sperm membrane preparations. This requires the definition of specific epitopes that will not cross-react with other tissues but which result in an effective and reversible mode of contraception.

Lectin binding studies of sperm membrane extracts have identified abundant amounts of 4 sugar moieties (mannose, galactose, N-acetyl-glucosamine, and fucose) which are common to many cell types (Kurpisz et al., 1989). Thus, it is not surprising that antisperm monoclonal antibodies often

cross react with antigenic determinants common to more than one cell type (Waibel et al., 1987; Anderson et al., 1987; Tsuji et al., 1988). In our proposed study we used a tolerization protocol to see if by tolerizing the mouse with these common sugar moieties, we can prevent the cross-reactivity with other cells; hence, we can develop more sperm specific monoclonal antibodies. This approach may also facilitate producing antibodies against surface proteins that are weakly immunogenic, even though these antigens may be involved in fertilization.

Seminal plasma-derived coating proteins and antigens are expressed not only on sperm but may also be present on somatic cells. These antigens often appear to be more immunogenic in the mouse than the gamete-specific components. In order to eliminate the reactivity to seminal plasma-derived coating protein, we also used an extract of human mature testicular tissue to immunize mice.

Antisperm antibodies that inhibit zona binding are also an area of interest in contraceptive research. In nonhuman models, antibodies to sperm antigens can inhibit fertilization by preventing zona binding (O'Rand, 1981). Whether the results obtained from animal studies can be applied directly to the human system is not yet clear. The new hemizona assay has been used as a clinical tool to evaluate patients and predict their fertilization potential. Our laboratory has presented data (Mahony et al. 1991)

showing that some antisperm monoclonal antibodies can inhibit zona binding as determined by the hemizona assay, although the antibodies may not inhibit any of the sperm functional assays other than hamster egg penetration. One aim of this study is to screen our antisperm monoclonal antibodies using this new hemizona assay.

The purpose of this study is to develop highly specific antihuman sperm MAbs and to characterize some that are functionally significant, so that in future study, these could be used in a cocktail of significant antisperm monoclonal antibodies to evaluate a cDNA library for production of sperm antigens in vitro. Definition and characterization of these antibodies will aid in the understanding of sperm function and fertilization and may provide a useful contraceptive approach.

CHAPTER 5

EXPERIMENTAL DESIGN AND METHODS

SPECIFIC AIM NO. 1

Production of murine monoclonal antibodies directed against human sperm surface antigens.

a. Sperm surface (coating and integral) antigens extraction from human ejaculated sperm and from human testicular sperm

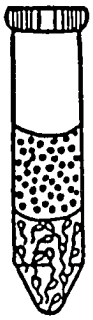
Extraction of human ejaculated sperm surface antigens

Researchers from our laboratory have recently investigated and reported (Gupta et al., 1988) a method of sperm antigen preparation to optimize production of relevant monoclonal antibodies. Human semen ejaculates, obtained from normal male donors or from men attending an infertility clinic (The Jones Institute for Reproductive Medicine, Norfolk), were allowed to liquefy at room temperature. After liquefaction, 0.5 mL of semen was placed beneath 2 mL Biggers, Whitten, Whittingham (BWW) medium (Biggers et al., 1971) and motile sperm were allowed to swim-up for a 90 min incubation period at 37°C. Motile sperm in BWW medium were pooled, counted with a Cell Soft

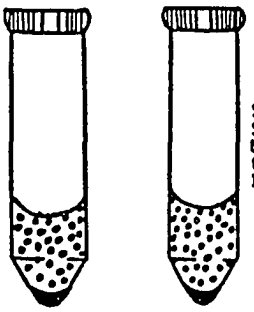
Automated Semen Analyzer (Cryo Resources Ltd, NY), washed three times in Tris-buffered saline (TBS) and stored at -70°C, (swim-up sperm, Fig. 6). A sperm pool was collected for protein extraction.

Sperm surface antigens were extracted from pools of swim-up sperm as follows: the volume of thawed sperm (500 million sperm) was doubled by addition of 0.6% (0.3% final conc.) of NP-40 in TBS (vol/vol), to which 1 mM (final conc.) phenyl methyl sulfonyl fluoride (PMSF) was added as a protease inhibitor. The suspension was incubated for 1 hour at room temperature with continuous end-to-end mixing. After extraction, the suspension was centrifuged for 30 minutes at 20,000 g at 4°C. The supernatant was collected, pooled, and dialyzed extensively against TBS in a 3,500 molecular weight exclusion dialysis membrane at 4°C. Protein concentration was measured by a BCA protein assay (Pierce Chemical Company) according to manufacturer's suggested protocol insert. Briefly, bicinchoninic acid, the key component in the BCA protein assay reagent, formed alkali metal salts which were soluble in water due to the polar carboxylic acid groups. The pyrole reaction product was measured spectrophotometrically at 562 nm. A flow diagram of sperm membrane antigen extraction procedure is shown in Fig. 7.

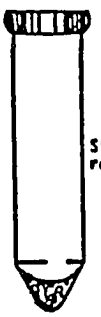
FIG. 6 Improvement of Semen Parameters by Swim-up (rise) Technique



Step 1 - Liquified semen sample was diluted with an equal volume of BWW

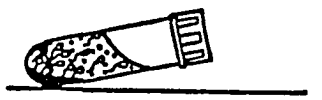


Step 2 - Sample was washed by centrifugation twice at 600g for 10 minutes.

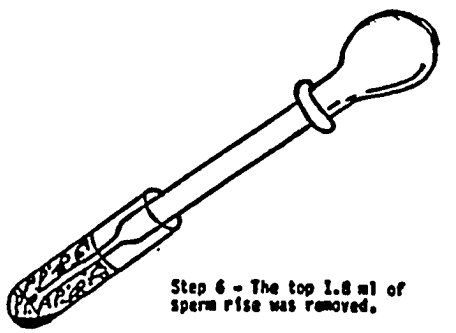


Step 3 - Sperm pellet was resuspended in 0.3 ml.

Step 4 - Concentrated sperm sample was underlayered beneath 2 ml of culture medium.

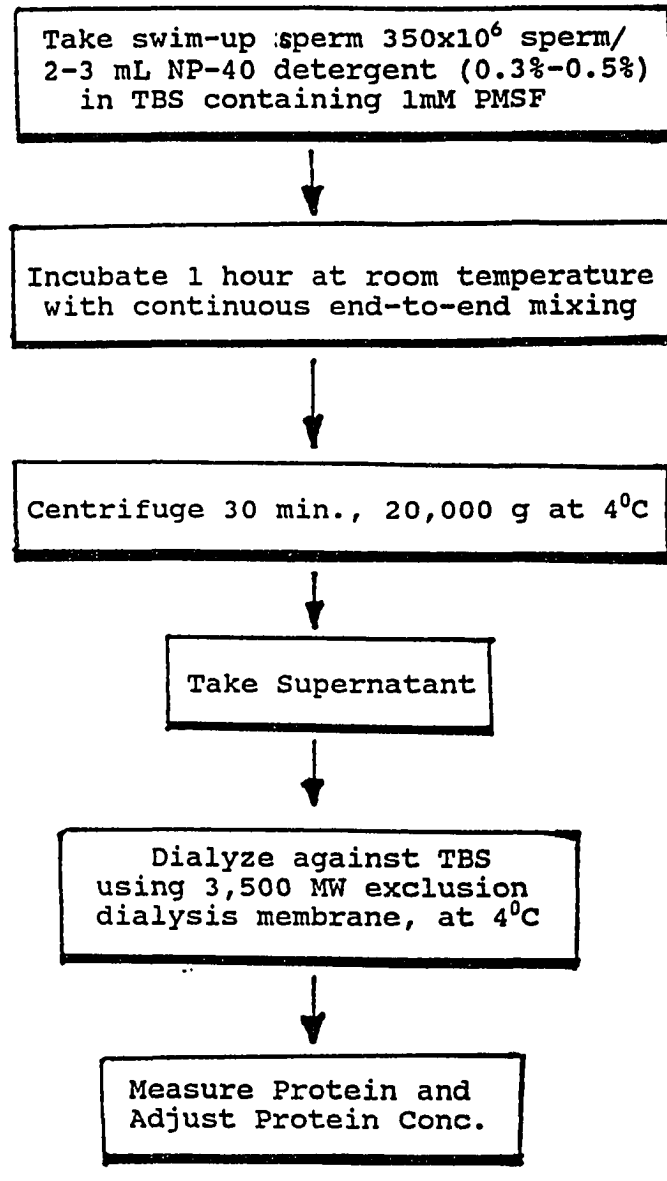


Step 5 - Tube was placed at a 60° angle in a 37°C incubator (5% CO_2 and air) for 1½ hours to allow sperm to "rise."



Step 6 - The top 1.8 ml of sperm rise was removed.

FIG. 7 A Flow Diagram of Sperm Membrane Antigen Extraction Process



Extraction of human testicular sperm antigen

The testicular sperm antigen preparation technique developed by Liu et al. (1989) was followed. Briefly, 10 grams of frozen human testes (obtained from National Disease Research Interchange, Philadelphia, PA) was homogenized in a glass homogenizer using 20 mL extraction buffer containing 50 mM Tris-HCl, 1 mM phenylmethyl sulfonylfluoride (PMSF), pH 8.0 at 4°C. After being centrifuged at 27,000 g for 20 minutes at 4°C, the supernatant was collected. The resulting pellet was resuspended and centrifuged again. The supernatants were pooled and subjected to ammonium sulfate fractionation. Solid ammonium sulfate (50 mg/mL) was added to the supernatant. This was followed by stirring for 10 minutes. The solution was centrifuged and the supernatant recovered. After additional ammonium sulfate (350 mg/mL) was added, the supernatant was stirred for 1 hour at 4°C. This was followed by centrifugation, after which the pellet was dissolved in a minimal amount of 10 mM phosphate buffer saline, pH 7.2 (PBS). The solution was dialyzed overnight against 4 liters of PBS. Protein concentration was measured by the BCA Protein assay method (Pierce Chemical Company) according to manufacturer's suggested protocol insert.

b. Immunization of Balb/c mice

i. by conventional immunization method

To generate mouse hybrid cell clones secreting monoclonal anti-human sperm antibody, a standard technique was used as described elsewhere (Kohler & Milstein, 1976). Briefly, Balb/c mice (Jackson Laboratories, Bar Harbor, ME) were immunized subcutaneously (day 0) with human sperm surface proteins extracted by NP-40 (50 ug/animal) in complete Freund's Adjuvant, followed by an intraperitoneal booster injection in incomplete Freund's Adjuvant on day 21 and 28. Mice with the highest titer of antisperm antibodies [determined using the enzyme-linked immunosorption assay (ELISA)] were given intravenous or intraperitoneal injection (100 ug protein in TBS) 4 days prior to fusion.

ii. by tolerization of Balb/c mice with common antigenic sugar moieties

Tolerogen: A mixture of sugars (galactose + fucose + mannose + N-acetylglucose) coated on glass beads.
Immunogen: NP-40 extracted human sperm surface antigens.

Tolerization was accomplished by intraperitoneal injection of sugar coated beads on weeks 0, 2, and 6. After each tolerogen injection, cyclophosphamide at 1 mg/mL was injected intraperitoneally thrice (10 minutes, 24 hours and 48 hours after each tolerogen).

Immunization against NP-40 extracted sperm antigens was accomplished by intraperitoneal injection, without adjuvant, at 7.5 hours and 4 weeks later.

c. Classical hybridoma technique to generate Mabs against extracted sperm surface antigens

Immunized spleen cells were fused with mouse myeloma cells (either SP2/0 or NS-1) in 3:1 ratio with 50% polyethylene glycol (PEG mol. wt. 1500). After fusion, cells were cultured in 96-well plates in selection medium containing RPMI-1640 (Gibco Laboratories Inc. Logan, UT), and HAT (Hypoxanthine, Aminopterin, Thymidine; Boehringer Mannheim, GmbH, West Germany). Ten to fourteen days after hybridization supernatant fluids from all wells were screened for antisperm antibodies against swim-up washed methanol fixed and swim-up washed motile human sperm with an ELISA technique. Positive hybridomas were expanded and subcloned at least twice under limiting dilution culture conditions (0.4 cells/wells). Following cloning, large amounts of culture supernatant were produced in vitro, and ascites fluids containing antibodies

were produced in vivo by intraperitoneal injection of 3-5 million hybridoma cells into balb/c mice 10 days after priming with 0.5 mL of pristane (2,6,10,14-tetramethyl-pentadecane, Sigma Chemical Company, St. Louis, MO). A flow diagram for production of anti-sperm MAbs is shown in Fig. 8.

d. Determination of class (isotype) and quantitation of antibody

Antibody heavy- and light-chain classes were determined using a mouse immunoglobulin subtype identification kit (Zymed Laboratories, South San Francisco, CA). MAb concentration in concentrated culture supernatant and ascites were quantitated by isotype-specific single radial immunodiffusion (Mancini et al., 1965) on standardized plates (Tago, Burlinghame, CA).

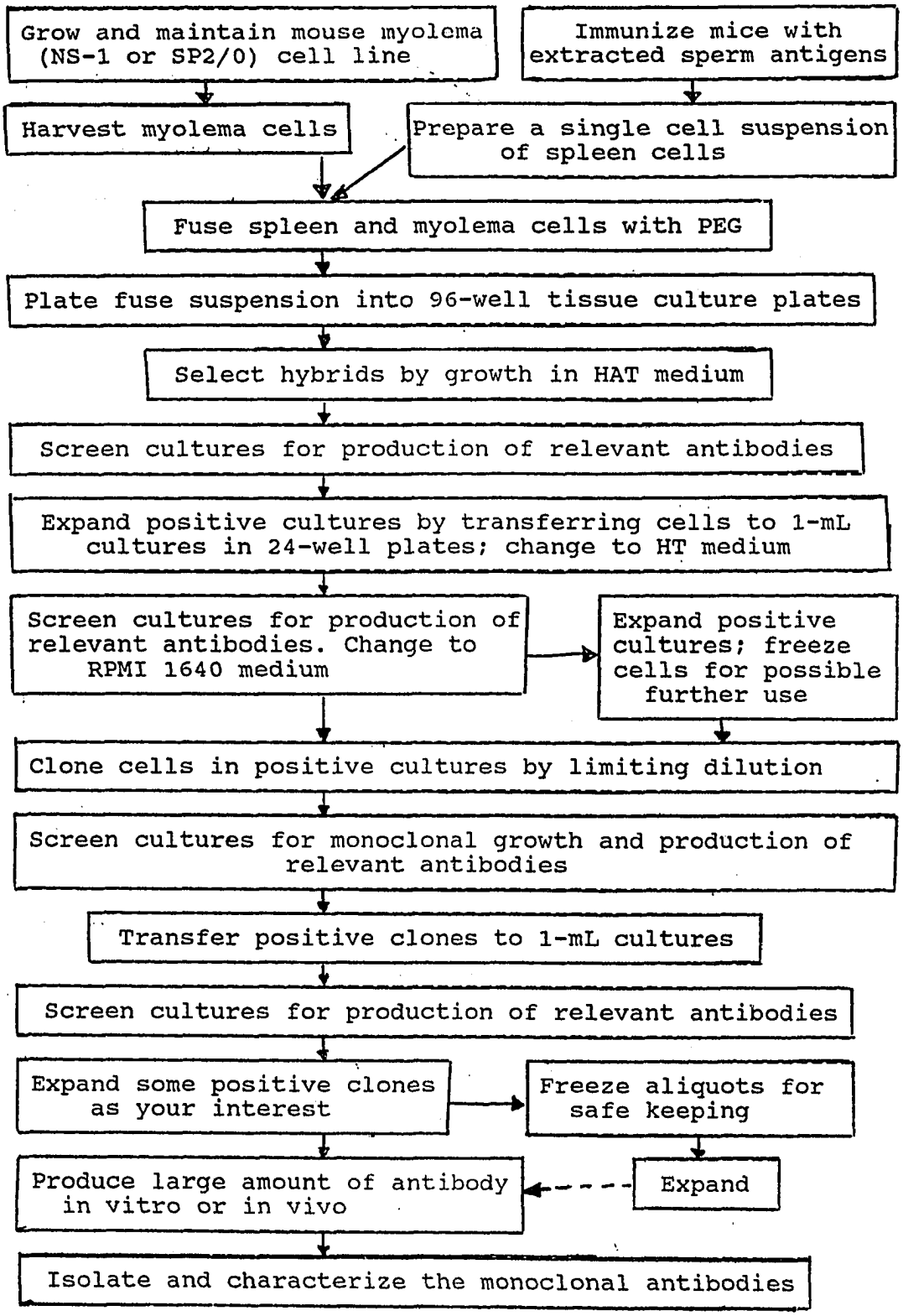
SPECIFIC AIM NO. 2

Localization of antigens reacting with a particular MAb restricted to the sperm surface

Immunofluorescence (IF)

To detect any cross reactivity of antisperm monoclonal antibodies with human spermatozoa both immunofluorescence (IF) (Hjort & Hansen, 1971) and immunochemical staining (ICS) were performed. For IF, swim-up human sperm were

FIG. 8 A Flow Chart for Production of Monoclonal Antibodies



washed three times by centrifugation with PBS and placed on teflon coated spot slides (Roboz, Washington, D.C.) at a concentration of 5 million sperm/mL. Slides were air dried, fixed for 10 minutes in methanol and frozen at -70^{0}C until use. The day that the test was performed, frozen slides were thawed at room temperature and washed with PBS and then blocked for 30 minutes with 10% normal goat serum, washed again with PBS, and then incubated for 60 minutes at room temperature or overnight at 4^{0}C with serial dilutions of antibody-containing ascites fluids. The slides were then washed in PBS, and further incubated for 60 minutes in the dark at room temperature with fluorescein isothiocyanate (FITC)- conjugated goat anti-mouse immunoglobulins (IgG, IgA, and IgM; Cappel Laboratory, Cochranville, PA). After three to four washings, the slides were mounted in mounting media, a coverslip placed over the tissue, and then examined under a Zeiss epifluorescence microscope. The principle of IF technique is shown in Fig. 9a.

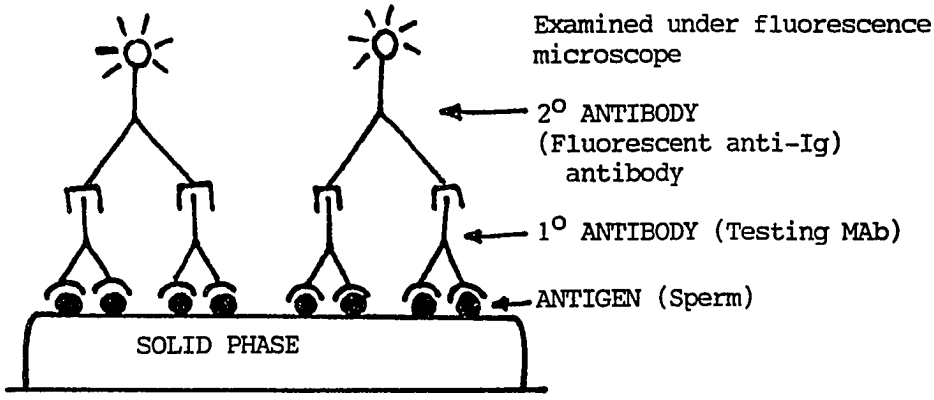
Immunochemical staining (ICS)

ICS technique was performed by using the Zymed Histo-stain-sp kit (Zymed Immunohistochemical Kit, Zymed Laboratories, Burlingame, CA). This enzyme immunostain system can be used in histochemistry and cytochemistry for localization and visualization of antigens as surface markers. The kit utilized a biotinylated second antibody, a horseradish

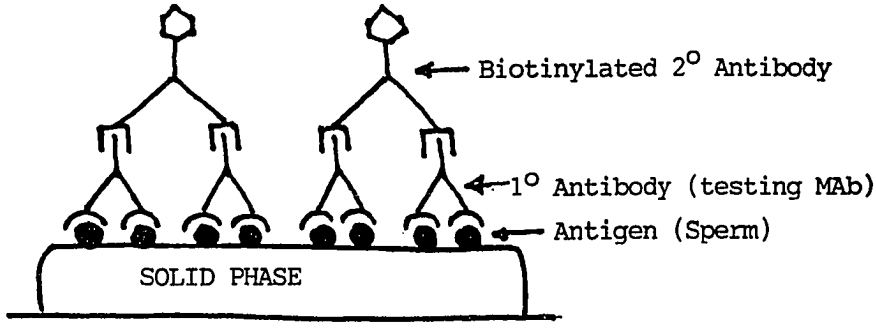
FIG. 9 A. Principle of Indirect Immunofluorescence (IF)

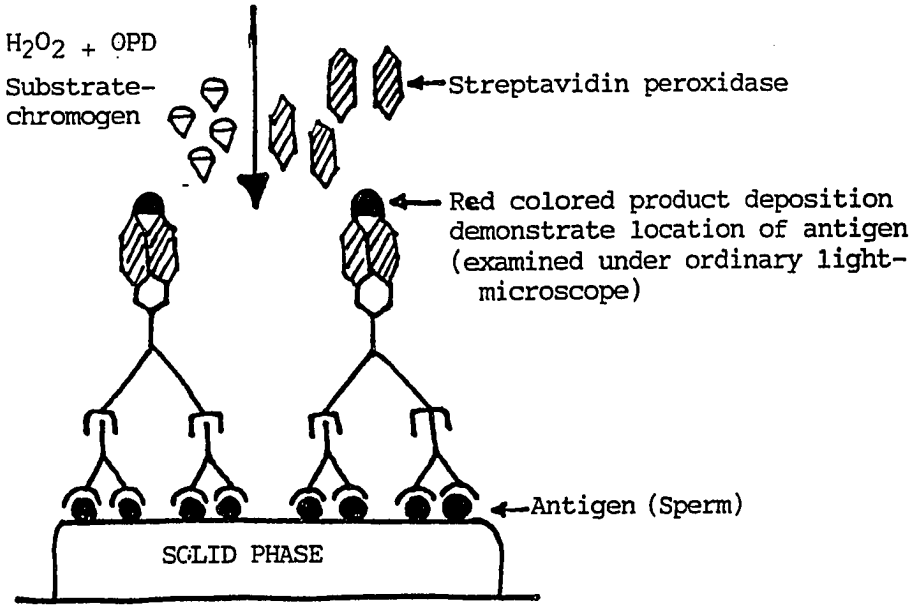
B. Principle of Immunochemical Staining (ICS)

A.



B.





peroxidase-streptavidin conjugate and a substrate chromogen mixture to demonstrate presence of the antigen in cells or tissue. The method of this staining system was as follows: Briefly, the methanol fixed human sperm coated spot slides were prepared the same way as described in IF technique above. Nonspecific background was eliminated by incubating sperm with nonimmune serum. The primary antibody to the specific antigen was incubated to target antigens. This was followed by addition of a biotinylated second antibody which served as the linker between the primary antibody and peroxidase-streptavidin conjugate. The signal generating reagent, streptavidin peroxidase was then added to bind to the biotin residues on the linking antibody. The presence of enzyme was revealed by addition of a mixture of substrate-chromogen solution. The enzyme, peroxidase, catalyzes the substrate hydrogen peroxide and converts it to chromogen, aminoethyl carbazole to a red colored deposit which demonstrates the location of the antigen. The principle of ICS technique is shown in Fig. 9b.

SPECIFIC AIM NO. 3

Determination of Molecular Weight of the Sperm Antigens by Sodium Dodecyl Sulfate Poly Acrylamide Gel Electrophoresis (SDS-PAGE) and Western Immunoblotting

To determine the molecular weight of sperm protein recognized by antisperm antibodies, the Western Immuno-blotting method was performed after SDS-PAG electrophoresis of the human sperm NP-40 extract as described by Laemmli et al. (1970). Briefly, a discontinuous gel composed of a running gel (10% polyacrylamide; pH 8.8) and a stacking gel (3% polyacrylamide; pH 6.8), both with 0.1% SDS, were made. Samples containing human sperm extract (100 ug protein) were diluted 1:1 with reducing sample buffer (20% glycerol, 3% SDS, 12.5% 0.5M Tris-HCl pH 6.8, 5% 2-mercaptoethanol, and 0.5% bromophenol blue) and boiled for 5 minutes. The electrophoresis buffer chamber contained 25mM Tris base, 0.19 M glycine, and 0.1% SDS. Electrophoresis was performed at 30 milliamps (constant current) at room temperature with a circulating cold water cooling system. Known pre-stained standards (BRL, Gaithersburg, MD) consisting of myosin, phosphorylase-B, bovine serum albumin, ovalbumin-chymotrypsinogen, B-lactoglobulin, and lysozyme with apparent molecular weights of 200, 97, 68, 43, 25, 18, and 14 KD were also run.

Proteins from the gels were electrophoretically transferred to a 0.22 uM nitrocellulose membrane (Bio Rad

Laboratories, Richmond, CA) by the blotting procedure of Towbin et al., (1979). In brief, the electrophoretic transfer of proteins was performed at 150 milliamps constant current in transfer buffer (25mM Tris, pH 8.4, 192 mM glycine, 20% v/v methanol) overnight at room temperature. Nonspecific binding sites were blocked by incubating the nitrocellulose membrane for 2 hours at room temperature with TBS containing 0.1% Tween-20 (TBS-Tween) to remove excess BSA and were allowed to react with individual monoclonal antibody in a miniblotter-45 (Immunetics, Cambridge, MA) for 4 hours at room temperature. After incubation, the nitrocellulose membrane was washed with TBS-Tween and incubated with a 1:300 dilution of peroxidase-labeled rabbit anti-mouse immunoglobulins for 1 hour at room temperature. The excess conjugate was removed by washing several times with TBS-Tween. The enzyme bound to the nitrocellulose was visualized by adding freshly prepared substrate solution (30 mg of chloronaphthol (Bio-Rad Laboratories, Richmond, CA) dissolved in 10 mL methanol and diluted 1:4 with TBS, to which hydrogen peroxide was added at a final concentration of 0.06%). The reaction was terminated by washing the nitrocellulose membrane with distilled water, followed by a 10 minute incubation and the reaction stopped with a stopping solution (0.1% NaN_3). The nitrocellulose blot was washed again with distilled water and stored in distilled water until photographed.

SPECIFIC AIM NO. 4.

Determination of reactivity of MAbs with fresh, capacitated, and acrosome reacted human sperm

A pool of swim-up human spermatozoa derived from normal donors was divided in three groups. The first group was washed with PBS (used as fresh sperm), the second group was incubated in BWW media 3-5 hours at 37 ^{0}C in a humidified 5% CO_2 incubator (used as capacitated sperm), and the third group was capacitated for 3-5 hours and then treated with a 10 uM calcium ionophore A23187 in dimethyl sulfoxide for 30 minutes (used as acrosome reacted sperm). After treatment, sperm were washed three times by centrifugation with PBS and reactivity of the MAbs with fresh, capacitated, and acrosome reacted human sperm were determined by using ELISA and ICS techniques.

Enzyme Linked Immunosorbent Assay (ELISA)

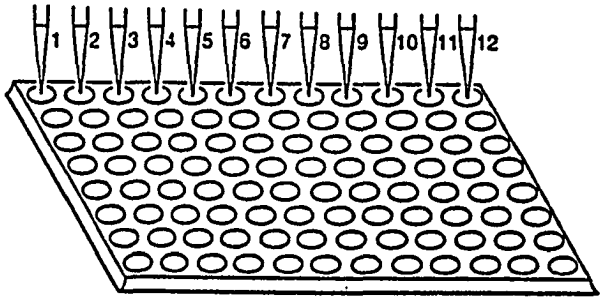
An indirect ELISA with whole sperm was used to screen the culture supernatant and ascites fluids for antibody activity. Washed human sperm cells (2.5 x 10^5/well) were resuspended in carbonate/bicarbonate buffer, pH 9.6, were added to wells of 96-well plates, allowed to settle over-night at 4°C, and then methanol-fixed and stored at -70°C until use.

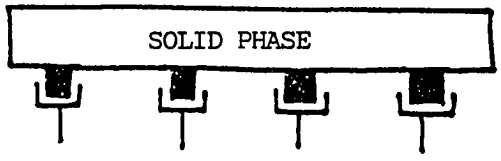
Frozen coated plates were thawed and washed thrice with PBS-Tween buffer to remove methanol and then blocked by incubation for 30 minute with PBS containing 1% polyvinyl

alcohol and 0.05% Tween-20 (Sigma) to remove any nonspecific binding. Culture supernatant or diluted ascites fluids as primary antibodies were reacted overnight at 4°C in wells of cell-coated plates. The negative control (NS-1 myeloma cell culture supernatant or ascites), a known positive control and a blank (reagent buffer) were used with each plate to validate the assay. Unbound immunoglobulins were removed during three washes with PBS-Tween buffer. Rabbit anti-mouse immunoglobulins (IgG, IgA, IgM) conjugated with horseradish peroxidase enzyme (Dako Chemical) were used as secondary antibody and reacted with bound primary antibodies by incubating 60 minutes at 37°C. After washing thrice with PBS-Tween, the bound peroxidase activity was measured by adding 150 uL/well of orthophenyldinitro (OPD) diluted in 0.1 M citrate buffer (pH 4.2, containing 0.03% hydrogen peroxide), incubating 10 minutes at room temperature, and stopping the reaction by adding 30 uL/well of 4 M sulfuric acid. The absorbance was read at 510 nM. The principle of sperm ELISA technique is shown in Fig. 10.

FIG. 10 Principle of Enzyme-linked Immunosorbent Assay (ELISA)

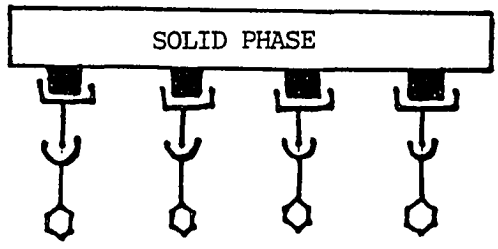
A 96-WELL PLATE COATED WITH SWIM-UP WASHED WHOLE SPERM (ANTIGEN)



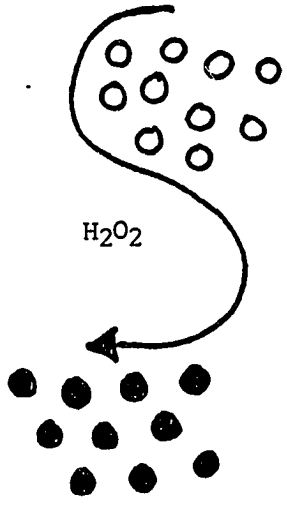


ANTIGEN (Sperm)

1° ANTIBODY (Testing)
MAb



2° ANTIBODY
(rabbit anti-mouse Igs
conjugated with horse
radish peroxidase enzyme)



SUBSTRATE (colorless)

PRODUCT (colored)

SPECIFIC AIM NO. 5

Determining whether these MAbs are against human sperm surface antigens

To test whether these MAbs were reactive against sperm membrane surface or internal antigens, these MAbs were tested by ELISA and IBT (immunobead test) on live sperm.

For ELISA, swim-up washed human spermatozoa were incubated with MAb for 1 hour at 37^0C in a humidified 5% CO_2 incubator, washed 3-4 times with PBS by centrifugation and used to coat the sperm plate for ELISA. Then a regular ELISA technique was performed as described previously.

For the indirect immunobead test (IBT), semen from normal fertile donors was processed to obtain the motile fraction by swim-up technique. The concentration of the motile fraction was adjusted to 2 million motile sperm/mL and incubated with the MAb for one hour at 37^0C and 5% CO_2 in air and then excess antibody was removed by centrifugation. Antibody coated sperm were mixed on a microscope slide with rabbit anti-mouse immunoglobulins (IgG and IgM; Zymed, San Francisco, CA) and the mouse Ig coated beads (polyacrylamide beads conjugated with highly purified anti-mouse antibodies of IgG and IgM class) and the motile sperm were examined for number and location of beads attached.

SPECIFIC AIM NO. 6

Evaluation of sperm specificity by cross-reactivity studies of the newly generated panel of Mabs

Washed human sperm cells (2.5 x 10^5/well), red blood cells (2.0 x 10^5/well), Raji cells (1.5 x 10^5/well), lymphocytes (1.5 x 10^5/well), and bacteria (5 x 10^5/well) were resuspended in carbonate/bicarbonate buffer, pH 9.6, were then added to wells of 96-well plates, allowed to settle overnight at 4°C, and then methanol-fixed and stored at -70°C until use. To test any cross reactivity of antisperm MAbs with these cells, regular ELISA was performed as described previously.

SPECIFIC AIM NO. 7

Localize the origin of sperm antigens in the reproductive system reacting with a particular MAb

To detect any cross-reactivity of antisperm monoclonal antibodies with human reproductive tissue sections (prostate, epididymis, vas, seminal vesicle, testes), both immunofluorescence (IF) (Hjort & Hansen, 1971) and immunochemical staining (ICS) were performed. For IF, methanol fixed frozen human reproductive tissue sections (available in our laboratory stored at -70°C.) were washed in PBS and then blocked for 30 minutes with 10% normal goat serum, washed again with PBS, and then incubated for 60 minutes at room temperature or overnight at 4^0C with serial dilutions

of antibody-containing ascites fluid. The slides were then washed in PBS, and further incubated for 60 minutes in the dark at room temperature with fluorescein isothiocyanate (FITC)- conjugated goat anti-mouse immunoglobulins (IgG, IgA, and IgM; Cappel Laboratory, Cochranville, PA). After three to four washings, the slides were mounted in mounting media, a coverslip was placed over the tissue, and then the slides were examined under a Zeiss epifluorescence microscope.

ICS technique was performed by using the Zymed Histostain-sp kit (Zymed Immunohistochemical Kit, Zymed Laboratories, Burlingame, CA) as described previously.

SPECIFIC AIM 8

Localization of antigens reacting with particular Mabs restricted to human and other mammalian sperm surfaces

To detect whether or not these anti-human sperm antibodies cross-react with other animal sperm, methanol-fixed sperm (dog, mouse, rat, rabbit, monkey, hamster, bull, baboon etc.) smears on spot slides were tested according the sperm immunofluorescence (IF)/or immunochemical staining (ICS) procedures as described previously.

SPECIFIC AIM NO 9

Determine whether these antisperm antibodies have functional significance

To evaluate whether antisperm antibodies to these antigens have any functional significance, sperm immobilization tests, sperm agglutination tests, cervical mucus penetration tests, hamster egg penetration tests, and hemizona tests were done.

a. Sperm Immobilization assay

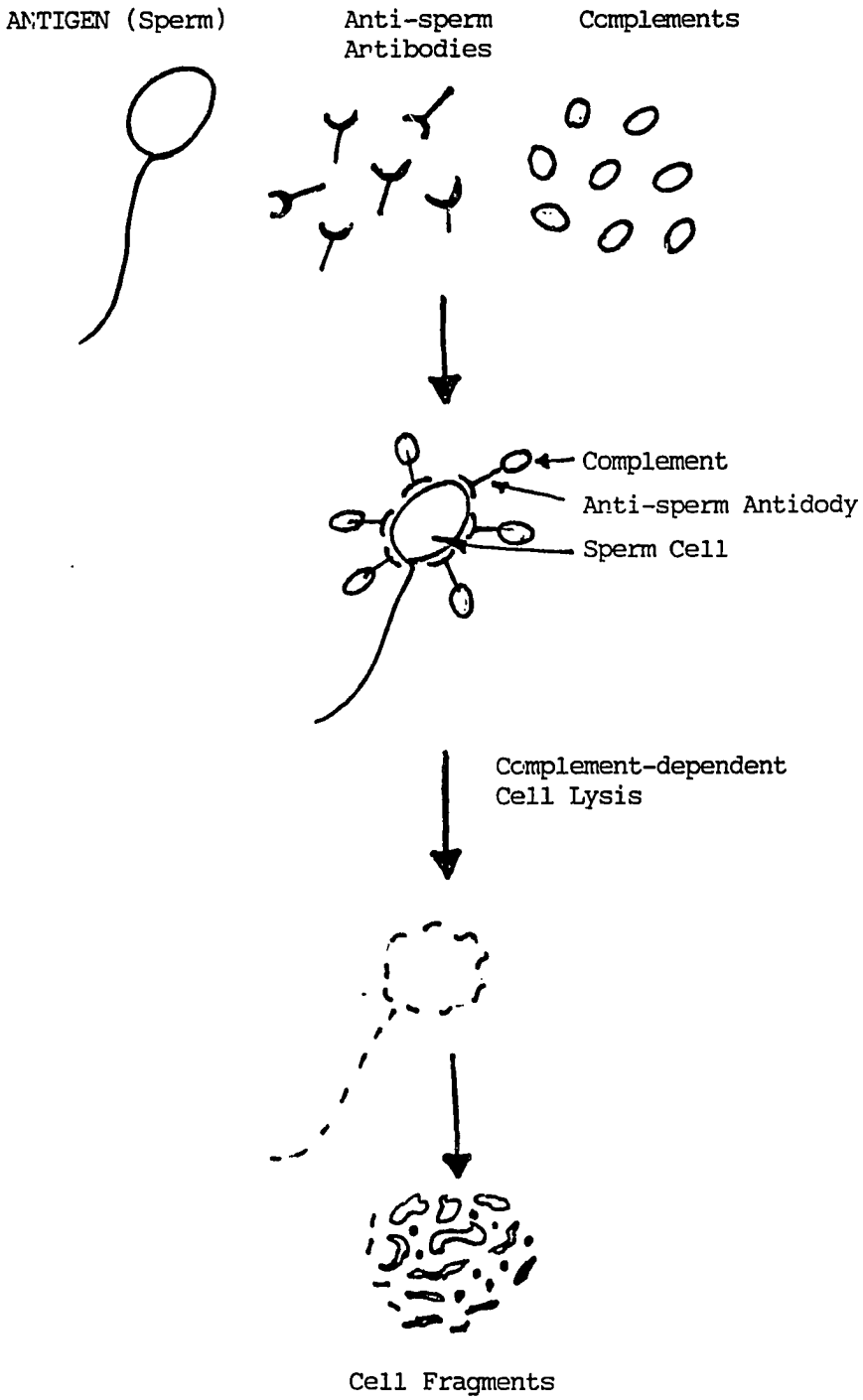
Monoclonal antibodies were evaluated for their ability to agglutinate and immobilize sperm with a modification of the methods described by Rose (Rose et al., 1976) and the Isojima sperm immobilization test (Isojima et al., 1968) respectively. The agglutination and immobilization assays were run concomitantly. For the immobilization test, swim-up washed human sperms were collected and resuspended to 40 million motile sperm/mL in BWW buffer pH 7.5. An aliquot of normal guinea pig sera (Cappel, Worthington, PA) was heat inactivated at 56°C for 30 minutes to remove complement activity and the assay was carried out in flexible flat bottom 96-well plates. Serial doubling dilutions of monoclonal antibodies were prepared for the immobilization assay by delivering 20 uL normal guinea pig sera to 6 wells in the 96-well tray and a parallel number of wells received 20 uL

of heat inactivated guinea pig sera. Into the first well of each series were delivered 20 uL monoclonal antibodies and 20 uL was propagated through the next 5 wells to give a 1:2 through 1:64 dilution series. Two uL of sperm were injected into each well and then all wells were covered with 2 drops of liquid paraffin oil and placed in a 37°C incubation chamber for 30 minutes. A proven positive and negative control ascites or serum diluted in saline were used to validate each assay. The sperm immobilization was determined by using a normal light microscope and immobilization values were recorded at which titer there were 0-5% mobilization. Fig. 11 illustrates the principle of complement-dependent sperm cell lysis (immobilization).

b. Sperm agglutination assay

The sperm agglutination test was run employing a similar scheme and similar materials. In a 96-well microtiter plate 12 series spots were prepared by adding 36 uL BWW to the first well and 20 uL BWW to all subsequent wells. Four uL of monoclonal antibodies were then delivered to the first well and after mixing, 20 uL of this mixture were propagated through the next 11 wells to give a 1/10 doubling dilution series. Two uL of sperm were delivered to each well followed by an overlay of two drops of liquid paraffin oil and incubated 2 hours at

FIG. 11 Principle of Complement-dependent Sperm Cell Lysis (Sperm Immobilization Assay)



37°C. Titer of 1:20 or higher giving agglutination was considered positive. Microscopic examination of the sperm also provided the information on the location of the antibody binding on the surface, eg on the head, tail, midpiece, or tail tip. The Fig. 12 illustrates some common sperm agglutination patterns.

c. Cervical Mucus Penetration Test

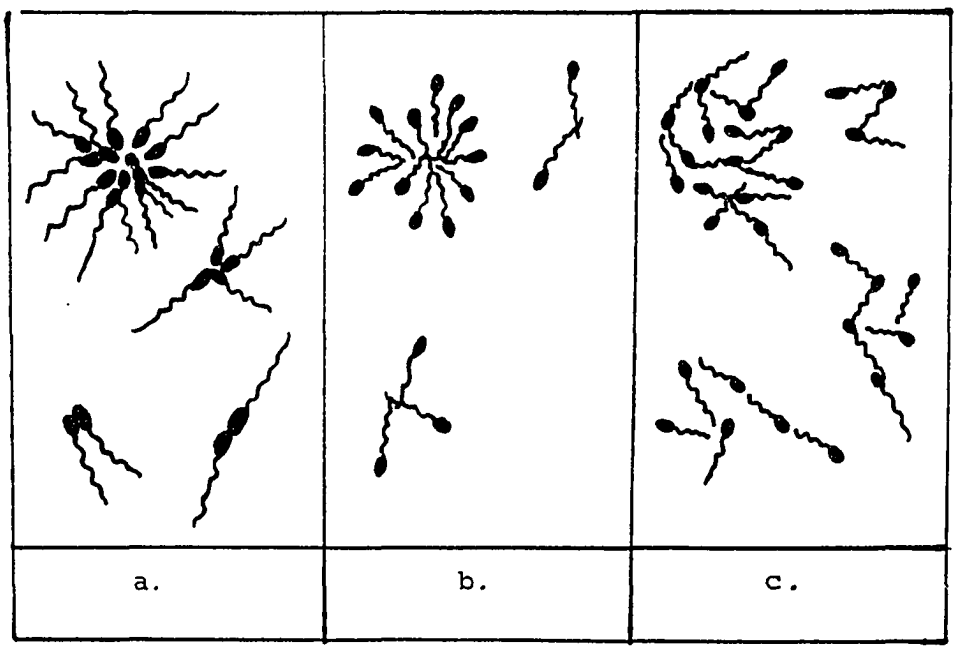
The procedure as described by Serono Diagnostics for the cervical mucus penetration assay (PENETRAK) was modified for the assessment of antibody interaction. Briefly, sperm (60×10^6/ml) were treated for one hour at 37°C in a humidified 5% CO_2 incubator with optimal antibody titer (in the case of agglutinating antibodies the same or one titer step beyond the agglutination titer of the antibody was used). Then the normal protocol for cervical mucus penetration was performed according to the manufacturer's directions (Serono Diagnostics, Braintree, MA).

d. Hamster Egg Penetration Assay (SPA)

The hamster egg penetration assay was used as an in vitro method for assessment of the sperm ability to undergo capacitation and the acrosome reaction, and to, subsequently bind and penetrate the oocyte plasma membrane and then to undergo decondensation within the

FIG. 12 Sperm Agglutination Patterns

a. head-to-head
b. tail-to-tail
c. head-to-tail



oocyte. The SPA technique was originally reported by Yanagimachi et al. (1976). Our laboratory has modified the procedure to test for the ability of antisperm antibodies to affect capacitation events as well as to post capacitation events. In brief, human sperm were incuba-ted with monoclonal antibodies during or after their capacitation period. All agglutinating MAbs were papain digested to collect the Fab fragments according to manufacturer's guidelines (Pierce, Rockford, IL). The medium used for handling sperm and oocyte prior to insemination was N-2-hydroxyethylpiperazine-N'-2-ethane-sulfonic-acid-buffered Biggers, Whitten, Whittingham (BWW) medium supplemented with BSA (Sigma Chemical Company, St. Louis, MO); penicillin; and streptomycin. Oocytes were obtained from mature hamsters given intraperitoneal injections of 40 IU of pregnant mare serum (Gestyl, Organon, Holland) 2 hours after initiation of the light phase of a 14:10 H light:dark cycle on the day of estrus (day of ovulation). Fifty-six hours later, the hamsters were injected IP with 40 IU of human chronic gonadotrophin hCG, (Sigma Chemical Company, St. Louis, MO). On the same day a fresh semen sample was collected and allowed to liquify at 37°C for 30 minutes. A one-half milliliter aliquot of semen under 1 mL BWW was distributed into 15 mL conical centrifuge tubes and incubated for 60-90 minutes at 37°C (swim-up procedure). The BWW

fractions were removed and evaluated for sperm motility and grade of progression. A sperm count was performed and the concentration of the mixture was adjusted to 10 million sperm with BWW media. Sperm were incubated overnight in 5% CO_2 and humid air at 37°C as the conical centrifuge tube was resting at a 30-40° angle.

Oocytes from superovulated hamsters were then collected from the oviducts 15-16 hours after the hCG injection. The surrounding cumulus cells were removed by treatment of the eggs with 0.1% hyaluronidase (Sigma Chemical Company, St. Louis, MO) in BWW medium. The zona pellucida were removed by treatment with 0.1% trypsin in BWW media (twice crystallized; Sigma Chemical Company, St. Louis, MO). The zona-free oocytes were then passed through five BWW washes to remove enzymes.

Concomitant with the egg processing, the sperm samples were evaluated and adjusted to 3.3 million motile sperm/mL of BWW media. A 100 ul drop of sperm was placed in a sterile plastic petri dish and covered with paraffin oil; 20-30 oocytes in 50 uL were deposited in the sperm drop (for a final concentration of 2.5 million motile sperm/mL) and incubated at 37°C in 5% CO_2 in humid air for 3 hours. Oocytes were evaluated for penetration after transfer of the eggs into fresh BWW medium to wash excess sperm from the egg and then placement of 10 eggs onto a slide (approximately 50 uL drop) and compression

of the eggs under a 22 x 22 cm coverslip. Penetration was evaluated by phase-contrast microscope at 400X. An egg was considered fertilized when it contained at least one swollen sperm head with an accompanying sperm tail.

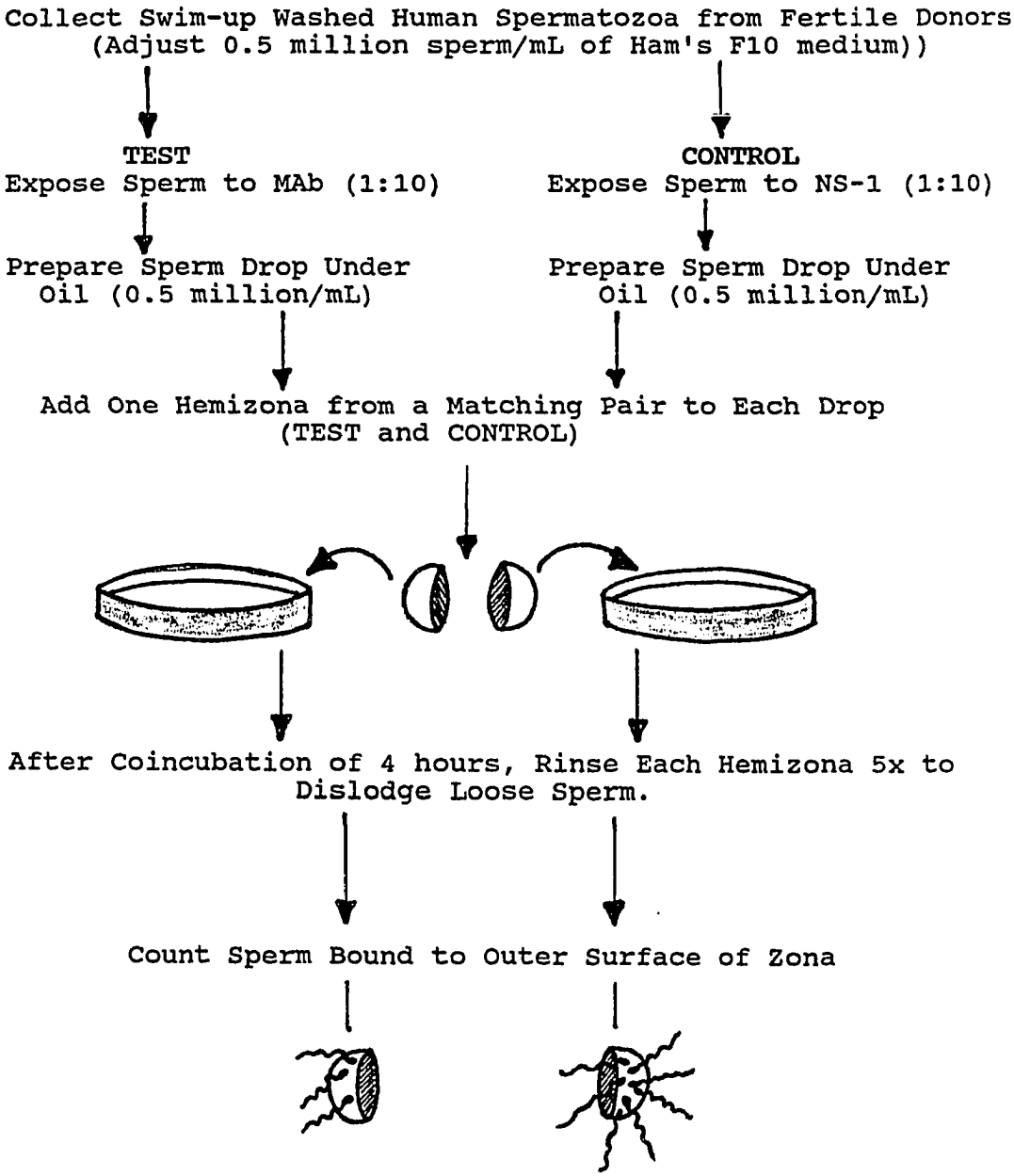
e. **Hemizona Test (HZA)**

The hemizona assay (HZA) has been used as a clinical tool to evaluate patients and predict fertilizing potential. Overstreet et al. (1976) first suggested the use of zona intact nonliving human oocyte to examine the sperm-zona pellucida binding. Researchers at our Institute have further modified this technique by taking the nonliving oocytes, cutting them in half by micromanipulation, removing the ooplasm and the two zona pellucida almost identical halves called the "hemizona". These halves are identical and therefore one half can provide an excellent internal control for the examination of various aspects of sperm-zona pellucida binding, a necessary prerequisite for human fertilization. Our purpose is to see whether the developed antisperm monoclonal antibodies could block the sperm-zona attachment, and thus prevent possible fertilization.

The procedure, as it is used for clinical testing, has been described in detail elsewhere (Franken et al. 1989). Briefly, human oocytes were obtained from cadaver tissues or surgically excised ovarian tissue. The

oocytes were stored until use at 4°C in a solution of 1.5 M magnesium chloride with 0.1% polyvinyl pyrolidone (PVP, MW 36,00) and 40 mM HEPES buffer to stabilize the pH at 7.0. One day prior to use, the salt stored oocytes were rinsed copiously in BWW supplemented with 0.35% BSA. The oocytes were cut in half using Narishige micromanipulator (Narishige, Tokyo, Japan) mounted on a phase-contrast microscope (Nikon Diaphot, Garden City, NY). The ooplasm was removed by pipetting each hemizona through a finely pulled pipette. Each pair of hemizona was stored overnight in a droplet of medium under mineral oil. The following day, the hemizona were rinsed in Ham's F-10 supplemented with human fetal cord serum and incubated at 37°C and 5% CO_2 in air. Semen specimens obtained from healthy proven fertile donors, were overlayed with medium to allow the sperm to swim-up into media. The motile fraction was collected and adjusted to a concentration of 0.5 million sperm/mL. One of the pair of hemizona was added to a control drop of sperm, while the other was added to the experimental drop (swim-up motile sperm incubated with specific monoclonal antibodies). After incubation, each hemizona was rinsed in fresh medium to dislodge loosely attached sperm, and the number of sperm tightly bound to the surface of the zona pellucida was counted. A flow diagram of the hemizona assay is shown in Fig. 13.

FIG. 13 The Hemizona Assay (HZA) Flow Diagram



SPECIFIC AIM NO. 10

Determine whether a carbohydrate moiety on the sperm surface may be involved in antigen.

Sodium Meta-Periodate Treated Sperm ELISA

To determine whether destruction of the carbohydrate moieties on sperm surface could affect antibody binding, human sperm aliquots were first treated with 0.5%, 1.0%, and 2.0% sodium meta-periodate solution and then the regular ELISA technique was performed as described previously. With each monoclonal antibody a control assay (untreated sperm ELISA) was also done to compare the percent of inhibition.

SPECIFIC AIM NO. 11

Determine whether a reduction in fertility occur in mice in vivo.

Passive Immunization of Mice With Monoclonal Antibodies

The purpose of this experiment was to determine the effects of passive immunization with monoclonal antisperm antibodies on mouse fertilization in vivo. The experimental method was followed according to the protocol described by Saling et al. (1985c). Briefly, female mice 5-8 weeks of age were superovulated by the standard protocol. At the time both the PMSG and hCG injections were given, each

female was given an additional IP injection of 0.5 mL monoclonal antibody. Following the hCG injection, the females were housed with a male (2 females and 1 male/cage). The following morning each female was checked for vaginal plugs. Only those females with vaginal plugs were considered in the subsequent experiments. Females were sacrificed 40-44 hours post-hCG injection and their oviducts were excised. Normal fertilization was assumed to have occurred when embryos (two cells or later stage) were recovered. The number of embryos and the number of uncleaved (one cell) oocytes were recorded. Four different doses of antibody were used (0.4, 1.0, 2.0, 4.0, mg/0.5 mL). The number of mice at each dose were 6 females and 3 males; so each test series involved 24 females and 12 males.

CHAPTER 6

RESULTS

A total of five hybridizations were done by fusing SP/O-Ag1.4 or NS-1 mouse myeloma cells with splenocytes from mice immunized with human sperm membrane antigens. Two extraction techniques were used for sperm membrane antigen isolation. In the first technique NP-40 detergent was used for antigen extraction from a pool of swim-up washed human spermatozoa collected from several donors. In the second technique mechanical homogenization of human testis was used for antigen extraction.

Among the 130 hybrid cell clones secreting monoclonal antisperm antibodies that were produced, 15 clones, based on their sperm specificity, antibody titers, antibody class, site of antigen recognition on the sperm surface, and the molecular weight of their binding antigens, were selected for further characterization. These hybridoma cell lines along with their antibody class (isotype) are given in Table 1. Eight out of fifteen were of the immunoglobulin (Ig) M and seven were IgG class. Among the seven IgG monoclonals; three were IgG_1, three were IgG_3, and one was IgG_{2b}.

TABLE 1 PROPERTIES OF FIFTEEN MONOCLONAL ANTIBODIES RAISED AGAINST HUMAN SPERM SURFACE ANTIGENS

Mabs	Ag prep	Ab Class	Ag location	Mol Wt. KD
DH3	NP-40	IgM	T,N, Ac	Sup. 68,25,14+/-
DH4	NP-40	IgM	T,N,PA	Sup. 65,25
DH13	NP-40	IgM	T,PA	Sup. >200
DH14	NP-40	IgM	T,N,PA	Sup. -
DH21	NP-40	IgM	N,PA,Eq	Asc. 68,45,37,23,14
DH22	NP-40	IgM	PA,T,N,Eq(+/-) *[Ac,PA,T(+/-)]	Asc. 68,14+/-
DH113	NP-40	IgG2b	Eq,N,PA	Sup. 65,50,14
DHF5	NP-40	IgG_3	T,PA	sup. 65,50
DH200	NP-40	IgG_3	WS	Sup. -
DH201	NP-40	IgG_1	T,PA	Sup. 14
DH202	NP-40	IgG_1	H	Sup. 14
DH203	NP-40	IgG_3	Ac,T	Asc. -
DH205	NP-40	IgG_1	H(rim),Eq,T	Sup. 14,20-25
DHTB11	Testicular extract	IgM	N,PA	Sup. 65
DHTol P3C9	NP-40	IgM	N,PA	Sup. 68, 65

T(tail), Ac(acrosome), N(neck), PA(postacrosome), H(head), WS(whole sperm), Eq(equatorial), Sup(culture supernatant), Asc(ascities), Mabs(monoclonal antibodies)
* on acrosome reacted sperm

Antigen localization

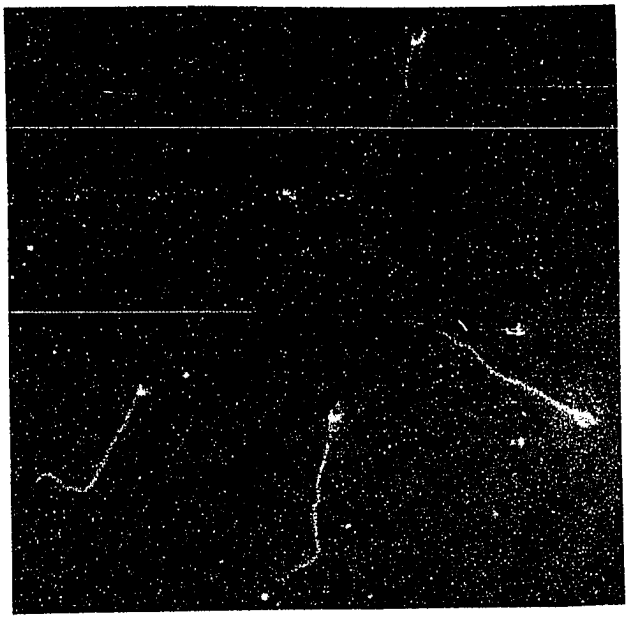
The locations of antigens recognized by the Mabs were examined using immunofluorescence (IF) and immunochemical staining (ICS) of methanol-fixed sperm from humans (Table 1). The fifteen antibodies all showed staining when biotin-avidin ICS and IF staining procedures were employed. These monoclonals often appeared to bind to multiple sites on human sperm. DH3 reacted with tail, neck, and acrosome regions; DH200 reacted with whole sperm; DH201 with tail and post-acrosome; whereas DH202 reacted with the entire head region of sperm. Among the others, DH4 and DH14 reacted with the tail, neck, and post-acrosome; DH13, DH201, and DHF_5 with the tail and post-acrosome; DH21 with the neck, post-acrosome and equatorial region; DH22 with the post-acrosome, tail, neck, and equatorial (some) of non-acrosome reacted sperm and acrosome, post-acrosome, and tail (+/-) of acrosome reacted sperm; DH113 with the equatorial, neck, and post-acrosome; DH203 with the acrosome and tail; DH205 with the head rim, equatorial and tail; $DHTB_{11}$ and $DHTolP_3C_9$ with the neck and post-acrosome region of human spermatozoa. Some of these MAb binding patterns with human sperms are shown in Fig. 14(a-g).

FIG. 14

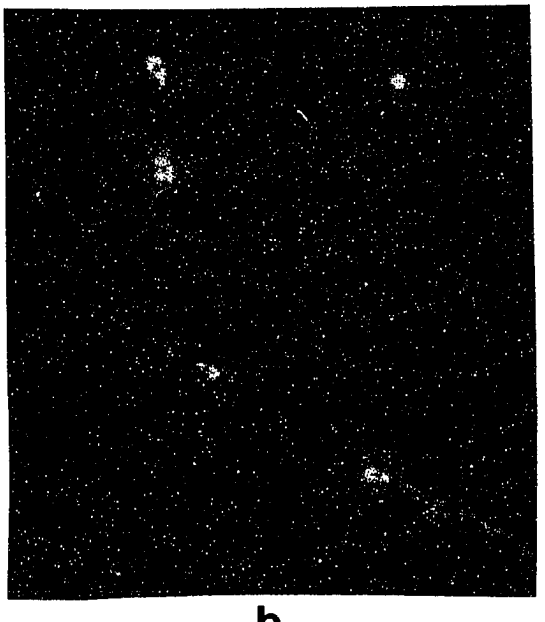
a. Antisperm MAb DH14 bound to postacrosome, neck and tail of human sperm (by IF).

b. Antisperm MAb DH22 bound to acrosome, post acrosome, and tail (+/-) of acrosome-reacted human sperm (by IF).

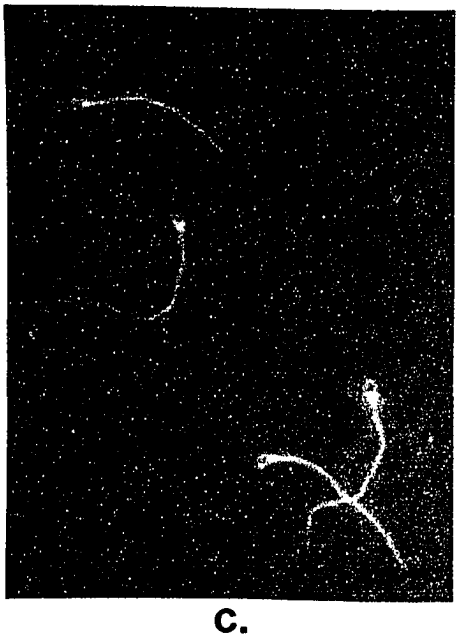
c. Antisperm MAb DH22 bound to postacrosome, equatorial (+/-), neck, and tail of fresh (not acrosome-reacted) human sperm (by IF).



a.



b.



c.

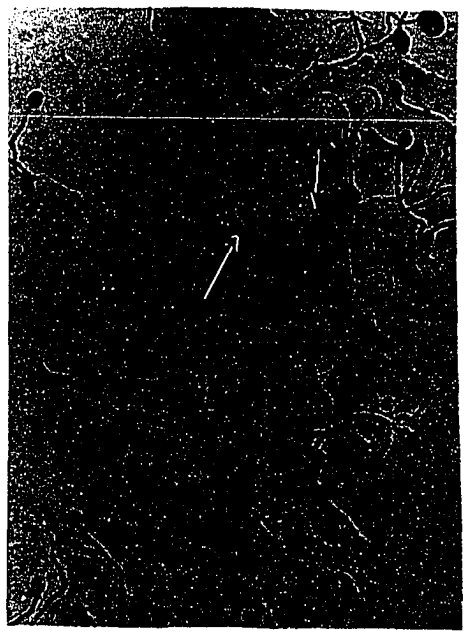
FIG. 14

d. Antisperm MAb DH13 bound to postacrosome and tail of human sperm (by ICS).

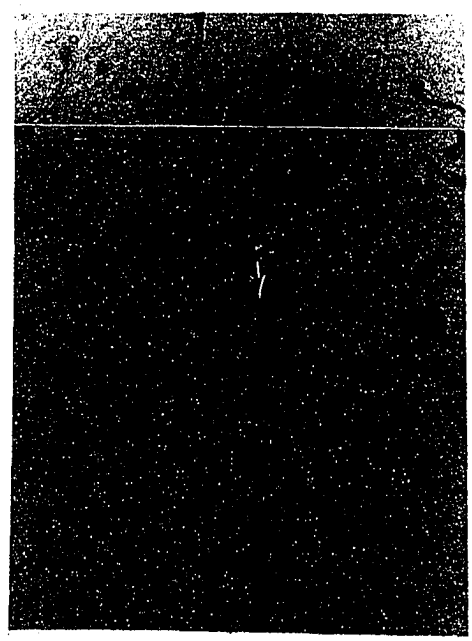
e. Antisperm MAb $DHTB_{11}$ bound to postacrosome and neck of human sperm (by ICS).

f. Antisperm MAb DH202 bound to the entire head of human sperm (by ICS).

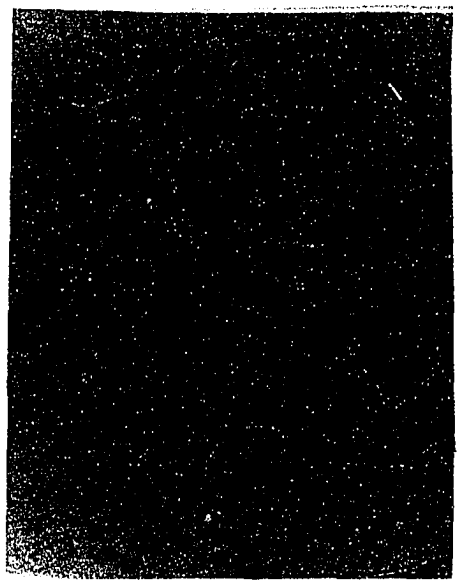
g. Antisperm MAb DH200 bound to all regions of human sperm (by ICS).



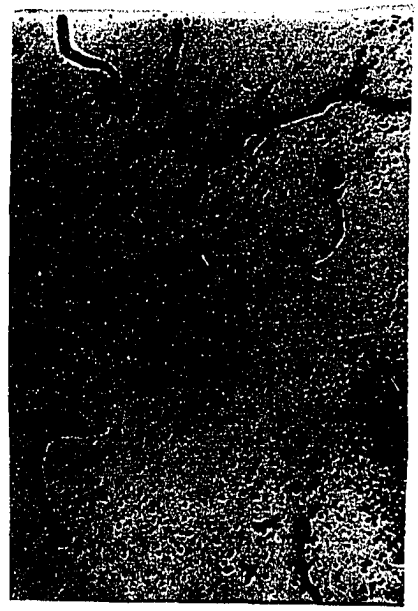
d.



e.



f.



g.

Molecular Weight of Corresponding Antigens

These monoclonal antibodies revealed multiple bands by immunoblotting procedures. DH3 and DH4 recognized antigens having apparent molecular weights of 68 and 25 KD. In addition to the 68 and 25 KD bands, DH3 also recognized a 14 KD region. DH13 recognized antigens with a 273 KD, and DH21 recognized 68, 45, 37, 23, and 14 KD bands. DH 22 recognized two bands, 68 and 14 KD, DH 113 recognized three bands of 65, 50, and 14 KD, DHF_5 and DH201 each recognized one band of 14 KD, and DH205 recognized two bands of 20 and 14 KD. Immunoblot of antisperm MAbs binding to sperm protein extract is shown in Fig. 15.

Reactivity of the Monoclonals with Fresh, Capacitated, and Acrosome Reacted Human Sperm by ELISA and ICS

Reactivity of the monoclonals with fresh, capacitated, and calcium ionophore treated (acrosome reacted) human sperm was evaluated by ELISA and ICS. The results are shown in Table 2. One monoclonal, DH22, showed higher reactivity with acrosome reacted spermatozoa than to fresh and capacitated human spermatozoa. All others showed higher reactivity with fresh and capacitated spermatozoa in contrast to acrosome reacted human spermatozoa.

FIG. 15

Immunoblot of antisperm MAbs binding to NP-40 extract proteins of human sperm. Sperm extract was electrophoresed under reduced conditions.

Lane 1 Known molecular weight markers of lysosome, 14KD; beta lactoglobin, 18KD; alpha chymotrypsinogen, 25KD; ovalbumin, 43KD; bovine serum albumin, 68KD; phosphorylase b, 97KD; and myosin 200KD.

Lane 2 Antisperm MAb DH3 recognized 68, 25, and 14 KD.

Lane 3 Antisperm MAb DH4 recognized 65 and 25 KD.

Lane 4 Antisperm MAb DH13 recognized >200 KD.

Lane 5 Antisperm MAb DH14 did not recognized any protein band.

Lane 6 Antisperm MAb DH21 recognized 68, 45, 37, 23, and 14 KD.

Lane 7 Antisperm MAb DH22 recognized 68 and 14 KD.

Lane 8 Antisperm MAb DH113 recognized 65, 50, and 14 KD.

Lane 9 Antisperm MAb DHF5 recognized 65 and 50 KD.

Lane 10 Antisperm MAb DH200 did not recognized any protein band.

Lane 11 Antisperm MAb DH201 recognized 14 KD.

Lane 12 Antisperm MAb DH202 recognized 14 KD.

Lane 13 Antisperm MAb DH203 did not recognized any protein band.

Lane 14 Antisperm MAb DH205 recognized 14 and 20-25 KD.

Lane 15 Antisperm MAb DHTB11 recognized 65 KD.

Lane 16 Antisperm MAb DHTolP3C9 recognized 68 and 65 KD.

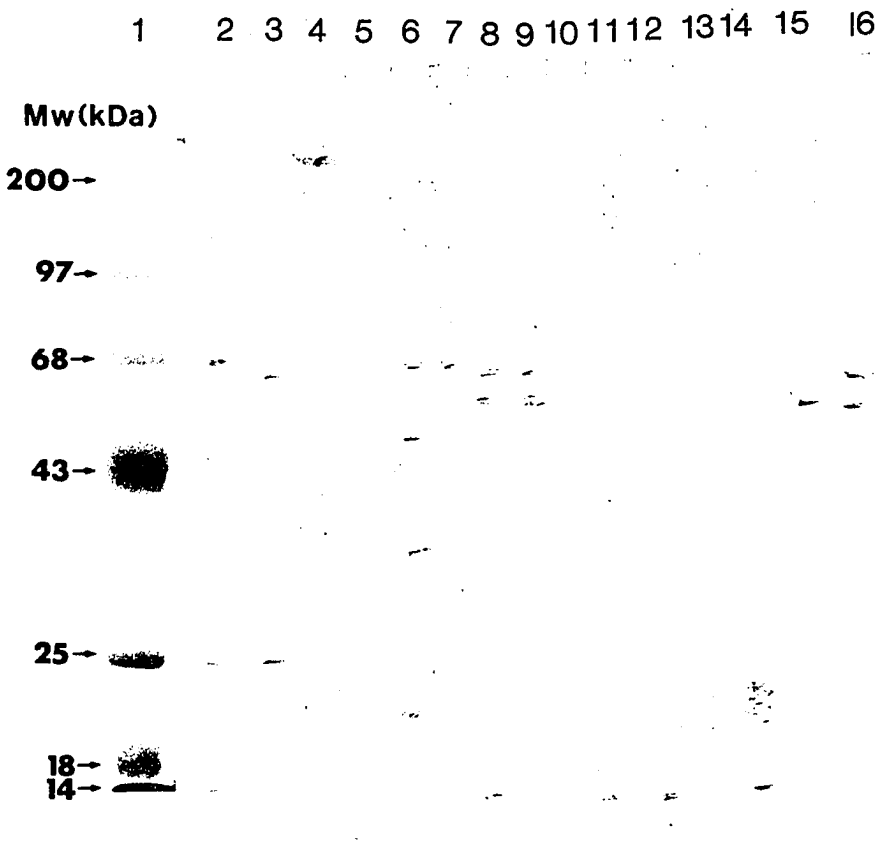


TABLE 2 REACTIVITY OF MONOCLONAL ANTIBODIES WITH FRESH, CAPACITATED, AND ACROSOME REACTED SPERMATOZOA

MAbs	Fresh ELISA/ ICS	Capacitated ELISA/ ICS	Acrosome reacted ELISA/ ICS
DH3	++/ T,N,Ac	++/ Eq,T	+/ T*,Eq*
DH4	++/ T,N,PA	++/ T,N	+/ T*,N*
DH13	++/ T,PA	++/ WS	+/ T*,Eq*
DH14	++/ T,PA	++/ WS	+/ WS*
DH21	++/ Eq,PA,N	++/	+
DH22	++/ PA,T,N,Eq	++/PA,T,N,Eq	+++/ Ac,PA,T,N,
DH113	++/ Eq,PA,N	++	+/
DHF5	++/ T,PA	++/ T,PA	+/ -
DH200	++/ WS	++/ N,Eq	+/ -
DH201	++/ T,PA	++/ WS	+/ -
DH202	++/ H	++/	+/
DH203	++/ Ac,T	++/ H	+/T,H*
DH205	++/ H,T	++/ Eq,T	+/-
DHTB11	++/ PA,N	++/ Eq,T	+/
DHTolP3C9	++/ PA,N	++/	+/

\+ optical density 2-3 times above background
++ optical density 8-10 times above background
+++ optical density >10 times above background
Fresh swim-up washed human sperm coated on spot slide and air dried
Capacitated swim-up sperm with BWW media incubated overnight at 37°C, 5% CO_2 then washed and coated on spot slide and air dried.
Acrosome-reacted swim-up sperm with BWW media and calcium ionophore are incubated overnight at 37°C, 5% CO_2 then washed and coated on spot slide and air dried.
ELISA Enzyme Linked Immuno Sorbant Assay
ICS Immunocytochemical Staining
* Faint staining
Ac(acrosome), Eq(equatorial), H(head), N(neck), PA(postacrosome), T(tail), WS(whole sperm)

Studies on Surface vs Internal Antigens

To test whether these Mabs are reactive against the sperm membrane surface or internal antigens, we tested them with a live sperm ELISA and with immunobead assays (Table 3). All of the fifteen MAbs showed positive binding with live sperm. Selecting antibodies which reacted only to surface antigens was one of the goals of our study.

Specificities of Monoclonal Antisperm Antibodies

Sperm specificity of these monoclonal antibodies was investigated by ELISA with white blood cells (WBC), red blood cells (RBC), lymphoblastoma (LBO) cell line, bacteria, human seminal plasma, human albumin, and human lactoferrin. As shown in Table 4, none of these monoclonals reacted with either WBC, RBC, LBO, bacteria, human albumin, or human lactoferrin. DH 200-203 slightly and DH205 moderately reacted with seminal plasma.

Cross reactivity of these monoclonals with various frozen human somatic tissue sections (myometrium, adrenal, thyroid, liver, lungs) was investigated by immunofluorescence and immunochemical staining. Results are shown in Table 4. None of these monoclonals reacted with any of these human somatic tissue sections tested.

TABLE 3 STUDIES ON SURFACE VS INTERNAL ANTIGENS

MAbs	Live sperm ELISA	Immunobead Test (IBT)
DH3	++	N,T
DH4	++	N,T
DH13	++	H,T
DH14	++	H,T
DH21	++	H,N
DH22	++	H,T
DH113	++	H,N
DHF_5	++	H,T
DH200	++	H,T
DH201	++	T
DH202	++	H
DH203	++	H,T
DH205	++	H,T
$DHTB_{11}$	++	H,N
$DHTolP_3C_9$	++	H,N

++ 8-10 times above background
H (head), N (neck), T (tail)
ELISA (enzyme-linked immunosorbant assay)
MAbs (monoclonal antibodies)

TABLE 4 SPECIFICITY OF ANTISPERM MONOCLONAL ANTIBODIES

MAbs	WBC	RBC	LBO	Bact.	Hu Sem. Plasma	lacto.	Hu. Alb
DH3	-	- *	- *	-	-	-	-
DH4	-	- *	- *	-	-	-	-
DH13	-	- *	- *	-	-	-	-
DH14	-	- *	- *	-	-	-	-
DH21	-	- *	- *	-	-	-	-
DH22	-	- *	- *	-	-	-	-
DH113	-	- *	- *	-	-	-	-
DHF5	-	- *	- *	-	-	-	-
DH200	-	-	- **	-	+/-	-	-
DH201	-	-	- **	-	+/-	-	-
DH202	-	-	- **	-	+/-	-	-
DH203	-	-	- **	-	+/-	-	-
DH205	-	-	- **	-	+	-	-
DHTB11	-	- *	- **	-	-	-	-
DHTol P_3C_9	-	- *	- **	-	-	-	-

* hybridoma supernatant tested as 1:100 dilution
** hybridoma supernatant tested as 1:50 dilution (all other tests were done as neet by using hybridoma supernatant)
+/- when ELISA optical density twice than negative control
WBC (white blood cell), RBC (red blood cell),
LBO (lymphocyte), Bact (bacteria staphylococci, streptococci, salmonella, klebsiella), lact. (lactoferrin), Alb. (human albumin)

Localization of Antigenic Epitopes Corresponding to MAbs in Reproductive System

To determine the possible origination of the antigenic epitopes corresponding to these MAbs in the reproductive system, immunofluorescence and immunochemical staining techniques were used for staining human frozen testes, epididymis (caput, corpus, cauda) vas, seminal vesicle, and prostate tissue sections (Table 5). DH3, DH113, DH200-203, and $DHTB_{11}$ bound to spermatozoa and spermatid in the testis; DH4, DH14, and DH205 bound to epithelium of the seminal vesicle; DH13, DH21, DH22 and DHF_5 bound to glandular epithelium of the cauda and sperm in the cauda; and DHTol-P_3C_9 bound to sperm in the testes, the epididymis (caput, corpus, and cauda), and the vas defferens. Some of these MAbs binding patterns on different tissue sections are shown in Fig. 16 (a-h).

Cross-Reactivity of Monoclonal Antibodies With Other Species' Spermatozoa

Reactivity of these antisperm monoclonal antibodies with other species' spermatozoa (monkey, dog, rabbit, mouse, rat, and bull) was done by immunochemical staining and immunofluorescence study (results are shown in Table 6). Most of these monoclonal antibodies showed reactivity with sperm of several species; DH 3 reacted with all species tested except monkey sperm; DH4 with dog and bull sperm; DH13 reacted with

TABLE 5
CROSS REACTIVITY STUDY AGAINST HUMAN TISSSUES OF ANTI-HUMAN SPERM MONOCLONAL ANTIBODIES BY IMMUNOFLUORESCENCE STUDY

MAbs	Prost	Myomet	Adren	Thyroid	Liver	Lungs	Testes	Caput	Cauda	Vas	sem
DH3	-	-	-	-	-	-	+	-	-	-	-
DH4	-	-	-	-	-	-	-	-	-	-	+
DH13	-	-	-	-	-	-	-	-	+	-	-
DH14	-	-	-	-	-	-	-	-	-	-	+
DH21	-	-	-	-	-	-	-	-	+	-	-
DH22	-	-	-	-	-	-	-	-	+	-	-
DH113	-	-	-	-	-	-	+	-	-	-	-
DHF5	-	-	-	-	-	-	-	+	+	-	-
DH200	-	-	-	-	-	-	+	-	-	-	-
DH201	-	-	-	-	-	-	+	-	-	-	-
DH202	-	-	-	-	-	-	+	-	-	-	-
DH203	-	-	-	-	-	-	+	-	-	-	-
DH205	-	-	-	-	-	-	-	-	-	-	+
DHTB11	-	-	-	-	-	-	+	-	-	-	-
TolP3C9	-	-	-	-	-	-	+	+	+	+	-

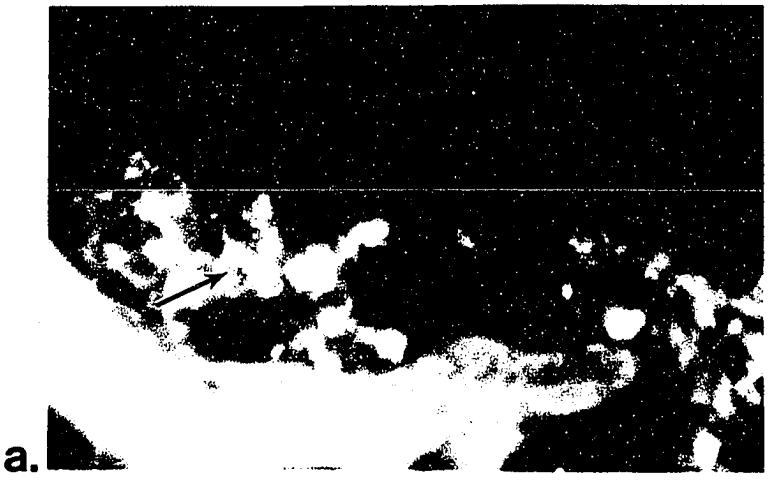
prost(prostate gland), myomet(myometrium), adren(adrenal gland), sem(seminal vesicle)

FIG. 16

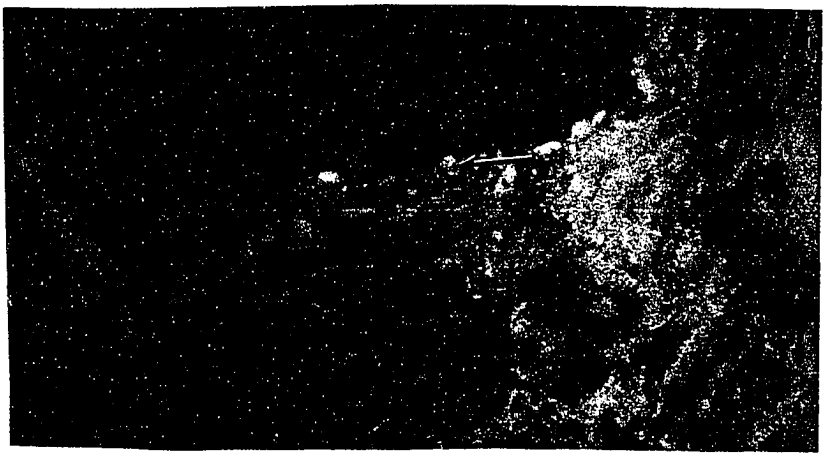
a. Antisperm MAb DHF_5 bound to caudal region of human epididymis (by IF).

b. Antisperm MAb DH13 bound to caput region of human epididymis (by IF).

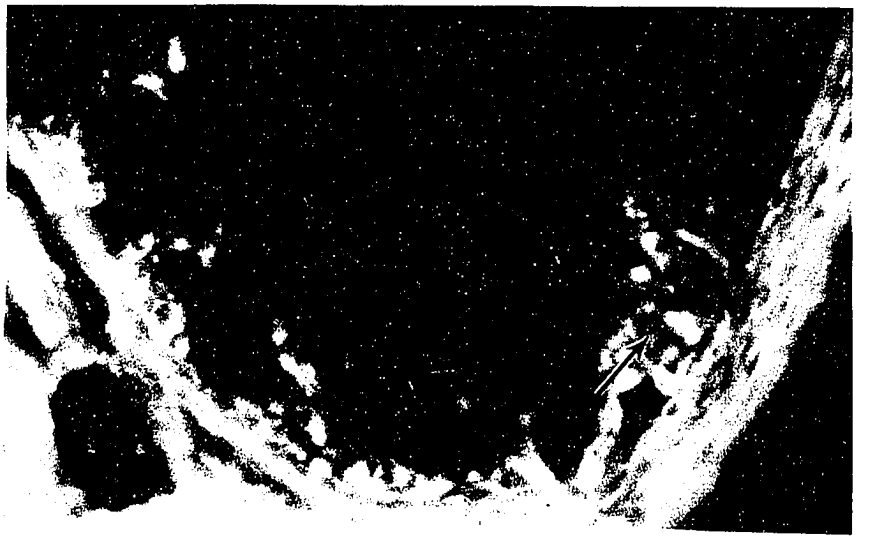
c. Antisperm MAb DHP_3C_9 bound to caput region of human epididymis (by IF).



a.



b.



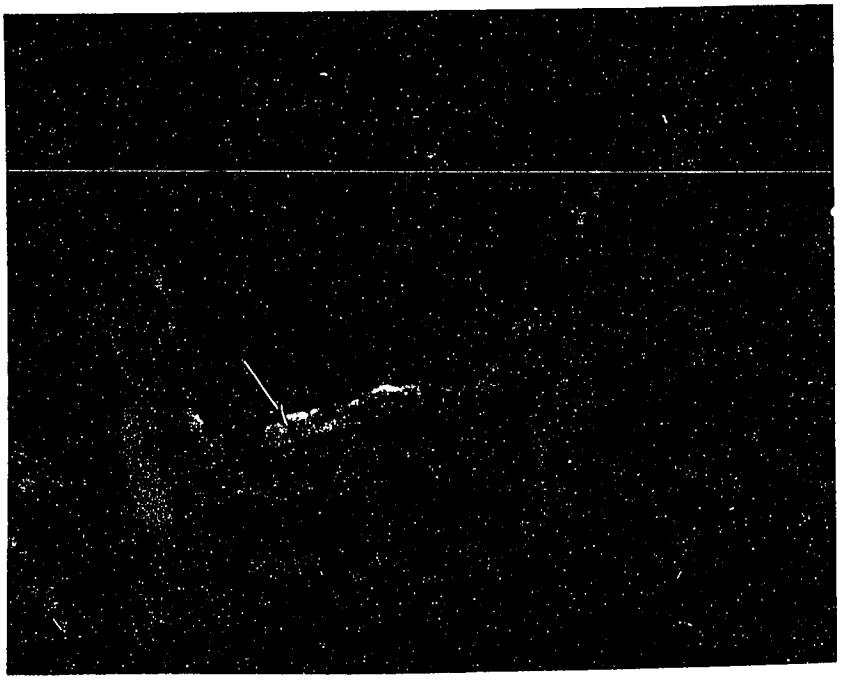
c.

FIG. 16

d. Antisperm MAb DH4 bound to human seminal vesicle components (by IF).

e. Antisperm MAb DH14 bound to human seminal vesicle components (by IF).

d.



e.

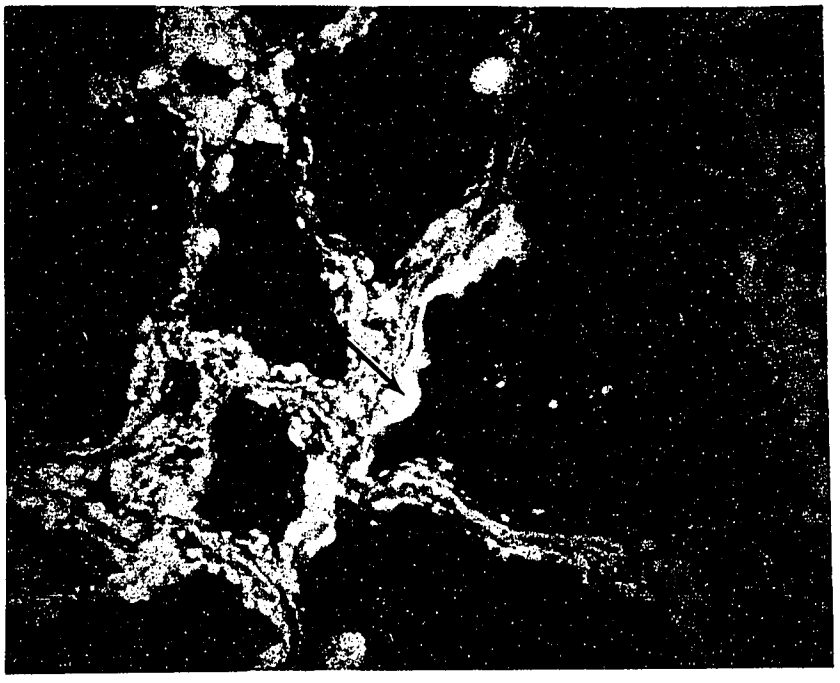
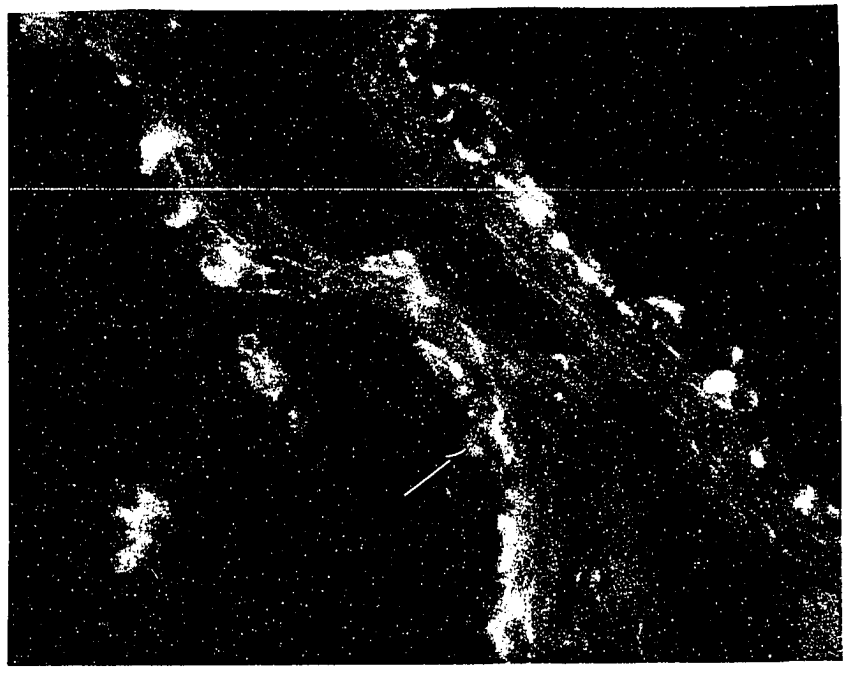


FIG. 16

f. Antisperm MAb DH3 bound to human testis components (by IF).

g. Antisperm MAb DHTolP_3C_9 bound to human testis components (by IF).

f.



g.

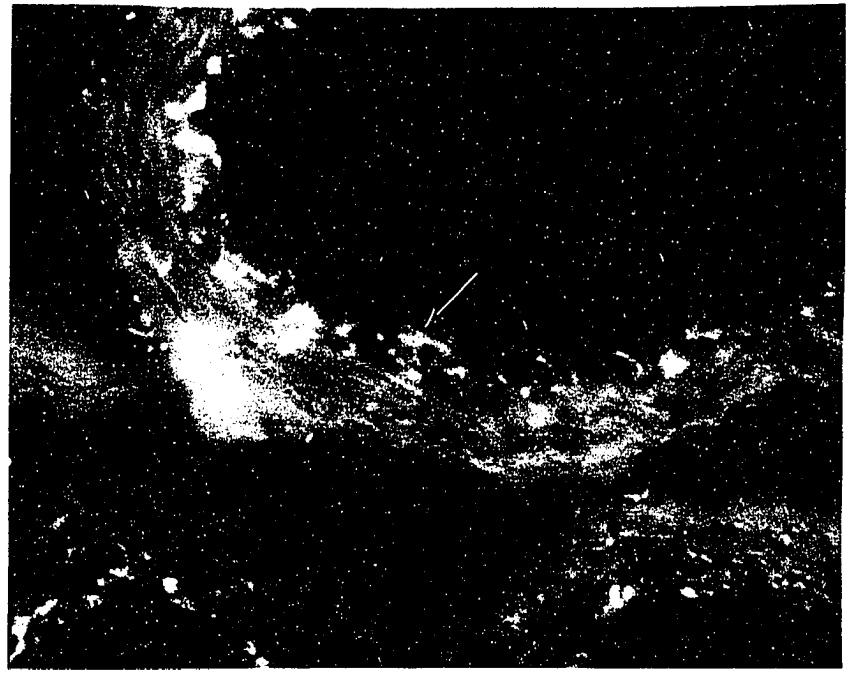
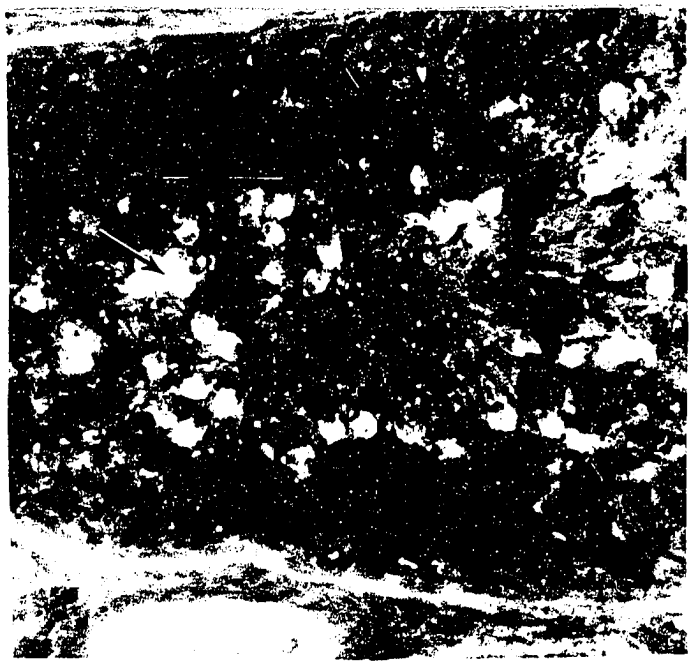


FIG. 16

h. Antisperm MAb $DHTB_{11}$ bound to human testicular spermatozoa and spermatids (by IF).



h.

TABLE 6 REACTIVITY OF ANTI-HUMAN SPERM MONOCLONAL ANTIBODIES WITH DIFFERENT SPECIES SPERM BY IMMUNOCHEMICAL STAINING (ICS) STUDY

MAbs	Monkey	Dog	Rabbit	Mouse	Rat	Bull
DH3	-	Ac,N	Ac	Ac,T	WS	Ac
DH4	-	Ac	-	-	-	Ac
DH13	Ac,T	Ac,T	Ac,T	Ac,T	ND	Ac
DH14	-	Ac	Ac+/-	-	-	Ac
DH21	-	Ac	-	-	-	Ac
DH22	Ac,Eq,T	-	ND	Ac,N	ND	Ac
DH113	ND	Ac	ND	ND	ND	Ac
DHF5	-	ND	Ac	T	Mp	Ac
DH200	-	-	-	T	ND	Ac
DH201	Ac	Ac	Ac	Ac	ND	Ac
DH202	Eq,T	-	Ac,N,Mp	H	-	Ac
DH203	Ac	-	Ac	H	-	Ac,T
DH205	-	Eq	ND	-	-	ND
$DHTB_{11}$	-	Ac,Eq	Ac,N	T	Mp	Ac,N
$DHTolP_3C_9$	Ac,T	Ac,Mp	ND	Ac,Mp	Ac(tip), Mp	Ac,Mp
(-)control	-	-	-	-	-	-

Ac(Acrosome), Eq(equatorial), N(neck), T(tail), Mp(midpiece), H(head), ND(notdone)

all species' sperm; DH14 reacted with dog, rabbit, and bull sperm; DH21 reacted with dog and bull sperm. DH22 reacted with all except dog sperm; DH113 and DHF_5 reacted with rabbit, mouse, rat, and bull sperm; DH200 with mouse and bull sperm; DH201 reacted with all species' sperm; DH202 and DH203 reacted with all except dog and rat sperm; DH 205 reacted with dog and bull sperm; and $DHTolP_3C_9$ reacted with monkey, dog, mouse, rat, and bull sperm. Some of these MAbs binding patterns with different species' sperm are shown in Fig. 17 (a-k).

Sperm Agglutination And Immobilization Assay

To see whether any inhibition of sperm functional tests (SA/SI) can occur by these monoclonal antibodies, micro sperm agglutination and immobilization assays were done (data shown in Table 7a). Among fifteen monoclonal antibodies; DH200, DH201, DH202, and DH203 caused agglutination and DH200, DH202, and DH203 immobilized human spermatozoa in the presence of complement. Furthermore, these same four monoclonals caused agglutination and immobilization of mouse spermatozoa (Table 7b). The pattern of agglutination was chain with DH200, cluster with DH201 and DH202, and mixed with DH203.

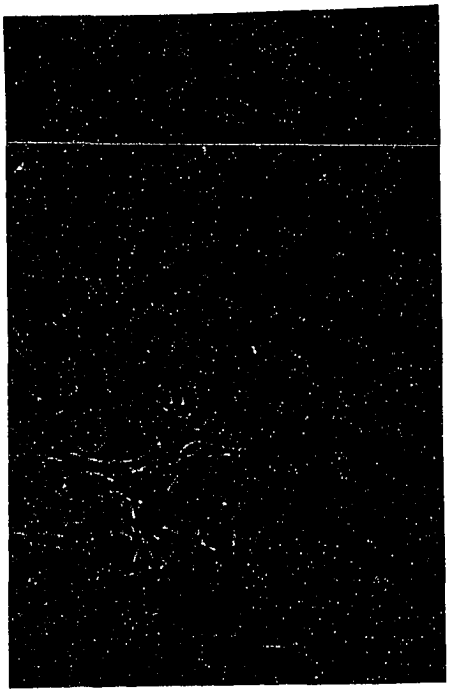
FIG. 17

a. Antisperm MAb DHTolP_3C_9 bound to acrosome and midpiece of bull sperm (by ICS).

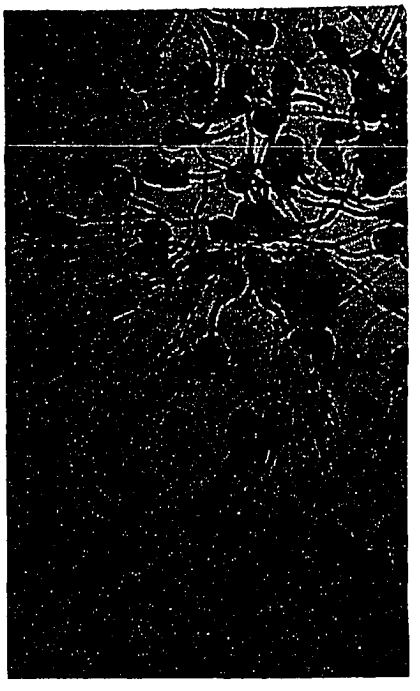
b. Antisperm MAb DH3 bound to acrosome of bull sperm (by ICS).

c. Antisperm MAb DH13 bound to acrosome of bull sperm (by ICS).

d. Antisperm MAb $DHTB_{11}$ bound to acrosome and neck of rabbit sperm (by ICS).



a.



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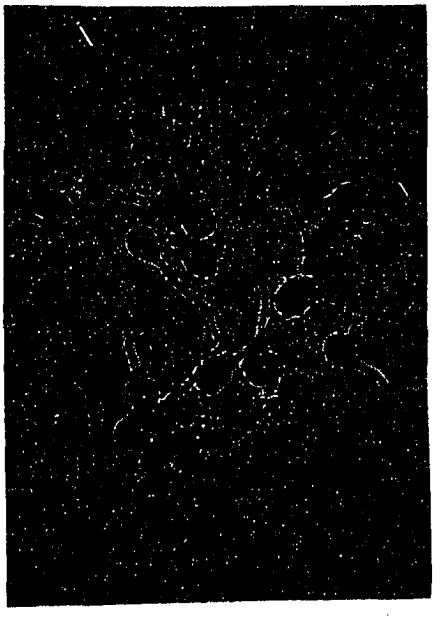
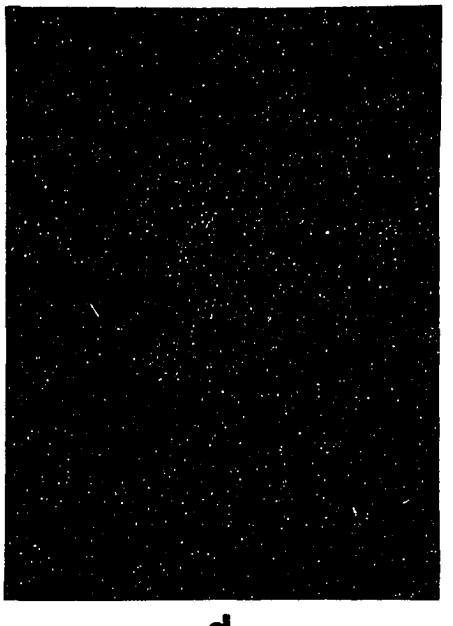


c.



d.

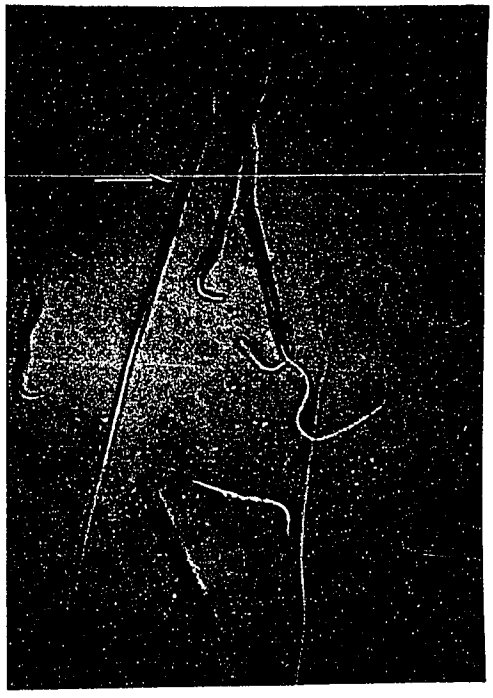
FIG. 17

e. Antisperm MAb $DHTB_{11}$ bound to midpiece of rat sperm (by ICS).

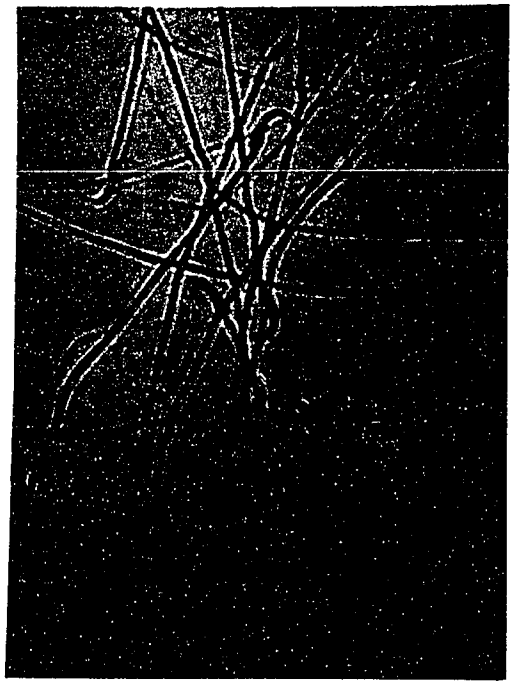
f. Antisperm MAb DH3 bound to all regions of rat sperm (by ICS).

g. Antisperm MAb DH3 bound to acrosome and tail of mouse sperm (by ICS).

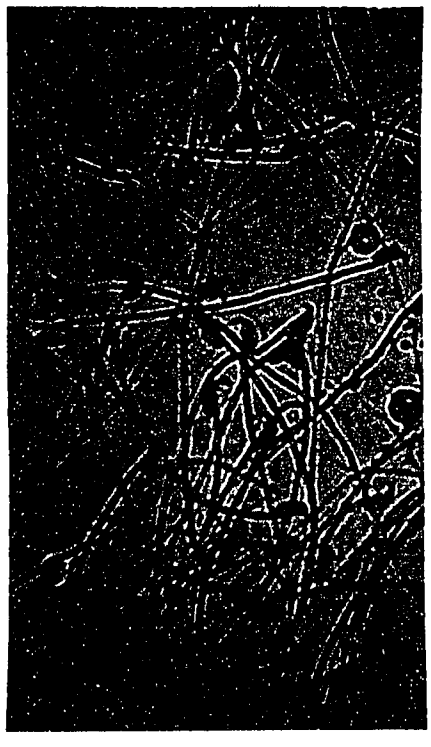
h. Antisperm MAb $DHTolP_3C_9$ bound to acrosome and midpiece of mouse sperm (by ICS).



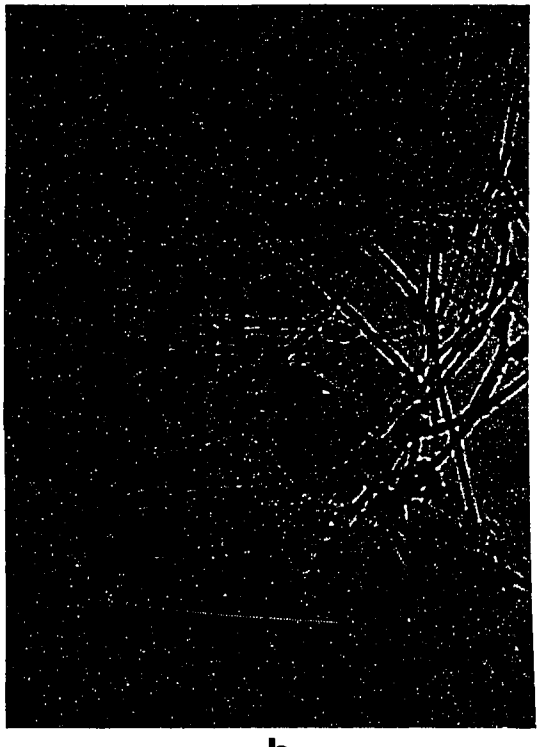
e.



f.



g.



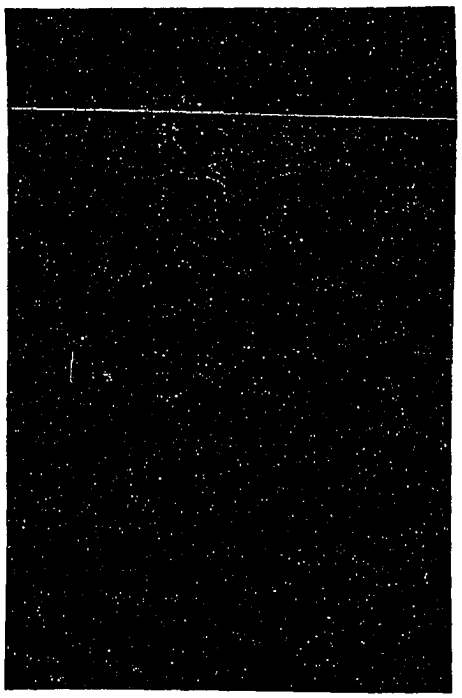
h.

FIG. 17

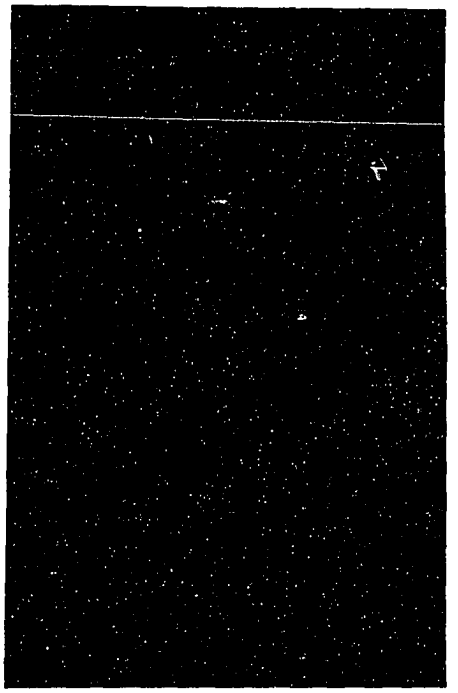
i. Antisperm MAb DH3 bound to acrosome and neck of dog sperm (by ICS).

j. Antisperm MAb DH202 bound to acrosome, neck, and midpiece of rabbit sperm (by ICS).

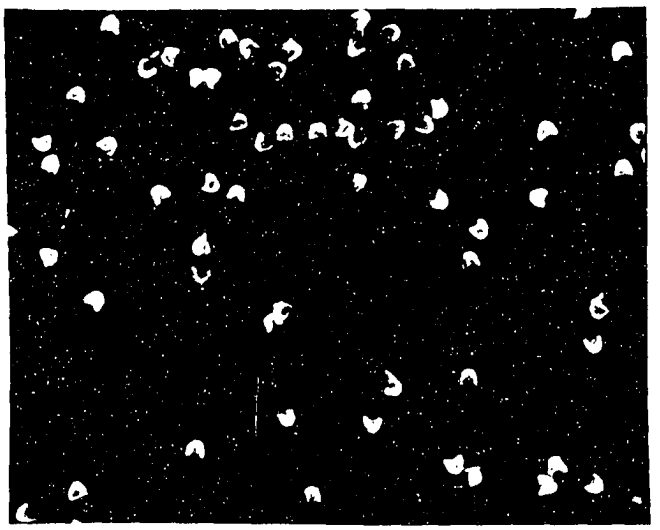
k. Antisperm MAb DH203 bound to acrosome of monkey sperm (by IF).



i.



j.



k.

TABLE 7a

REACTIVITY OF MONOCLONAL ANTI-HUMAN SPERM ANTIBODIES IN SPERM IMMOBILIZATION AND SPERM AGGLUTINATION ASSAYS WITH HUMAN SPERM

MAbs	Immobilization	Agglutination	
DH3	-	-	
DH4	-	-	
DH13	-	-	
DH14	-	-	
DH21	-	-	
DH22	-	-	
DHF_5	-	-	
DH113	-	-	
DH200	>64	>20480	(chain)
DH201	-	>20480	(cluster)
DH202	16	>20480	(cluster)
DH203	>64	>20480	(mixed)
DH205	-	-	
$DHTB_{11}$	-	-	
$DHTolP_3C_9$	-	-	

TABLE 7b

REACTIVITY OF MONOCLONAL ANTI-HUMAN SPERM ANTIBODIES IN SPERM IMMOBILIZATION AND SPERM AGGLUTINATION ASSAYS WITH MOUSE SPERM

MAbs	Immobilization	Agglutination
DH3	-	-
DH13	-	-
DH22	-	-
DHF_5	-	-
DH200	>64	>20480
DH201	>64	>20480
DH202	>64	20480
DH203	>64	>20480
$DHTB_{11}$	-	-
$DHTolP_3C_9$	-	-

- (negative), 64 (at 1:64 dilution), 20480 (at 1:20480 dilution)

Hamster Egg Penetration Assay

The ability of these antisperm monoclonal antibodies to prevent human spermatozoa to penetrate zona free hamster oocyte was investigated and results are given in Table 8. Among these fifteen monoclonals, DH3, DH13, DH14, DH22, DH205, and $DHTolP_3C_9$, significantly inhibited hamster egg penetration. Only DH3 and DH13 inhibited human sperm penetration of zona free hamster eggs when treated with precapacitated sperm but not post-capacitated sperm. On the other hand, MAb DH22 showed stronger inhibition when incubated with post-capacitated sperm.

Hemizona Assay

The hemizona assay (sperm functional test) was done with MAbs to evaluate inhibition of sperm egg attachment due to binding of these antibodies. Two to four hemizona were assessed for each MAb. These MAbs were also sent to another laboratory (Jane Rogers, Vanderbilt University, TN) to repeat the assay and to confirm the test results. A hemizona index (HZI) calculated by counting the number of tightly bound sperm on the test zona and dividing by the number of tightly bound sperm on control zona. This number was multiplied by 100 to obtain the percentage. A HZI of less than 50% was considered to represent significant inhibition of sperm egg attachment probably due to the presence of antisperm antibodies. Of the 15 MAbs tested, 7 inhibited

TABLE 8 ZONA FREE HAMSTER EGG PENETRATION ASSAY** (SPA)

	Normal Donor	DH3	DH4	NS-1
SPA	100%	34%, *92%	78%,*88%	100%
PI	4.5	0.55, 3.5	1.6, 5.2	6.6
%Inhib.	0%	66% 8%	22%,*12%	0%
	Normal Donor	**DH13**	**DH14**	**NS-1**
SPA	92%	30%, *90%	32%,*25%	100%
PI	3.6	0.4, 2.5	1.25	4.5
%Inhib.	8%	70%, *10%	68%,*75%	0%
	Normal Donor	**DH22**	**DHF_5**	**NS-1**
SPA	100%	56% *25%	66%	93%
PI	3.0	0.87	0.83	3.5
%Inhib.	0%	44%,*75%	34%	7%
	Normal Donor	**$DHTolP_3C_9$**	**DH201**	**NS-1**
SPA	100%	42%	71%	86%
PI	3.0	0.5	1.1	
%Inhib.	0%	56%	29%	14%
	Normal Donor	**DH21**	**DH205**	**NS-1**
SPA	95%	54%	59%	96%
PI	3.3	0.75	0.95	3.8
%Inhib.	5%	46%	41%	4%
	Normal Donor	**DH203**	**DH113**	**NS-1**
SPA	100%	75%	70%	93%
PI%	5.7	1.2	0.95	3.5
%Inhib.	0%	25%	30%	7%

* short preincubation in presence of antibody (1.5 hour)

$$\text{SPA\% (percent of sperm penetration)} = \frac{\text{no. of eggs penetrated}}{\text{no. of eggs inseminated}} \times 100$$

$$\text{PI (penetration index)} = \frac{\text{total no. of swollen head}}{\text{no. of egg inseminated}}$$

%Inhib. (percent of inhibition) = 100 - %SPA

** Some of these MAbs were sent to another laboratory (Jane Rogers, Vanderbilt University, TN) for SPA to confirm the test results

tight binding by at least 50% (Table 9). Eight MAbs did not significantly reduce sperm binding, although the average number of sperm bound was decreased compared to the control.

In Vitro Cervical Mucus Penetration Assay

In vitro cervical mucus (CM) penetration tests were conducted to examine whether any inhibition of penetration occurs due to antibody binding to sperm. A significant reduction of penetration was considered when the distance of penetration was less than 20 mm. None of these MAbs impeded sperm CM penetration (Table 10).

Determination of Whether a Carbohydrate Moiety was involved on the Sperm Surface

Destruction of carbohydrate components on the sperm surface was done by treating methanol-fixed sperm with sodium metaperiodate; then ELISAs were performed by the usual procedure and the effect of damage to carbohydrate antigen on MAb binding was examined (Table 11). A concentration dependent response to periodate oxidation was observed with three MAbs (DH200, DH202, and DH203), although the antigenicity of these MAbs was retained in some degree.

TABLE 9 HUMAN SPERM/ZONA TIGHT BINDING TEST: HEMIZONA ASSAY (HZA)

	MAbs	HZA[a] index[b]	HZA inhibition
INHIBITORS OF ZONA BINDING[c]	DH3	19%	81%
	DH4	27%	73%
	DH13	17%	83%
	DH14	46%	54%
	DH21	41%	59%
	DHTB11	40%	60%
	DH205	33%	67%
NONINHIBITORS OF ZONA BINDING[d]	DH22	80%	20%
	DHF5	66%	34%
	DHTolP3C9	53%	47%
	DH113	57%	43%
	DH200	80%	20%
	DH201	84%	16%
	DH202	85%	15%
	DH203	71%	29%

[a] The hemizona assay-two to six pairs of HZ were assessed for each MAb

$$[b]\text{HZI(hemizona index)} = \frac{\text{no. of tight bound sperm on test zona} \times 100}{\text{no. of tight bound sperm on control zona}}$$

[c] INHIBITING: HZI< 50
[d] NONINHIBITING: HZA> 50

These MAbs were also sent to another laboratory (Jane Rogers, Vanderbilt University, TN) to repeat the HZA assay and to confirm the test results.

TABLE 10 IN VITRO CERVICAL MUCUS PENETRATION ASSAY

MAbs	Ig class	Distance penetrated**
DH3	IgM	33mm
DH4	IgM	25mm
DH13	IgM	25mm
DH14	IgM	27mm
DH21	IgM	45mm
DH22	IgM	22mm
DHF5	IgG_3	25mm
DH113	IgG_{2b}	25mm
DH200	IgG_3	25mm
DH201	IgG_1	25mm
DH202	IgG_1	25mm
DH203	IgG_3	25mm
DH205	IgG_1	26mm
DHTB11	IgM	26mm
DHTolP3C9	IgM	25mm
*Control	-	30mm

* sperm incubated with ascites from NS-1 (non-antibody secreting cell line) and used as negative control.

** distance penetrated >25mm means no inhibition.

TABLE 11
PERCENT OF INHIBITION OF ANTI-HUMAN SPERM ANTIBODIES BINDING AFTER DESTRUCTION OF CARBOHYDRATE MOIETY ON SPERM MEMBRANE BY
TREATING SPERM WITH SODIUM-META-PERIODATE

MAbs	I N H I B I T I O N					
	Untreated control	Treated				
		10mM	25nM	50mM	100mM	200mM
DH3	0%	0%	0%	0%	0%	0%
DH4	0%	0%	0%	0%	0%	0%
DH13	0%	0%	0%	0%	0%	0%
DH14	0%	0%	0%	0%	0%	0%
DH21	0%	0%	0%	0%	0%	0%
DH22	0%	0%	0%	0%	0%	0%
DH113	0%	0%	0%	0%	0%	0%
DHF5	0%	0%	0%	0%	0%	0%
DH200	0%	18%	28%	68%	84%	84%
DH201	0%	0%	0%	0%	0%	0%
DH202	0%	70%	80%	84%	86%	86%
DH203	0%	13%	39%	40%	63%	63%
DH205	0%	0%	0%	0%	0%	0%
DHTB11	0%	0%	0%	0%	0%	0%
DHTolP3C9	0%	0%	0%	0%	0%	0%

$$\% \text{ inhibition} = 100 - \frac{100 \times \text{test O.D}}{\text{control O.D}}$$

[Three separate assays were done and MAbs ran in duplicate in each time, then average of all test results was taken for percent of inhibition calculation].

Passive Immunization

Two MAbs were selected for a passive immunization in order to evaluate whether they reduced fertility in mice. These two antibodies were selected for passive immunization experiment based on their cross reactivity with mouse sperm, location of reacting antigens on sperm, and inhibition of any other sperm functional tests in vitro (sperm immobilization and sperm agglutination). No significant reduction of fertility was found compared to control (Table 12).

TABLE 12 IN VIVO SPERM FUNCTIONAL TEST: PASSIVE IMMUNIZATION

MAbs	Ag location human/ mouse	SA/SI human	SA/SI mouse	Passive Immunization fertility inhibition
DH201	T / Ac	+ /-	+/+	no significant inhibition compared to control
DH203	Ac,T / H	+ /+	+/+	no significant inhibition compared to control

T(tail), Ac(acrosome), H(head), SA/SI(sperm agglutination assay/sperm immobilization assay), Ag(antigen)

CHAPTER 7

DISCUSSION

In this study, splenic plasma cells from immunized mice producing human antisperm antibodies were successfully fused with myeloma cells to produce monoclonal antibodies (MAbs). Two extraction techniques were used for sperm membrane antigen isolation. The first technique involved NP-40 detergent for antigen extraction from swim-up (motile fraction) human ejaculated sperm and the second technique employed homogenized human testis for antigen extraction. We used pooled ejaculates from several normal human donors for antigen extraction procedures to reduce some possible individual variations of sperm membrane components. Swim-up sperm were all motile and likely to be those sperm associated with fertilization. By using swim-up washed sperm rather than only washed sperm, we did eliminate some non-motile sperm and also a population of round cells, including germ cells and white blood cells which are not important for fertilization but present in ejaculates of normal men. Another comparison study from our laboratory (Gupta et al., 1990) suggested that swim-up sperm rather than thrice-washed sperm are far more appropriate for studies of sperm antigens associated with motile sperm.

The results of ELISA and immunobead tests (both on live sperm) indicated that we successfully isolated human sperm membrane surface antigens from these extraction procedures, and by using these extracts as immunogens, we developed some sperm membrane specific monoclonal antibodies. The human testicular antigen extraction procedure used in this study for immunizing mice also showed a promising approach for developing antisperm MAbs. The one advantage of these MAbs is that since they reacted with testicular sperm, as well as ejaculated sperm, they can be used in the cDNA testis library available for large scale preparation of corresponding antigen for contraceptive vaccine development.

The results of the immunofluorescence and immunochemical staining studies on methanol-fixed sperm indicated that these antibodies recognized antigens on the plasma membrane overlaying different regions of the sperm. It has been reported by many researchers (Hansen and Hjort, 1971; Jeffery and Parish, 1972) that naturally occurring human antisperm antibodies bind to antigens located on the acrosome, post-acrosome, equatorial, midpiece, and tail. Our study also indicated the same profile. Since sperm acrosomal region plays the central role in sperm-egg interactions, acrosomal antigens seemed of greatest importance. Three MAbs (DH3, DH22, and DH203) developed in this study reacted to sperm acrosomal antigens and among these MAbs, two (DH3 and DH22) impeded sperm-egg interactions.

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When these antisperm monoclonal antibodies were subjected to immunohistological and immunofluorescence evaluations against an extensive panel of human tissues or cells, no cross-reactivity to somatic tissues was observed. Most of the antisperm antibodies with sperm functional inhibitory properties reported by many researchers have cross-reacted with some human somatic tissues or cells and therefore are disqualified for contraceptive vaccine use. The absence of cross-reactivity to somatic tissues with MAbs developed in this study meet one of the important criteria of a primary candidate for contraceptive vaccine development as established in a WHO workshop.

Human reproductive tissue sections treated with our various antisperm MAbs were tested by employing IF and ICS. Staining was found on spermatozoa and spermatids in the testis; on epididymal epithelium and the sperm in caput-, corpus-, and cauda epididymis; components of seminal vesicle; and sperm in the vas deferens. This gives us information as to the possible origination of these corresponding antigens in the human reproductive system. It has been reported (Myles and Primakoff, 1983; Phelps and Myles, 1987) that even as early as in the testis, some antigenic sites are localized by observing antibody binding to the whole cell, whole head, anterior tail, and posterior tail. Eddy (1990) and Cameo et al. (1990) reported that surface sites and molecules of defined sperm surface domains are complex.

Binding can occur at different times and perhaps via different mechanisms. Also, these arrangements are not static but can be altered at different stages in sperm maturation. Rearrangements of surface molecules can occur as a result of external signal and include the creation or uncovering of new epitopes on preexisting surface molecules. Our data showed that DH13, DH21, DH22, and DHF5 reacted with the epididymal factors and that DH4, DH14, and DH205 reacted with the seminal vesicle factors. DH3, DH113, DH200-203, and DHTB11 recognized testis originated antigens but did not recognize any epididymal factors. These testicular antigens were probably altered or masked at different stages of epididymal sperm maturation and then unmasked again on ejaculated sperm. On the other hand, DHTolP3C9 did bind to a testicular originated antigen, but the antigen appeared to remain unchanged during different stages of sperm maturation.

When species cross-reactivity was assessed for a variety of other species' spermatozoa, six MAbs cross-reacted with monkey sperm and all MAbs reacted with a variety of other species' spermatozoa. This wide species-cross-reactivity indicates shared antigens in these species and the possibility of employing experimental animal models to test the use of antigenic determinants recognized by these MAbs as birth control vaccines. Such a finding is reasonable in light of other investigators' studies. Lopo and Vacquir (1980) have

reported that antisera against sea urchin sperm cross-react with surfaces of sperm of 28 species from seven animal phyla. Other investigators have also detected the presence of common sperm antigens among mammalian species by means of MAbs (Feuchter et al. 1981; Gaunt, 1982). They also observed cross-reactivity to mouse-, rat-, rabbit-, and guinea pig sperm. Analysis of antibody binding pattern in the IF test indicated that segregation of similar sperm-specific antigenic determinants into identical domains in the tested species might be related to some common sperm function. One such sperm antigen is testis-specific lactate dehydrogenase ($LDH\text{-}C_4$) which has been reported to be antigenically and functionally similar in several mammalian sperm (Erickson et al., 1975). However, it should kept in mind that MAbs recognize only a region of a particular molecule. It is therefore possible that other parts of the surface components recognized by the cross-reacting MAbs may differ among the species, thus altering the function of these components.

When these monoclonals were tested on fresh, capacitated, and acrosome reacted spermatozoa, DH22 showed differential reactivity with swim-up sperm as compared to Ca^{++} ionophore treated sperm (acrosome reacted) in ELISA. This finding suggests that changes in the recognition of spermatozoa surface antigens during capacitation allowed increased or shifted antigen expression. The pattern of antibody binding to the sperm also changed. It reacted heavily with

acrosomal region on acrosome reacted sperm. Primakoff et al., (1988) also reported an antigen, PH-20, localized on both plasma membrane and inner-acrosomal membrane depending on the sperm status. Before the acrosome reaction, this antigen was localized to the posterior head surface of the sperm. After the acrosome reaction, however, PH-20 migrates from the posterior head surface onto the inner acrosomal membrane and this migration increases the total amount of PH-20 protein on the sperm surface approximately threefold. Further studies on antigen migration or unmasking would be useful for DH22. Since DH22 showed a brighter binding by IF to acrosomal cap region of acrosome reacted sperm, this MAb can be used as a marker to identify acrosome reacted sperm among the different stages of sperm population. When our antibodies were compared with fresh (noncapacitated) and capacitated sperm, changes in surface composition were not observed as sperm under capacitation since antigens were equally detectable on fresh and capacitated sperm, which probably suggests that these monoclonals recognize antigens common to both fresh and capacitated spermatozoa.

Immunoblot analysis indicated that most of our anti-sperm MAbs reacted against sperm membrane proteins ranging from 14 to 68 KD molecular weight. Primakoff and Myles (1983) reported guinea pig sperm head antigens ranging from 18 to 70 KD. These results may indicate that proteins within this molecular weight range are commonly associated

with sperm plasma membrane. Isahakia and Alexander (1984) reported a mouse antihuman sperm MAb, MA3, that reacted against human sperm acrosomal antigen with >200 KD. MA3 showed inhibition of sperm functional assay (agglutination and immobilization). Saling (1985) also reported an anti-sperm MAb, M42, which significantly inhibited mouse fertilization in vitro. This M42 also recognized a high molecular weight doublet, 220-240KD, that was specific to spermatozoa. In our study we also observed one MAb, DH13, that reacted with post acrosome and tail region of human sperm, and recognized a high molecular weight protein, >200 KD, however it did not show any agglutinating or immobilizing properties although it inhibited both the SPA and HZA. Perhaps this MAb is associated with sperm zona receptor or egg fusion receptor. Four antisperm MAbs that reacted with low and medium molecular weight antigens associated with human sperm have been reported by different authors, MA24, 20-35 KD (Naz et al., 1984a), MA4, 20-35 KD (Isahakia and Alexander, 1984), C11H, 50-60 KD and 20-35 KD (Kallojoki and Suominen, 1984), and AF-1, 80-90 KD and <20 KD (O'Rand and Irins, 1984). All of these were against human sperm except AF-1 which was against rabbit sperm antigen. Among these AF-1 and MA-24 demonstrated inhibition of at least one sperm functional assay. In our study we also observed some MAbs that reacted with low molecular weight proteins ranging 14-37 KD. Among these MAbs, DH3, DH4, DH21, DH22, DH201, DH202

also inhibited at least one sperm functional assay. Some of these MAbs also reacted with medium molecular weight protein bands (50-68 KD) in addition of lower molecular weight protein bands (14-45 KD). The WHO task force of antisperm MAb provided limited data on the molecular weights of the relevant antigens (Anderson et al. 1987). Among those antisperm MAbs identified for further investigation as antifertility vaccine candidates, S20 (against testicular germ cells and intraacrosomal cap) reacted with a low molecular weight protein ranged from 18-34 KD (Herr et al. 1991). S20 inhibits sperm-egg interaction. Lee et al. (1991) reported two antisperm MAbs reactive against many mammalian species' sperm acrosome. MAb HS-11 recognized two polypeptide bands in the 35-50 KD range and MAb HS-63 recognized 3 bands in the 42-50 KD range. Both HS-11 and HS-63 inhibited sperm-egg interaction in mouse in vitro. In our study MAb DH3 reacted with the acrosome, neck, and tail region of human sperm and recognized 14, 25, and 68 KD protein bands. DH 22 reacted with an intraacrosomal protein and recognized 14 and 68 KD bands. Both DH3 and DH22 inhibited sperm/ hamster egg interaction and human zona binding. Comparison of the data from the WHO report (Anderson et al. 1987) with that of our study, it seems as if many lower molecular weight proteins are associated with sperm-egg interaction but some medium and high molecular wight proteins also can participate. It is not surprising that a MAb raised against

a given antigen can give multiple bands. These results can indicate that a MAb could share some common antigenic epitopes of different proteins involved with different sperm functions. Moreover, SDS can cause breakage to molecules thus antigens can be of reduced size in SDS, i.e., 48 KD and a 24 KD molecule could indicate such a phenomenon. One of our MAb DH21 recognized five bands, 68, 45, 37, 23, and 14 KD. Here 45 KD band might reduced in size and produced another band of 23 KD. This can further studied by using nonreducing gels. Perhaps purification and a more detailed biochemical characterization of these antigens are necessary to illustrate more information.

Employing hybridoma technology, monoclonal antibodies developed against human spermatozoa have demonstrated the multiplicity of the antigens having a role in the process of fertilization. We found that MAbs, DH3, DH13, DH14, DH22, and DHTolP$_3$C$_9$, significantly inhibited the standard sperm penetration assay (SPA) that has been used in most investigations of mammalian sperm-egg fusion. Among our antisperm MAbs DH3 and DH13 showed inhibition only when they were treated with precapacitated spermatozoa but not with postcapacitated spermatozoa. These results support the hypothesis that antibody directed against the plasma membrane of sperm can affect the mobility of membrane particles, thus impeding or possibly hastening capacitation and the acrosome reaction process necessary for sperm-egg

fusion. Another possibility would be that these antigens coated sperm in epididymis but were removed during capacitation so these MAbs did not inhibit SPA when treated with postcapacitated sperm. Other researchers also reported (Alexander 1988) that some antisperm monoclonal antibodies can prevent sperm fusion with hamster eggs when exposed to precapacitated human spermatozoa. Although some researchers suggested that the sperm acrosomal region is the most potent for egg binding and penetration, Naz and associates (1984) and Primakoff et al. (1987) reported that MAbs binding to the post-acrosome and tail can also block binding and penetration of zona-free hamster eggs by sperm. In our study we developed two MAbs (DH13 and DHF5) that recognized the post-acrosomal and tail regions on sperm and did inhibit sperm-egg fusion. DH3 was found to react to the acrosome, tail and neck regions on human sperm, as well as to significantly inhibit hamster egg penetration by human spermatozoa (SPA). This result suggests that the sperm post-acrosome and tail regions are composed of multiple antigens that may have different sperm functions in the fertilization process. MAb DH22 showed strong inhibition when post-capacitated sperm was incubated with this Mab. This result supports our previous finding that DH22 strongly reacted with acrosome-reacted sperm, particularly on the acrosomal region of sperm. DH22 moderately reacted with the equatorial region with pre-capacitated and capacitated sperm. Sperm that are

capable of fusing with eggs are apparently acrosomereacted (Yanagimachi, 1981). Some laboratories have also reported that the equatorial region of the plasma membrane may also be involved in sperm-egg fusion (for review, Yanagimachi, 1981). Our study supports this concept because DH22 reacted with the equatorial region and showed some inhibition of sperm-egg fusion with precapacitated sperm.

The hemizona assay (HZA) was used to measure the efficiency of sperm function to achieve tight binding to the zona pellucida in the presence of these MAbs. Use of NS-1 as a control ascites fluid demonstrated that peritoneal fluid proteins did not interfere with the sperm-zona interactions. Use of Fab fragments with the HZA was employed to answer any lingering question regarding the effects of agglutination. The initial screening procedure used here permits the evaluation of large numbers of Mabs. Those that are found to have shown some inhibitory effects can be evaluated extensively at a later date. Additional studies could be done including the antibody dose dependency, the removal of excess antibodies and other components (Mahony et al., 1990). Seven of our antisperm MAbs (DH3, DH4, DH13, DH14, DH21, DHTB11, and DH205) impeded the HZA; all were against coating antigens. MAbs DH4, 21, TB11, and 205 did affect sperm performance in the HZA but did not affect the SPA. These results support our previous findings that these corresponding antigens are available only during the process

of capacitation but are unrecognizable or absent from sperm head once the sperm are predominately acrosome reacted. These MAbs may be specific for a portion of the zona binding receptor. Such antibodies are thought to reduce zona binding of sperm by obscuring the receptor site/steric hindrance (Singer et al., 1985; Alexander, 1990). MAbs DH3 and DH13 strikingly impeded HZA binding as well as decreased the SPA when precapacitated sperm were used. These results indicate that these MAbs may be covering a portion of the receptor involveed in zona binding; sperm penetration can be inhibited by preventing the acrosome reaction as capacitation occurrs. However, the SPA assesses other events i.e., sperm-egg fusion and decondensation of nuclear material in addition to the ability to spontaneously acrosome react over time.

Cervical mucus (CM) penetration is another important sperm function known to be inhibited by sperm antibodies (Fjallbrant, 1969). Kremer (1982), Hendry et al (1982), and Clarke (1988) reported that only sperm bound antibodies of IgA class are associated with poor CM penetration in vitro, but sperm bound IgG class antibodies do not appear to be associated with poor CM penetration. Sperm surface IgA class immunoglobulins adhere to the glycoproteins of the cervical mucus and impair sperm penetration (Kremer and Jager, 1980). Wang et al. (1985) reported that, in the presence of sperm, surface tail-tip antibodies of either IgA

and IgG classes did not interfere with sperm penetration of human cervical mucus. Therefore, we can assume that probably both the immunoglobulin class and the region of binding on sperm are important in determining the degree of impairment of fertility in men with sperm antibodies. All of our fifteen MAbs were either IgM or IgG class, and none of these Mabs showed any inhibition of CM penetration.

When periodic acid treated sperm were used for ELISA, result showed that binding of MAbs DH200 and DH202 to sperm surface antigens was inhibited severely, and DH203 inhibited binding moderately, suggesting that these sperm antigens may be composed of carbohydrate as a major moiety, and peptide as a minor moiety. Isojima et al. (1990) reported three MAbs (H6-3c4, 2B6, and 2E5) against human sperm coating antigens. The antigenicity was completely destroyed by treating with periodate, but the antigenicity to MAbs 2C6 was retained in some degree compared to other MAbs. Their suggestion was the antigenic epitope of MAbs H6-4C4 may be glycolipid, but that of MAb 2C6 may be composed of carbohydrate and peptide as major and minor moieties, respectively. Other researchers from our laboratory have also reported that some complex carbohydrate antigens are associated with the sperm cell surface (Kurpisz et al., 1989).

Studies of a few female sera (Ingerslev, 1979; Isojima et al., 1972) have revealed that sperm-agglutinating antibodies and sperm-immobilizing antibodies can be either

of the IgM- or the IgG-class, although the majority of sperm immobilizing antibodies belong to the IgG class (Isojima, 1989). All our sperm agglutinating MAbs belonged to the IgG class, as did our three immobilizing MAbs. Since sperm SA/SI antibodies in patients and experimental animals are polyclonal antibodies, production of monoclonal antibodies with SA/SI activities is essential to analyze the antigenic epitopes of corresponding antigens. In 1980, Isojima et al., reported a rat-mouse heterohybridoma, IC4, against human seminal plasma which secreted a strong SI-Mab. But this antigen does not seem to be a good candidate for immunological contraception because the antigenicity is shared with human milk protein. The Isojima group also produced some mouse MAbs possessing SA- and SI-activities, but all were human seminal plasma antigen specific. In our study none of the four MAbs with SA- and SI- activities reacted with human seminal plasma, but did react with sperm membrane integral antigen. So, they are against a sperm membrane integral antigen rather than sperm coating antigen originating from seminal plasma.

The absence of cross-reactivity to somatic tissues coupled with inhibition of at least one sperm functional test by antisperm surface antibodies sperm is one of the important criteria of a primary candidate for contraceptive vaccine development as established by the WHO in a Monoclonal Antibody Workshop (Anderson et al., 1987). Eight

of our monoclonal antisperm antibodies were able to inhibit at least one in vitro sperm functional test and showed no cross reactivity with human somatic tissues. Further purification of these antigens and analyzation of the antigenic epitopes and biochemical characterization would be of interest to better understand their immunocontraceptive potential.

CHAPTER 8

CONCLUSIONS

1. NP-40 detergent can extract surface protein (both coating and integral) from sperm membrane. Using this extracted protein as immunogen is a successful approach to produce sperm surface-specific monoclonal antibodies.

2. We have developed one hundred thirty monoclonal antisperm antibodies and, among these, fifteen have been characterized. These fifteen do not cross-react with human somatic cells but react with sperm and germinal cell components of the male reproductive system, indicating their sperm specificity. This fulfills one of the important criteria for contraceptive vaccine use reported by the WHO Monoclonal Antibody Workshop. Four of our MAbs are reactive to epididymal factors, three are reactive to seminal vesicle factors, and seven are reactive to testicular factors.

3. Eight of our monoclonal antisperm antibodies were able to inhibit at least one in vitro sperm functional test which also meets another criteria of immunocontraception. These MAbs can be used to isolate sperm

antigens for biochemical characterization or can be used for cDNA testis libraries for production of sperm membrane antigens to better understand their immunocontraceptive potential.

4. MAbs against a coating antigen affected the in vitro sperm penetration assay suggesting importance of sperm surface interaction with the egg and egg vestments.

5. Since a significant number of women with infertility of undetermined etiology have elevated titers of antisperm antibodies capable of inducing sperm agglutination or immobilization in their sera and cervical fluids, MAbs DH200-203 (sperm agglutinating and/or immobilizing antibodies) can be used for corresponding antigen isolation and biochemical characterization to better understand their role in immunocontraception.

6. Since one MAb (DH22) is capable of binding to the acrosomal cap region of acrosome reacted sperm, this MAb can be used as a marker to identify acrosomal reacted sperm among the different stages of sperm population.

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APPENDIX

TABLE A Spermatozoal Auto- and Iso-Antigen

Antigens	Localization and Characterization of Antigen
Membrane antigens	Two glycoproteins, widely distributed in the membrane. One antigen restricted to the tail end piece.
Sperm-specific enzymes	
LDH-C4	In the post-acrosomal area of the plasma membrane and in seminal plasma.
acrosin	In acrosome, including equatorial segment.
hyaluronidase	In acrosome.
DNA polymerase	In nucleus and seminal plasma.
Nuclear proteins	Protamine 1 and 2 in sperm nucleus
Various subsurface antigens, characterized mainly by their location	In acrosome, equatorial segment, postnuclear area, main tail piece, and tail end piece.

TABLE B

Monoclonal antibodies against human sperm.

WHO code	Source laboratory	Previous designation	Published references	Human sperm		Testicular[a]		Vx SP[b]	Sperm: other species		Somatic tissue cross-Rxn[c]
				Live	Fixed	GC	IC		Mouse	Primate	
S01	Alexander	MA14	35	+	±	+	+	–	±	–	+
S02	Alexander	MA17	35	±	+	–	+	–	±	–	+
S03	Alexander	MA19	35	±	+	+	+	–	+	+	±
S04	Alexander	MA20	35	+	+	+	+	+	–	±	+
S05	Alexander	MA27	35	+	+	–	–	–	–	±	±
S06	Alexander	MA28	35	±	+	+	+	+	–	+	±
S07	Alexander	MA29	35	±	+	+	–	+	±	+	+
S08	Alexander	MA31	35	–	±	–	–	+	–	+	±
S09	Alexander	MA32	35	±	+	+	–	+	–	+	±
S10	Alexander	MA34	35	±	+	+	–	+	±	+	±
S11	Alexander	MA36	35	+	+	–	+	+	–	–	+
S12	Alexander	Vx5	35	–	–	+	+	+	±	–	+
S13	Herr	MHS-1	36,37	±	+	+	+	+	+	+	+
S14	Herr	MHS-2	36,37	–	+	+	+	–	+	+	+
S15	Herr	MHS-4	36,37	±	+	–	–	+	–	±	– (L)
S16	Herr	MHS-5	36,37	–	+	±	–	+	–	±	– (L)
S17	Herr	MHS-6	36,37	–	–	+	+	+	–	–	+
S18	Herr	MHS-13	36,37	±	+	+	+	±	–	–	+
S19	Herr	MHS-8	36,37	+	+	+	+	+	±	±	+
S20	Herr	MHS-10	36,37	±	+	+	–	–	±	+	– (E)
S21	Herr	MHS-11	36,37	±	+	+	–	–	+	+	– (L)
S22	Isahakia	M5B1		±	+	+	–	–	+	+	+
S23	Goldberg	MJ-1		–	–	–	–	–	±	–	– (L)
S24	Goldberg	MJ-2		–	–	–	–	–	–	–	– (L)
S25	Goldberg	MJ-3		±	–	±	–	–	–	–	– (L)
S26	Goldberg	MJ-4		–	–	±	–	–	+	–	– (L)
S27	Goldberg	MJ-5		±	–	±	–	–	–	–	– (L)
S28	Goldberg	MJ-6		–	–	±	–	–	±	–	– (L)
S29	Goldberg	MJ-7		±	–	–	–	–	–	–	– (L)
S30	Talwar	C1		–	+	±	–	–	–	–	– (L)
S31	Talwar	6SPB6		–	+	±	–	+	–	–	±
S32	Talwar	F11G1		–	±	+	+	–	+	–	+

S33	Herr	MHS-3	36,37	+	±	±	–	+	–	–	±
S34	Isojima	2C6 2GBA11	38,39	±	–	±	–	–	–	–	– (E)
S35	Kolde	YWK-1	40,41,42	±	–	+	–	–	±	–	±
S36	Lee	11HS-11	43,44	±	+	+	+	±	+	+	±
S37	Lee	11HS-63	43,44	±	+	+	–	–	+	+	– (E)
S38	Lee	11HS-80	43,44	+	±	±	–	–	–	–	+
S39	Lee	11HS-85	43,44	+	+	±	–	–	–	–	+
S40	Wang	NRIFP-1	45,46	+	+	–	–	–	±	–	– (L)
S41	Wang	NRIFP-2	45,46	–	±	–	+	–	–	–	+
S42	Wang	NRIFP-3	45,46	–	±	–	–	+	–	–	±
S43	Wang	NRIFP-4	45,46	–	–	–	–	±	–	–	– (L)
S44	Wang	NRIFP-5	45,46	–	–	+	+	–	–	–	+
S45	Wang	NRIFP-6	45,46	–	–	–	+	–	–	–	+
S46	Wang	NRIFP-7	45,46	±	–	±	+	–	–	–	+
S47	Wang	NRIFP-8	45,46	–	–	–	–	–	–	–	– (L)
S48	Wang	NRIFP-9	45,46	–	–	–	–	–	–	–	±
S49	O'Rand	anti RSA	47–50	–	–	–	–	–	–	–	– (E)
S50	Alexander	NS.1		–	–	–	–	–	–	–	NT
S41	Alexander	antisheep RBC		–	–	–	–	–	–	–	NT
S52	(coded)			–	–	NT		–	NT	NT	NT
S53	(coded)			–	–	–	–	–	NT	NT	NT
S54	(coded)			–	–	NT		–	NT	NT	NT
S55	(coded)			–	±	–	–	–	NT	NT	NT
S56	(coded)			–	–	–	–	–	NT	NT	NT
S57	Saling	M29	51,52,53	–	–	NT		–	NT	NT	NT
S58	Mettler	SPAG11–13	54–58	–	+	–	+	+	NT	NT	+
S59	Mettler	SPAGV1–2	54–58	–	+	–	+	+	NT	NT	– (L)
S60	Johnson	H316	5,59	±	+	+	+	–	NT	NT	+
S61	Moore	18.6	60	+	±	+	–	–	NT	NT	– (E)
S62	Moore	60	60	–	–	–		–	NT	NT	– (L)
S63	Moore	64.24	60	–	–	–	+	–	NT	NT	– (L)
S64	Moore	76.11	60	–	–	–		–	NT	NT	– (L)
S65	Moore	97.25	60	–	–	–		–	NT	NT	– (L)
S66	Moore	C-5	60	–	–	–		–	NT	NT	– (L)
S67	De Ioannes	C2E5	61,62	–	–	+	+	±	NT	NT	– (L)
S68	De Ioannes	4G2	61,62	–	–	+	+	±	NT	NT	– (L)

[a]GC, germ cells; IC, interstitial cells.
[b]Vx, vasectomy; SP, seminal plasma.
[c]L, limited panel; E, extensive panel; NT, not tested.