Characterization of Antigens on the Surface of Human Spermatozoa Using Monoclonal Antibodies.

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Declaration

The experiments described in this thesis were the unaided work of the author except where acknowledgement is made by reference. No part of this work has previously been accepted for any other degree nor is any part of it being submitted concurrently in candidature for another degree. All experiments were performed at the Medical Reserch Council Reproductive Biology Unit in Edinburgh.

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Abstract

A panel of murine monoclonal antibodies was raised against human sperm plasma membrane in order to identify and characterize molecules important in fertilization. Twelve monoclonal antibodies were produced and of these nine belong to the IgM subclass while the remaining three are of the IgG_{2a} subclass. The antibodies were purified from bulk culture supernatants using affinity chromatography — the IgG antibodies on a protein A column and the IgMs on a column made using a commercially available anti-mouse IgM antibody coupled to an inert support. These purified antibodies were then used to characterize their respective antigens biochemically and assessed for their biological activity using a variety of *in vitro* tests of sperm function.

All twelve antibodies recognized a molecule which was present on the entire surface of human spermatozoa. Eight antibodies cross-reacted with both marmoset and boar spermatozoa as well as with human leucocyte sublasses. The other four immunoglobulins were highly specific for human sperm cells and did not cross-react with any of the other cells tested. In Western blotting experiments, the monoclonals recognized epitopes present on a variety of molecules. Only one antibody, spm 4/4, failed to recognize any proteins when transferred to nitrocellulose.

When tested for their ability to interfere with the acrosome reaction, four antibodies significantly inhibited the percentage of spermatozoa in a population undergoing a spontaneous acrosome reaction. Subsequently, two of these antibodies, spm 1/1 and spm 1/2, were shown to cause a significant decrease in the percentage of sperm which were induced to undergo the acrosome reaction by the calcium ionophore A23187. The interaction between spermatozoa and the zona pellucida was assessed using salt-stored human zonae and two immunoglobulins, spm 1/2 and spm 4/6, significantly reduced the mean number of spermatozoa bound to the zona pellucida. These two antibodies also caused a marked inhibition of sperm-oolemmal fusion as

assessed using the hamster egg penetration test. When this test was performed at various sperm concentrations, both of these antibodies exhibited a dose dependent decrease in the penetration rate. One further antibody, spm 4/7, was found to have biological activity in that it stimulated the interaction between spermatozoa and the oolemma.

The gene encoding the antigen recognized by one of the inhibitory antibodies, spm 1/2, was isolated from a human testicular cDNA library cloned into the expression vector λgt11. The insert DNA was amplified using the polymerase chain reaction and then subcloned into the phagemid pBluescript SK- for sequencing. The whole insert was sequenced and found to be 1997 nucleotides in length. It contains an open reading frame from nucleotide 1 to nucleotide 660 which encodes for the carboxy-terminal 219 amino acids of the antigen recognized by the antibody. It has a long 3' untranslated region (1337 nucleotides) which contains a messenger RNA degradation signal, two polyadenylation signals and a poly A tail. Homology searches of the Genebank and Owl databases were made and no significant homology identified.

Thus from the initial panel of twelve monoclonal antibodies, three which recognize molecules of particular interest were identified. Two have inhibitory effects on fertilization and may be of use in producing a contraceptive vaccine. The other immunoglobulin has a stimulatory effect on fertilization and may prove to have an clinical application in the treatment of subfertile men.

(approximately 40 000 words in main text)

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Abbreviations

AR	acrosome reaction
β-gal	β-galactosidase
bp	base pairs
BWW	Biggers, Whitten and Whittingham medium
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
ECL	enhanced chemiluminescence
EtBr	ethidium bromide
FCS	foetal calf serum
HEPT	hamster egg penetration test
HOS	hyperosmotic swelling
Ig	immunoglobulin
IPTG	isopropyl-1-thio-β-D-galactoside
mRNA	messenger RNA
ORF	open reading frame
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
ppm	parts per million
SDS	sodium dodecylsulphate
TBS	tris buffered saline
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside

APAAP	mouse alkaline phosphatase anti-alkaline phosphatase complex
BCA	bicinchoninic acid
BSA	bovine serum albumin
cDNA	copy deoxyribonucleic acid
dNTPs	deoxyribonucleoside triphosphates
EDTA	ethylenediamine tetraacetic acid
ELISA	enzyme linked immunoabsorbant assay
FCA	Freund's complete adjuvant
HAT	hypoxanthine, aminopterin, thymidine selection medium
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethane sulphonic acid
HGPRT	hypoxanthine guanine phosphoribosyl transferase
HIV	human immunodeficiency virus
HRP	horseradish peroxidase
kD	kiloDalton
m	metre
mm	millimetre
mg	milligram
ml	millilitre
mM	millimolar
MTM	mixed thymocyte medium
µl	microlitre
µM	micromolar
nm	nanometre
pfu	plaque forming unit
PMSF	phenylmethyl sulphonyl fluoride
RAM	rabbit anti-mouse immunoglobulins
RGD	arginine glycine aspartate

RNA	ribonucleic acid
RPMI	Roswell Park Memorial Institute
TAE	Tris/acetate/EDTA buffer
TBE	Tris/borate/EDTA buffer
TE	Tris/EDTA buffer
TK	thymidine kinase
Tris	Tris-[hydroxymethyl]aminomethane
XL1-B	*Escherichia coli* cells strain XL1-Blue

1 Introduction

Global population growth has started to slow and is now less than 2% a year but this still means that there will be approximately 90 million more births than deaths this year (Potts, 1990). Over 90% of these births will be in developing countries and clearly for the survival of the human race on Earth with its limited resources, this 'population explosion' must be controlled and curtailed.

In order to achieve this aim new methods of contraception have to be developed. Among the novel ideas is the concept of a contraceptive vaccine which would provide a safe and reliable method of birth control. There are three main target areas for a possible contraceptive vaccine — (1) endocrine signals, (2) products of the conceptus and (3) gamete specific antigens. The production of a vaccine based on gamete specific antigens, in particular spermatozoal antigens, is the long term aim of the work described in this thesis and therefore the other areas will not be considered further.

Spermatogenesis is not initiated until puberty, long after immunological tolerance to self antigens is established and therefore antigens unique to spermatozoa are not included in this recognition process (Haas & Beer, 1986). The process of spermatogenesis is therefore sequestered from the immune system. Tight junctions between Sertoli cells form the protective 'blood-testis' barrier which isolates spermatozoa within the seminiferous tubules from blood components such as lymphocytes and immunoglobulins (Johnson, 1973). Throughout the rest of the male reproductive tract, spermatozoa are sequestered behind tight junctions between epithelial cells. Disruption of this blood-testis barrier can lead to the triggering of an immune response (Dondero *et al*, 1990). Such conditions include vas obstruction (Hendry *et al*, 1982), biopsy study (Hjort *et al*, 1974), malignancy (Guazziero *et al*, 1985) and vasectomy (Alexander & Anderson, 1979). The most common cause of

antigen leakage from the protected reproductive tract is vasectomy (Haas & Beer, 1986) and approximately 50% of men develop antibodies to their spermatozoa after vasectomy (Talwar, 1980). Evidence for the feasibility of a contraceptive vaccine is provided by a group of people who suffer from idiopathic autoimmunity against gamete-specific antigens (Bronson *et al*, 1984). Such immunological problems are a well recognized cause of human infertility and this affects approximately 10% of men who are spontaneously infertile (Dondero *et al*, 1990).

Further advances in the development of new contraceptive regimes occur hand-in-hand with advances in the understanding of basic processes involved in reproduction (Mastroianni *et al*, 1990). Not all antibodies that react with the sperm surface may affect fertility (Jager *et al*, 1987) therefore a detailed understanding of the events of the fertilization process is imperative both for contraceptive vaccine development and for the diagnosis and treatment of patients with putative immune-mediated infertility (Alexander, 1989). The structural and functional characteristics of the human sperm surface are still poorly understood and monoclonal antibody technology provides important probes for these studies (Anderson & Alexander, 1983). As monoclonal antibodies recognize a specific epitope on a particular molecule, they are valuable tools for dissecting the process of fertilization. They can be used for the identification and characterization of the important molecules and subsequently for the identification of clones of the gene encoding the antigen of interest, ultimately permitting the production of the antigen by recombinant DNA technology in sufficient quantities for trials *in vivo*.

In the work described in this thesis, monoclonal antibodies were produced against a sperm plasma membrane preparation (Chapter 4), purified and biochemically characterized (Chapter 5), assessed for their cell- and species-specificity (Chapter 6) and biologically characterized (Chapter 7). On the basis of the results obtained in the

preceeding experiments, one of the monoclonal antibodies was chosen for further investigation and used in molecular studies which are described in Chapter 8.

Each experimental chapter has a specific introductory section which aims to discuss briefly literature relevant to the work described in that chapter. Thus the overview provided in Chapter 2 does not set out to review the whole literature pertinent to all the material contained in this thesis. It does however aim to provide a foundation for the understanding and appreciation of the studies described in the rest of the thesis. Methods common to more than one chapter which are integral to the whole thesis are described in Chapter 3 but those relevant to the work described within only one chapter are detailed in the materials and methods section of that particular chapter. Each chapter also contains a discussion of the results obtained in the context of those reported by other investigators and the limitations of the methods used, and concludes with a short summary of the results reported in that chapter. The final chapter, Chapter 9, comprises a general discussion of the experiments and results described in the thesis and highlights areas which hold particular promise for the future.

2 Background

2.1 Historical Aspects

A Sanscrit treatise on ancient Indian embryology thought to have been written in 1416BC refers to the process of fertilization saying "From the conjugation of blood and semen, the embryo comes into existence" (Moore, 1989). Thus as early as 1416BC it was recognized that for fertilization to occur semen from a male was required and that once fertilization had happened menses ceased although it was wrongly believed that blood and semen combined to produce the embryo. However it was not until the middle of the 17th century when early microscopes were developed, that much progress was made in understanding the process of fertilization. In 1673, Malpighi studied what he believed to be unfertilized hens' eggs which had in fact been fertilized (Gwatkin, 1977). He thus observed early chick embryos and concluded that a hen's egg contained a miniature chick which enlarged when stimulated by a spermatozoon. Four years later in 1677, Hamm and Leeuwenhoek were the first people to observe human spermatozoa (Moore, 1989). However they misconstrued the role of spermatozoa in fertilization and concluded that the human spermatozoon contained a miniature human being which enlarged when it entered an egg. These studies led to the formulation of the 'Theory of Preformation' although the proponents of this theory were divided. Some believed that the spermatozoon contained the homunculus (the name given to the preformed organism) and were thus called 'spermatists' while others, the 'ovists', believed that the egg contained the homunculus (Gwatkin, 1977). A copy of a drawing by Hartsoeker, a spermatist, showing the homunculus as it was believed to be positioned inside the sperm head is shown in Figure 2.1. It was not until approximately 200 years later when Schleiden and Schwann proposed the 'Cell Theory of Tissue Organization' that these ideas were really questioned. In 1840, strongly

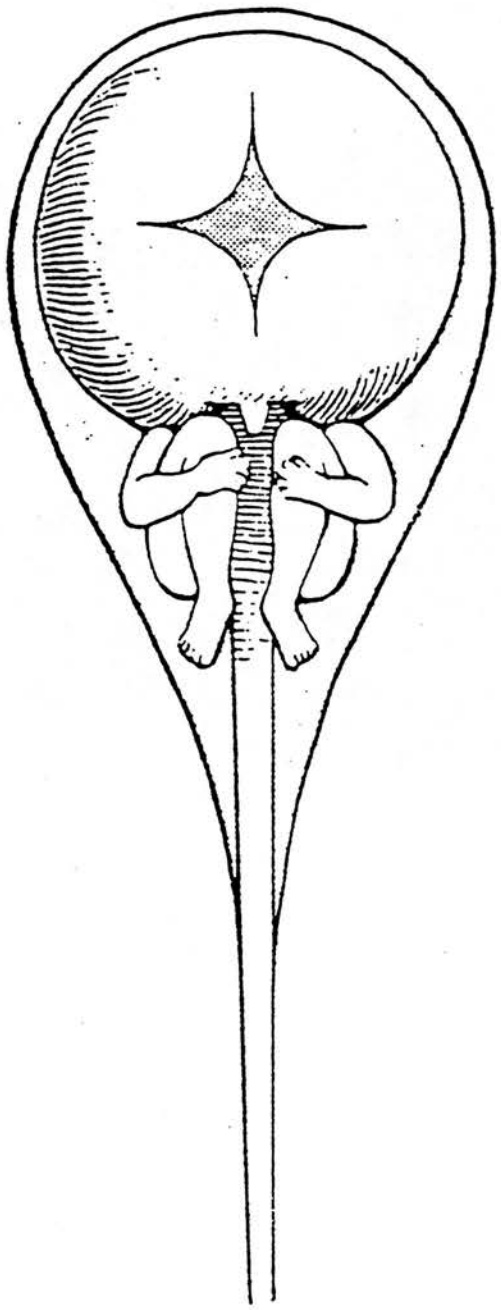

Figure 2.1 Drawing by Hartsoeker showing how 17th century spermatists believed the homunculus to be positioned within the sperm head.

influenced by this new theory, van Koelliker looked for the cellular origin of spermatozoa in the testis (Roosen-Runge, 1977; Bremner, 1981). He concluded that spermatozoa developed in the testicular tubules by processes analagous to the development of cells. Thus spermatozoa did not contain young developing animals as held by the advocates of the preformation theory, but were the results of a variation on ordinary cell production. The Swiss embryologist, Fol, was the first person to observe the process of fertilization as it took place by watching a starfish spermatozoon attach to and fertilize a starfish egg in 1877 (Epel, 1977) and the following year Flemming observed chromosomes and suggested a probable role for them in fertilization (Moore, 1989). By the end of the 19th century, van Beneden had shown that the chromosomes of the first cleavage were derived equally from spermatozoon and egg (Gwatkin, 1977). Mendel's laws of heredity were rediscovered by Correns in 1900 and their significance realized soon afterwards (Moore, 1989; Stent & Callendar, 1987). Thus by the turn of the century, the haploid/diploid status of the gametes and zygote were finally appreciated and the process of fertilization realized.

During the first half of this century, much progress was made in the study of fertilization mechanisms occurring in organisms which practise external fertilization such as marine animals (Wassarman, 1987a). Studies on mammalian fertilization lagged behind and to a large extent this can be attributed to the difficulties encountered when studying animals which practise internal fertilization. This situation changed in 1954 when Dauzier *et al* were the first people to successfully fertilize a mammalian egg *in vitro* by incubating rabbit eggs with spermatozoa recovered from the uterus and observing cleavage of the resulting zygotes (Dauzier *et al*, 1954; Gwatkin 1977).

2.2 Fertilization

At the time of ejaculation some 200-500 million spermatozoa are released into the upper fornix of the vagina. Spermatozoa travel from here, through the female reproductive tract, to the ampulla of the fallopian tube which is the site of fertilization. The interaction between spermatozoon and egg is dynamic, as spermatozoa must recognize the egg, bind to and penetrate through the egg investments and fuse with the oolemma. Once penetrated by a spermatozoon, the egg must respond by modifying its investments so that no further spermatozoa can fertilize the egg in order to prevent a polyspermic fertilization from occurring. An overview of the processes involved in fertilization is provided in Figure 2.2.

2.2.1 Sperm Transit Through the Female Reproductive Tract

Sperm transit from the vagina to the upper female genital tract is complex and poorly understood (Stone, 1989). The vaginal environment is highly acidic (pH3-4), despite partial neutralization by semen which is alkaline, and thus is highly deleterious to spermatozoa (Chretien, 1989). After ejaculation, spermatozoa rapidly move from the hostile acidic vaginal environment into the cervix. The lumen of the cervical canal is filled with a complex cervical secretion, cervical mucus, which is a hydrogel composed of macromolecular glycoprotein filaments arranged in a meshlike three-dimensional network containing more than 90% water (Pommerenke, 1964; Odeblad, 1968). This viscous gel which fills the lumen of the cervix, acts as a filter which allows sperm penetration (under certain conditions) while maintaining an effective barrier against the infiltration of microorganisms into the uterine cavity (Haas & Beer, 1986). During the luteal phase of the menstrual cycle and throughout pregnancy spermatozoa are trapped within this glycoprotein network, as under progesterone dominance the mucus framework shrinks and forms an impervious clot of gelatinous material (Chretien &

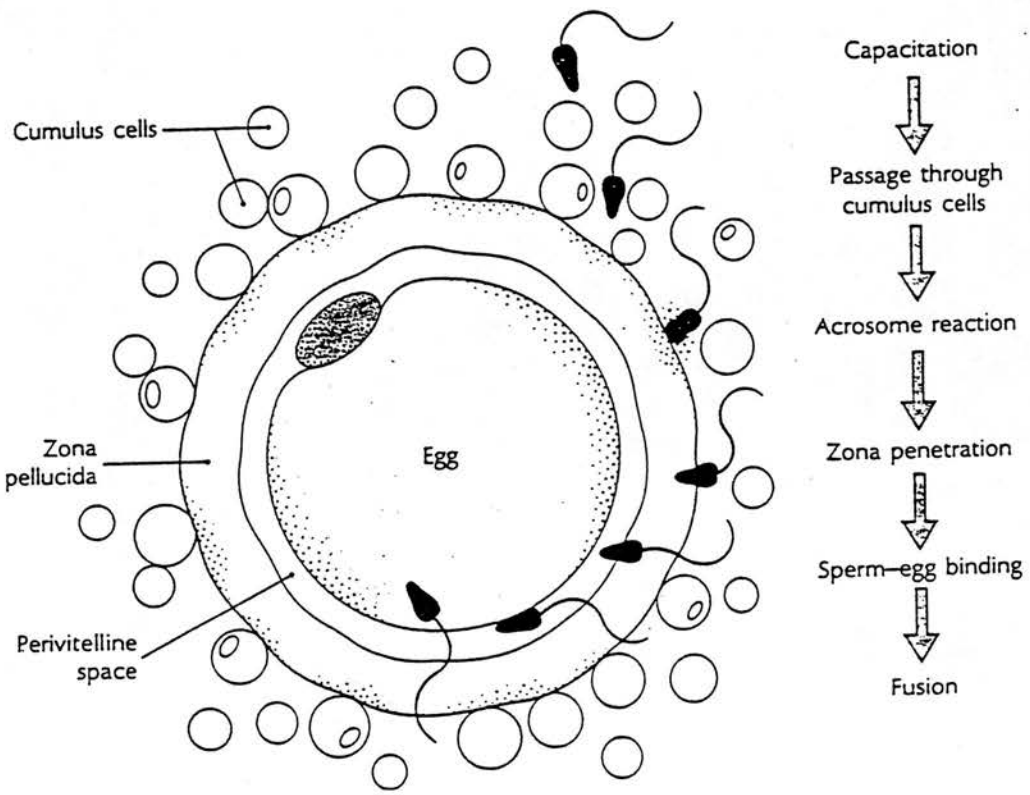

Figure 2.2 Diagrammatic overview of fertilization. See text for details.

David, 1980). However at ovulation, under oestrogen dominance, the meshwork widens progressively and midcycle mucus is readily penetrated by spermatozoa (Chretien, 1989). Under such conditions, many spermatozoa move rapidly from the hostile vaginal environment to the protective cervical mucus, although sperm loss in the vagina is thought to be high (Barratt & Cooke, 1991). Settlage *et al* (1973) suggested that only one in every two thousand ejaculated spermatozoa will enter the cervical mucus. There is a constant flow of cervical mucus down the canal to the vagina and thus only spermatozoa with sufficient dynamic properties to overcome its resistance successfully penetrate the mucus (Chretien, 1989). Many morphologically abnormal spermatozoa are excluded from cervical penetration (Hanson & Overstreet, 1981; Katz *et al*, 1990) or become trapped in the meshwork and are rapidly expelled by the mucus flow (Chretien, 1989). Thus a very reduced population of spermatozoa successfully pass through the cervix and enter the uterine cavity (Chretien, 1989; Stone, 1989; Zhu *et al*, 1992). The arrival of spermatozoa in the uterus induces a leucocytic response and an increase in phagocytosis (Austin, 1957; Hafez, 1976; Stone, 1989) and many spermatozoa are lost in the numerous folds and crypts which cover the uterine endometrium (Hafez, 1976; Chretien, 1989). Several workers have recovered only a few hundred spermatozoa from flushings of human fallopian tubes (Settlage *et al*, 1973; Croxatto *et al*, 1975; Zhu *et al*, 1992) and Ahlgren (1975) calculated that the number of spermatozoa reaching the site of fertilization in the oviduct did not exceed 200, a figure which is regularly quoted (Harper,1982; Wassarman, 1987a; Chretien, 1989; Ralt *et al*, 1991). Thus of the millions of spermatozoa released upon ejaculation, only a few hundred reach the fertilization site in the fallopian tube and this may contibute to the prevention of polyspermy (Stone, 1989).

There is some evidence that passive transport aids the movement of spermatozoa through the female genital tract (Settlage *et al* 1973; Stone, 1989). Brown (1944) found that the time taken for spermatozoa to reach the fallopian tubes after

insemination indicated that the velocity of sperm movement *in vivo* was greater than that measured *in vitro* thus suggesting that the female reproductive tract offered some assistance to the movement of spermatozoa. Although passage of inert particles from the vagina to the oviduct has been described (Egli & Newton, 1961), dead spermatozoa are unable to ascend the tract (Stone, 1989) indicating that active sperm movement is also required.

It has been suggested that spermatozoa found the egg by rheotaxis where they swam against the current in a moving body of fluid. As the flow of fluid in the female genital tract is outward towards the vagina, spermatozoa move upstream to the fallopian tubes. However, it has been demonstrated in many animal species, including mammals, that the process of fertilization is preceeded by a chemical signal from the oocyte to the male sperm cells (Garbers *et al*, 1989) and some evidence suggests that sperm passage to the site of fertilization is synchronized with ovulation to ensure successful gamete interaction occurs (Cooper *et al*, 1979; Smith *et al*, 1987; Suarez, 1987). In many species, spermatozoa have been shown to accumulate in various regions of the female reproductive tract after insemination and these sites may serve as regions of sperm storage or reservoirs (Overstreet, 1983). The cervix (Overstreet *et al*, 1988; Zinaman *et al*, 1989) and the intramural segment of the fallopian tubes (Hunter, 1987) have both been implicated as sperm reservoir sites in humans. Human follicular fluid has also been shown to contain a chemoattractant (Herriot *et al*, 1986; Bryant *et al*, 1988). Villaneuva-Diaz *et al* (1990) and Ralt *et al* (1991) showed that spermatozoa were attracted to human follicular fluid but that this response was quite variable. Ralt *et al* (1991) also demonstrated that there was a strong correlation between the ability of follicular fluid from a particular follicle to attract spermatozoa and the ability of the egg obtained from the same follicle to be fertilized *in vitro*. Cell surface receptors for progesterone have been identified on the head of human spermatozoa (Blackmore & Lattanzio, 1991) and this coupled with the evidence that progesterone can induce the

acrosome reaction in human spermatozoa (Osman *et al*, 1989) made progesterone a strong contender for the chemoattractant. However Ralt *et al* (1991) showed that the chemoattractant ability of follicular fluid was not correlated with levels of progesterone or oestrogen. Recently Parmentier *et al* (1992) have demonstrated the presence of transcripts belonging to a putative olfactory receptor gene family in mammalian germ cells and although it does not follow that the corresponding proteins are produced, it is tempting to speculate that these transcripts might encode the receptors implicated in the chemotaxis of sperm cells during fertilization.

2.2.2 Capacitation

Freshly ejaculated spermatozoa are not able to directly fertilize an egg but first require a period of maturation in the female reproductive tract. These prefertilization changes in spermatozoa were first reported independently by Austin (1951) and Chang (1951) and subsequently termed 'capacitation' (Austin, 1952). The original definition of capacitation included all maturational changes which occurred in spermatozoa after they left the male reproductive tract which rendered them capable of penetrating zona-intact eggs (Austin, 1952; Chang, 1984). As spermatozoa recovered from the fertilization site often had modified acrosomes or lacked them entirely (Austin & Bishop, 1958a), it was initially thought that the capacitation process included the loss of the acrosome during the acrosome reaction (Section 2.2.3). However, it was discovered that capacitated spermatozoa could be 'decapacitated' by treatment with seminal plasma and that decapacitated spermatozoa could be 'recapacitated' in the oviducts (Chang, 1957) indicating that capacitation is a reversible phenomenon (Bedford & Chang, 1962; Zaneveld *et al*, 1991). In contrast, the acrosome reaction is an irreversible event and thus capacitation was redefined to include all the prefertilization changes occurring in spermatozoa which prepare them for the acrosome reaction but excluding the acrosome reaction itself (Bedford, 1970). Capacitation is therefore a general term which includes

many physiological and morphological changes that occur in spermatozoa during transit through the female reproductive tract but which is still poorly understood (Chang, 1984; Sidhu & Guraya, 1989).

The majority of changes in capacitating spermatozoa occur in an asynchronous manner (Perreault & Rogers, 1982; Fraser & Ahuja, 1988) and as the physiological synchronization of capacitation in large numbers of spermatozoa is difficult to achieve, biochemical analysis of these changes is also difficult (Fraser & Ahuja, 1988). Many alterations to the plasma membrane occur during capacitation (O'Rand, 1979) including the loss of decapacitation factors (Chang, 1957; Eng & Oliphant, 1978), loss or movement of antigenic sites (Koehler, 1976; Oliphant & Brackett, 1973; Myles & Primakoff, 1984) and lectin binding changes (Schwarz & Koehler, 1979; Talbot & Franklin, 1978; Gordon *et al*, 1975). Some changes in lipid composition have also been reported (Schwarz & Koehler, 1979; Davis *et al*, 1980; Evans *et al*, 1980) which can result in restricted movement of some antigens (O'Rand, 1977, 1979).

Spermatozoa also exhibit a progressive increase in oxygen uptake and glycolytic activity during capacitation (Hamner & Williams, 1963; Mounib & Chang, 1964; Murdoch & White, 1967; Black *et al*, 1968; Iritani *et al*, 1969). These changes in the metabolic activity of spermatozoa during capacitation are manifested in altered movement characteristics of spermatozoa during the latter stages of capacitation (Robertson *et al*, 1988). This different motility pattern of spermatozoa before and after capacitation was first noticed in hamster spermatozoa and described by Yanagimachi as 'activation' (Yanagimachi, 1969, 1970) but subsequently renamed 'hyperactivation' to distinguish this type of movement from the initiation of sperm motility which occurs at ejaculation in many species (Yanagimachi, 1981). Hyperactivated motility has since been shown to occur in many other species (Yanigimachi, 1988b; Burkman, 1991) including guinea pig (Yanagimachi, 1972a), mouse (Fraser, 1977), rabbit (Brackett & Oliphant, 1975) and humans (Gould *et al*, 1983; Burkman, 1984; Mortimer *et al*,

1984). Prior to capacitation, spermatozoa swim in linear trajectories (Yeung & Wooley, 1984) but spermatozoa exhibiting hyperactivated motility move in a non-progressive circular manner (Suarez *et al*, 1983) and the sperm heads trace erratic trajectories (Katz & Yanagimachi, 1980; Robertson *et al*, 1988). The biological importance and significance of hyperactivated motility is still a subject of debate (Katz *et al*, 1989b; Mortimer & Mortimer, 1990; Barratt & Cooke, 1991) although it is generally agreed to be a necessary part of capacitation (Bedford, 1983; Chang, 1984; Yanagimachi, 1988b). Burkman (1984) showed that subfertile human specimens exhibited significantly less hyperactivation when compared to samples proven fertile during IVF and subsequently demonstrated that sperm populations with low hyperactivation show decreased binding to hemi-zonae pellucidae and have significantly lower IVF success rates (Burkman *et al*, 1990; Coddington *et al*, 1991).

It has been proposed that capacitation may facilitate the migration of spermatozoa by avoiding entrapment in the female tract and thus increasing the possibility of contact with the egg (Katz *et al*, 1989a) and/or that hyperactivated movement is required for penetration of oocyte investments in particular the cumulus oophorus and the zona pellucida (Fleming & Yanagimachi, 1982; Fraser, 1981). The alterations to the sperm membrane, together with the increase in sperm metabolism and consequent development of hyperactivated motility, that occur during capacitation and enable spermatozoa to undergo the acrosome reaction, are thus thought to be mechanisms which ensure that the spermatozoon's ability to fertilize an egg occurs at the correct time and place (O'Rand, 1979; Wassarman, 1987a; Sidhu & Guraya, 1989; Kopf & Wilde, 1990).

2.2.3 Acrosome Reaction

The acrosome is a membrane bound organelle which occupies the anterior region of the sperm head, just above the nucleus and beneath the plasma membrane (Wassarman,

1987a) and its name derives from the Greek for 'sharp body' (Epel, 1977). The acrosome reaction is a specialized calcium-dependent exocytosis event which occurs during the fertilization process and involves fusion of the outer acrosomal membrane with the overlying plasma membrane and the concomitant release of the acrosomal contents (Russell *et al*, 1979; Yanagimachi, 1981). One of the first reports of an acrosome reaction was made by Popa in 1927 who noticed that a droplet of 'sticky substance' was extruded from the tip of sea urchin spermatozoa when they were treated with 'egg water' (Popa, 1927 cited in Austin, 1968) but it was not until the 1950's that the acrosome reaction of sea urchin spermatozoa which occurs following contact with the jelly coat of the egg was fully described (Dan, 1952, 1956). The role of the acrosome reaction in mammalian spermatozoa was originally studied in rodents by Austin & Bishop (1958a&b) who concluded that "the acrosome becomes modified in sperm passing through the female genital tract and is detached before the sperm penetrates the zona pellucida".

The importance of the acrosome in human fertilization is highlighted by the impaired fertility of men whose spermatozoa have acrosomal defects (Liu *et al*, 1989a; Schill, 1991) such as globozoospermia (Jeyendran *et al*, 1985; Aitken *et al*, 1990), crater defect syndrome (Baccetti *et al*, 1989) and decapacitated spermatozoa syndrome (Perotti *et al*, 1981; Baccetti *et al*, 1984). Globozoospermic spermatozoa possess no acrosomal vesicle and are not able to bind to the zona pellucida (Aitken *et al*, 1990) nor to fuse with the oocyte (Weissenberg *et al*, 1983; Syms *et al*, 1984; Lalonde *et al*, 1988). The lack of an acrosome is incompatible with the participation of spermatozoa in the whole process of fertilization because a normal acrosome reaction is a prerequisite for sperm penetration of the egg investments (Moore & Bedford, 1983; von Bernhardi *et al*, 1990) particularly the zona pellucida (Tesarik *et al*, 1988; Tesarik, 1989) and the oolemma (Yanagimachi, 1988a; Tesarik & Testart, 1989).

The human sperm acrosome reaction is thought to proceed in a slightly different manner to that observed for spermatozoa from other species although the end result is the same (Nagae *et al*, 1986). In the typical acrosome reaction, multiple fusions occur between the plasma and outer acrosomal membranes and the acrosomal contents are dispersed through the fenestrated membranes (Piko & Tyler, 1964a&b; Barros *et al*, 1967; Sidhu & Guraya, 1989; Saling, 1991). Several investigators have shown that human spermatozoa follow a slightly different path (Roomans & Afzelius, 1967; Jamil & White, 1981; Nagae *et al*, 1986; Zaneveld *et al*, 1991). Initially the acrosomal matrix partially decondenses and the outer acrosomal membrane alone, or in combination with the plasma membrane, invaginates to form many vesicles in the acrosomal cap. After this the plasma and outer acrosomal membranes fuse. Membrane fusion always occurs at the anterior end of the equatorial segment and seldom occurs in the acrosomal cap region. These membranes then detach from the main body of the spermatozoon leaving numerous vesicles near the inner acrosomal membrane. Finally these vesicles disperse leaving the inner acrosomal membrane completely exposed (Nagae *et al*, 1986). The acrosome contains several hydrolytic enzymes including proacrosin/acrosin, neuramidase and hyaluronidase (Allison & Hartree, 1970; McRorrie & Williams, 1974; Morton, 1977; Stambaugh, 1978; Meizel, 1984) which are released during the acrosome reaction and thought to aid zona penetration (Meizel, 1978; Chang, 1984; Talbot, 1985; Zaneveld *et al*, 1991).

There is some controversy over the location of spermatozoa when they undergo the acrosome reaction. The main area of dispute is over whether it occurs before or after zona binding. Initially it was thought that only spermatozoa which had undergone the acrosome reaction could penetrate the cumulus oophorus (a layer of follicle cells embedded in a hyaluronic acid polymer matrix which surrounds ovulated eggs in most mammalian species), as spermatozoa observed in the cumulus matrix and associated with the zona pellucida of fertilized and unfertilized eggs *in vitro* had undergone the

acrosome reaction (Talbot & Di Carlantonio, 1984a&b; Wassarman, 1987a). Also hyaluronidase, one of the enzymes which is released from the acrosome during the acrosome reaction, was thought to degrade the hyaluronic acid in the cumulus matrix thus aiding sperm penetration of the cumulus layer (Austin & Bishop, 1958b; Bedford, 1972; Talbot & Franklin, 1974a&b; Perreault *et al*, 1979; Huang *et al*, 1981a). Furthermore many substances have been shown to induce spermatozoa to undergo the acrosome reaction (Wassarman, 1987a; Meizel, 1985) including oviductal fluid, follicular fluid and cumulus cells (Gwatkin, 1977; Green, 1978; Meizel, 1978, 1984; Yanagimachi, 1981; Boatman & Robbins, 1991) although other reports have refuted these claims (Suarez *et al*, 1983; Jacobs *et al*, 1984) and the physiological relevance of some of this data has recently been contested (Mortimer & Camenzind, 1989; White *et al*, 1990; Zaneveld *et al*, 1991).

It is difficult to identify unambiguously whether acrosome reacted spermatozoa found in the cumulus matrix *in vivo* are capable of fertilizing the egg or whether spermatozoa associated with the zona pellucida *in vivo* underwent the acrosome reaction before or after zona binding (Wassarman, 1987a). Several investigators have now suggested that spermatozoa which spontaneously acrosome react *in vitro* are inferior sperm cells and thus are unable to fertilize an egg (Schill *et al*, 1988; White *et al*, 1990; Fenichel *et al*, 1991). Human spermatozoa can initiate binding to the zona pellucida after the acrosome reaction (Morales *et al*, 1989) but only those that undergo the acrosome reaction at the zona pellucida seem capable of penetration (Tesarik *et al*, 1988; Tesarik, 1989). Furthermore Topfer-Petersen *et al* (1985) reported that infertile men with polyzoospermia (ie with an increased number of spermatozoa in their ejaculate, usually >350 x 10^6/ml), have an abnormal rate of spontaneously occurring acrosome reactions. These data suggest that spermatozoa which spuriously acrosome react before interaction with the egg are not capable of completing the fertilization process. This indicates that the timing and location of the acrosome reaction are

important factors in determining the fertilizing ability of spermatozoa (Schill *et al*, 1988; Tesarik, 1989; Wolf, 1989; Tesarik *et al*, 1990). There have also been some reports that acrosome intact spermatozoa can penetrate the cumulus and bind to the zona pellucida (Anderson *et al*, 1975; Storey *et al*, 1984; Cherr *et al*, 1986; Menkveld *et al*, 1991) and that both uncapacitiated and acrosome reacted spermatozoa are incapable of entering the matrix (Cherr *et al*, 1986; Cummins & Yanagimachi, 1986). Talbot *et al* (1985) showed that sea urchin and frog spermatozoa which lack hyaluronidase can penetrate the cumulus matrix *in vitro* and reach the zona pellucida although they are unable to bind to or penetrate it. There have also been some reports which identified hyaluronidase associated with the plasma membrane of acrosome intact spermatozoa. It has therefore been questioned whether it is necessary for spermatozoa to undergo the acrosome reaction in order to penetrate the cumulus matrix (Talbot & Franklin, 1974a&b; Talbot, 1985; Zao *et al*, 1985). Although it must be remembered that these experiments were carried out *in vitro* and that under such conditions the state of the cumulus may differ from that found *in vivo*, these data suggest that the cumulus matrix is not a significant barrier to spermatozoa trying to reach the egg.

major to fertilization
The block appears to be at the level of the zona pellucida as spontaneously acrosome reacted spermatozoa are still able to fertilize an egg and lead to normal development and subsequent birth of normal young, but only if the zona pellucida is first removed from the oocyte (Naito *et al*, 1992). Experiments with 'denuded' eggs (which have had the cumulus cells removed) indicate that only acrosome intact spermatozoa bind to the zona pellucida (Wolf & Inoue, 1976; Saling *et al*, 1978; Saling & Storey, 1979; Phillips & Shalgi, 1980a; Bleil & Wassarman, 1983) and after binding they are induced to undergo the acrosome reaction (Saling *et al*, 1979; Florman & Storey, 1982; Bleil & Wassarman, 1983; Cherr *et al*, 1986). These observations are consistent with the results of further experiments which showed that acid- or heat-solubilized zona pellucida can induce the acrosome reaction (Florman & Storey,

1982; Bleil & Wassarman, 1983, Cherr *et al*, 1986). When the zona pellucida is dissociated into its component glycoproteins, one of these, ZP3, binds preferentially to acrosome intact spermatozoa (Bleil & Wassarman, 1986) and can stimulate the acrosome reaction (Bleil & Wassarman, 1983; Wassarman *et al*, 1985). Treatment of ZP3 with pronase destroys its acrosome reaction-inducing ability thus indicating that the protein component is important for this function (Florman *et al*, 1984) although Wassarman (1987b&c) subsequently demonstrated that both the polypeptide chain and the oligosaccharides are required. More recently, Leyton & Saling (1989) demonstrated that the cross-linking of receptors in spermatozoa by ZP3 is responsible for its acrosome reaction-inducing capability. Binding of ZP3 is thought to induce a fusogenic cascade involving guanine nucleotide binding proteins (Endo *et al*, 1987; Florman *et al*, 1989; Lee *et al*, 1992; Wilde *et al*, 1992). It is therefore now thought that acrosome intact spermatozoa penetrate through the cumulus oophorus matrix and bind to the zona pellucida where cross-linking of sperm receptors by ZP3 induces the spermatozoon to acrosome react.

Although much of this work has been carried out in the mouse, the same mechanisms are believed to occur in humans. Human spermatozoa can traverse the cumulus oophorus in an acrosome intact state and the human cumulus does not rapidly induce the acrosome reaction in these spermatozoa (White *et al*, 1990). Both acrosome intact and acrosome reacted spermatozoa can bind to the human zona pellucida (Morales *et al*, 1989) although Chen & Sathananthan (1986) demonstrated that only a small proportion of the spermatozoa which reach the zona of intact oocytes one hour post insemination had acrosome reacted. However after a further 1-2 hours, many spermatozoa were observed undergoing the acrosome reaction within the cumulus and on the zona surface (Sathananthan *et al*, 1982; Dvorak *et al*, 1984; Chen & Sathananthan, 1986). The zona pellucida has also been shown to play a significant role in the induction of the acrosome reaction in human spermatozoa (Cross *et al*, 1988;

Aitken, 1989b; Liu & Baker, 1990). Tesarik (1989) demonstrated that the number of spermatozoa within the zona pellucida corresponded with the number of acrosin deposits on the zona surface. This indicated that the timing of the acrosome reaction was synchronized to maximize the spermatozoon's potential to penetrate through the egg investments by releasing acrosin at the surface of the zona pellucida. These results have lead to the suggestion that the first human spermatozoa to penetrate the cumulus, react at the zona surface and that only later do a significant number begin to react within the cumulus matrix before reaching the zona pellucida (White *et al*, 1990). The action of the hydrolytic enzymes released by the acrosome reaction will be maximized if the timing of the acrosome reaction is coordinated to coincide with the binding of the spermatozoon to the layer which the enzymes aid penetration through (Zaneveld *et al*, 1991). Thus if the enzymes are involved in zona penetration (Section 2.2.4), it would be physiologically more advantageous if they were released after the spermatozoon had bound to the zona so that they exert their action specifically at the site where sperm binding takes place. Thus the induction of the acrosome reaction in 'later' spermatozoa while in the cumulus layer will result in a less efficient acrosome reaction and may be another protective mechanism by which the egg decreases the probability of a polyspermic fertilization occurring.

2.2.4 Sperm-Zona Pellucida Interaction

The zona pellucida is an extracellular matrix of glycoproteins which surround the oocyte (Bleil & Wassarman, 1980a; Dunbar *et al*, 1980; Dunbar & Wolgemuth, 1984; Wassarman *et al*, 1985). Mammalian zonae are typically composed of three major glycoprotein families which are named ZP1, ZP2 and ZP3 in order of decreasing molecular weight (Bleil & Wassarman, 1980a; Sacco *et al*, 1981). The members of each individual family are all related by the same polypeptide backbone but exhibit molecular weight and charge heterogeneity due to differential glycosylation which is

both N- and O-linked to the peptide core (Hendrick & Wardrip, 1981; Wassarman *et al*, 1985). The zona pellucida proteins are synthesized by the growing oocyte and secreted to form a filamentous zona matrix (Bleil & Wassarman, 1980b; Phillips & Shalgi, 1980b; Shimizu *et al*, 1983). This matrix is composed of ZP2 and ZP3 copolymers crosslinked by ZP1 (Greve & Wassarman, 1985; Wassarman, 1988b). Initially spermatozoa become loosely associated with the zona pellucida and this is referred to as 'attachment' (Hartmann *et al*, 1972; Hartmann, 1983). Subsequently, spermatozoa adhere more strongly and this is known as 'binding' (Hartmann *et al*, 1972; Hartmann, 1983). It is thought that the orientation of both gametes with respect to each other during the attachment phase, influences whether or not this progresses to the binding phase (Wassarman, 1987a).

As discussed in the previous section, it is currently thought that acrosome intact spermatozoa bind to the zona pellucida (via ZP3) and are induced to undergo the acrosome reaction. Spermatozoa bind to O-linked oligosaccharide chains of ZP3 (Bleil & Wassarman, 1980c; Florman *et al*, 1984; Florman & Wassarman, 1985; Bleil & Wassarman, 1988) and cross-linking of zona-receptors in spermatozoa by ZP3 results in the acrosome reaction (Leyton & Saling, 1989). The enzymes released from the acrosome as a consequence, are thought to aid zona penetration (Gaddum & Blandau, 1970; McRorie & Williams, 1974; Gwatkin, 1977; Hartree, 1977; Stambaugh, 1978). The zona protein ZP1, has been identified as the primary substrate for acrosin (Brown & Cheng, 1985; Dunbar *et al*, 1985; Urch *et al*, 1985). The digestion of ZP1 by acrosin destroys cross-linking between the ZP2 and ZP3 copolymers and this coupled with the propulsive force of sperm movement allows the spermatozoa to penetrate the zona pellucida (Bedford & Roger, 1983). ZP2 exhibits preferential binding for acrosome reacted sperm (Bleil & Wassarman, 1986; Mortillo & Wassarman, 1991) and is thought to act as a secondary sperm receptor for bound

spermatozoa after the induced acrosome reaction (Bleil & Wassarman, 1986; Bleil *et al*, 1988).

The zona pellucida conveys some species specificity to the fertilization process (Section 2.2.7), is involved in the block to polyspermy (Section 2.2.6) and is also thought to act as a filter which ensures that morphologically normal spermatozoa participate in the fertilization process (Krzanowska & Lorenc, 1983; Kot & Handel, 1987; Menkveld *et al*, 1991). Whether this is due to a rejection of abnormal spermatozoa by the zona pellucida or due to an inherent disability of the abnormal sperm cells to bind to the zona pellucida is unclear.

2.2.5 Sperm-Oolemma Interaction

Spermatozoa which penetrate the zona pellucida reach the perivitelline space where they interact with microvilli from the oocyte which attach to the sperm head at anterior and posterior positions (Koehler *et al*, 1982). Fusion of the spermatozoon with the egg is initiated by the plasma membrane overlying the equatorial segment (Bedford & Cooper, 1978; Bedford *et al*, 1979). This region of the plasma membrane acquires the ability to fuse with the oolemma only after the spermatozoon has undergone the acrosome reaction, as acrosome intact spermatozoa cannot fuse with the egg even if the zona pellucida is first removed (Yanagimachi & Noda, 1970). It is not thought that any of the contents of the acrosomal vesicle which are released during the acrosome reaction, alter the plasma membrane over the equatorial segment because acrosome intact spermatozoa cannot be induced to fuse with the oolemma after treatment with crude acrosomal extracts (Yanagimachi, 1988a). It has been suggested that an influx of Ca^{2+} into acrosome reacting or reacted spermatozoa may be one of the factors responsible for this modification of the sperm membrane (Monroy, 1985).

As soon as the spermatozoon contacts the egg plasma membrane, the sperm tail abruptly ceases to move (Yanagimachi, 1966; Sato & Blandau, 1979; Gaddum-Rosse,

1985) and unlike sperm-zona pellucida penetration, sperm movement is not required for sperm-oolemma fusion to occur in humans (Yanagimachi, 1981). This is demonstrated by spermatozoa from men who have Kartagener's syndrome, a condition where spermatozoa are completely normal except they lack dynein arms and therefore are completely motionless (Afzelius *et al*, 1975). If such spermatozoa are capacitated, acrosome reacted and micromanipulated so that they lie adjacent to the oolemma, they fuse with and fertilize the egg (Aitken *et al*, 1983).

The cortex of the egg, an area of cytoplasm just beneath the oolemma, contains the contractile proteins actin and myosin, which extend from the cortex into the microvilli. The microvilli interdigitate over the sperm head and the actin and myosin filaments contract upon fertilization, drawing the spermatozoon into the egg (Wassarman, 1987c). The sperm head decondenses within the egg and subsequently becomes the male pronucleus (Bedford & Roger, 1983). Fusion of the spermatozoon with the oolemma activates the egg which completes meiosis and extrudes the second polar body (Bedford & Roger, 1983). It also triggers the cortical reaction which contributes to the block to polyspermy (Section 2.2.6).

The amino acid sequence Arg-Gly-Asp (RGD) has been shown to be involved in a variety of cell-cell adhesion recognition systems (Ruoslahti & Piershbacher, 1986; Hynes, 1987) and the receptors that recognize RGD in their ligands are members of a supergene family known as the integrins (Edelman, 1987; Hynes, 1987). Fibronectin is an extracellular matrix protein which contains the RGD sequence in its cell binding domain (Yamada & Kennedy, 1985; Plow *et al*, 1985) and which has been detected on the surface of fixed human spermatozoa (Vuento *et al*, 1984; Glander *et al*, 1987). Fusi & Bronson (1992) confirmed the presence of fibronectin on the surface of unfixed living spermatozoa and demonstrated that its expression was increased on capacitated spermatozoa probably due to the unmasking of a cellular form of fibronectin on the sperm surface during the capacitation process. Preliminary experiments showed that

peptides containing the RGD adhesion peptide sequence are able to cause a concentration-dependent decrease in the adhesion of human spermatozoa to the oolemma of zona-free hamster eggs and their subsequent penetration whereas similar peptides which do not contain RGD cannot (Bronson & Fusi, 1990a&b). The use of immunobeads coupled to an RGD-containing peptide indicated that the integrin receptor is localized on the egg surface (Bronson & Fusi, 1990b). Recently Fusi *et al* (1992) provided further evidence that the human oolemma contains an integrin cell adhesion receptor by demonstrating that a specific RGD-binding moiety was present on the egg plasma membrane and that fibronectin was also able to bind to the oolemma. Furthermore anti-fibronectin antibodies interfere with the sperm-oolemma interaction as assessed using human sperm and the zona-free hamster eggs (Fusi & Bronson, 1992). Recently Blobel *et al* (1992) have isolated the gene for a guinea pig sperm protein, PH30, which is involved in guinea pig sperm-egg fusion and identified an integrin binding domain (disintegrin) within the sequence (see Section 2.3.3). No human homologue of this protein has yet been identified. Taken together these data strongly suggest that a fibronectin-integrin receptor system is involved in the human sperm-oolemma interaction.

2.2.6 Block to Polyspermy

The fertilization of the egg by more than one spermatozoon (polyspermy) is lethal for the embryo (Bedford & Roger, 1983). This is mainly prevented by the cortical reaction which leads to the zona reaction (Soupart & Strong, 1974; Bedford & Roger, 1983; Wassarman, 1987c; Ducibella, 1991). Corticle granules are membrane-bound, lysosome-like organelles which are located in the egg cortex. The cortical reaction is triggered by fertilization and involves fusion of the cortical granules with the plasma membrane of the egg (Nicosia *et al*, 1977; Schuel, 1978; Gulyas, 1980; Wassarman, 1987a). Membrane fusion initially occurs at the point of sperm-egg fusion and is

propagated as a wave over the entire surface of the egg (Wassarman, 1987a). The contents of the granules, including various hydrolytic enzymes, are released into the perivitelline space (Gwatkin, 1977; Whitaker & Steinhart, 1985) and permeate the zona pellucida which is highly porous (Gwatkin, 1977). The zona pellucida is then modified — the extracellular layer hardens and sperm receptors are inactivated (Barros & Yanagimachi, 1971; Bleil & Wassarman, 1980a; Wassarman, 1987a). ZP3 is modified to become $ZP3_f$ which no longer binds to spermatozoa nor induces the acrosome reaction and ZP2 is modified to become $ZP2_f$ which is also unable to bind spermatozoa (Bleil & Wassarman, 1980c; Bleil *et al*, 1981; Wassarman *et al*, 1986; Moller & Wassarman, 1989; Kopf & Wilde, 1990). This prevents further spermatozoa from interacting with the egg and provides some protection for the developing embryo.

2.2.7 Species Specificity

Several investigations into mammalian heterologous fertilization have been reported with varying results (Dickmann, 1962; Yanagimachi, 1972b; Hanada & Chang, 1972, 1976, 1978; Fukuda *et al*, 1979). In general, sperm penetration through the zona pellucida and sperm-egg fusion may occur between closely related species (Maddock & Dawson, 1974; Lambert, 1984; Roldan *et al*, 1985) but spermatozoa usually fail to attach to or penetrate into the zonae pellucidae of eggs from distant species or from different genera (Yanagimachi, 1977). Often fertilization does not occur if zona-intact eggs are used but spermatozoa can readily fuse with the oolemma if the zona pellucida is removed, indicating that much of the block to species specificity resides in the zona pellucida (Hanada & Chang, 1972; Yanagimachi, 1972b, 1977; Pavlok, 1979). Zona-free hamster eggs can be penetrated by spermatozoa from a wide variety of mammals including humans and this is used as the basis of an *in vitro* test of the fertilizing capacity of spermatozoa, the hamster egg penetration test (Yanagimachi *et al*,

1976; Binor *et al*, 1980; Pavlok *et al*, 1983; Hoffman & Curtis, 1984; Yanagimachi, 1984; Flechon & Pavlok, 1986; Warikoo *et al*, 1986; Lambert *et al*, 1991). However the plasma membrane does not totally lack species specificity. When zona-free hamster eggs are incubated with equal numbers of hamster, human and guinea pig spermatozoa, a greater number of homologous than heterologous spermatozoa penetrate the eggs (Yanagimachi, 1981) and the plasma membrane of mouse eggs will only fuse with mouse spermatozoa (Yanagimachi, 1984, 1988a). It is thus thought that the affinity of the egg plasma membrane is greatest for homologous spermatozoa although the mechanism of action of this species specificity is not known. Even in those few cases when interspecies fertilization does occur (Bedford, 1977; Lambert, 1984; Roldan *et al*, 1985), the heterologous combination of spermatozoa and oocytes results in fewer fertilized oocytes than the homologous combination, both *in vitro* and *in vivo* (O'Rand, 1988). Embryo abnormality in the heterologous situation is high and development is often arrested at the two-cell stage. Although a few mammalian hybrids do exist (eg horse and donkey cross to give mule or hinny and horse and zebra cross to give horsebras or zebrorses), these are the exception rather than the rule (Wassarman, 1987a; O'Rand, 1988). The fact that interspecies fertilization does occur shows that some of the mechanisms involved are similar between species and that the incompatability demonstrated by the abnormality of the resulting embryo or its arrested development is largely due to the incompatability of the genomes (O'Rand, 1988). This implies that if a molecule is shown to be important for fertilization in one species, a homologue may be found in other species (see Section 2.3).

2.3 Sperm Antigens

Numerous sperm antigens have now been identified and some have been well characterized. Of the many that have been reported, only a few have been identified as

playing a role in fertilization (Anderson *et al*, 1987). A selection of monoclonal antibodies which have identified molecules of particular interest, is discussed in the following sections and they have been categorized according to the event in the fertilization process at which they exert their effect. Special attention has been paid to antigens found on human spermatozoa and to those which are particularly relevant to the development of contraceptive vaccines.

2.3.1 Capacitation and the Acrosome Reaction

MA24 is a monoclonal antibody which was raised against human spermatozoa and is germ cell- but not species-specific (Naz *et al*, 1984). Its corresponding antigen, FA-1 has been isolated from both human and murine testis (Naz *et al*, 1984) and is homologous when purified from either source (Naz *et al*, 1986; Naz, 1987b). FA-1 exists as a monomer of 23kD that frequently dimerizes to a 47kD form (Naz *et al*, 1984; Naz & Bhargava, 1990). This glycoprotein contains 18.8% carbohydrate by mass (Naz *et al*, 1986) and localizes to the postacrosomal region of the sperm head and the mid and principle piece of the tail (Naz *et al*, 1984). MA24 inhibits murine and rabbit *in vitro* fertilization and the hamster egg penetration test performed using human spermatozoa (Naz *et al*, 1984; Naz, 1988). The antibody reduces the number of spermatozoa binding to the zona pellucida and abolishes sperm penetration through the zona. In the murine system, fertilization is inhibited by MA24 only in the presence of the zona pellucida as no block is seen when it is removed (Naz *et al*, 1984; Naz, 1988) suggesting that the block occurs at or before the level of the zona. As the antibody also completely inhibits the pentration of zona-free hamster oocytes by human spermatozoa, it is thought that FA-1 may be vital for capacitation or the acrosome reaction (Gould *et al*, 1983; Naz *et al*, 1984; Naz, 1988). Fertilization was blocked in female rabbits when they were artificially inseminated with MA24-treated spermatozoa (Naz *et al*, 1984). Active immunization studies using purified FA-1 in female rabbits have been

performed and resulted in a complete block of fertility in the majority of animals (Naz, 1987b). Antisera from these rabbits recognized the specific monomeric (23kD) and dimeric (47kD) forms of FA-1 in Western blots and were tissue specific. This indicates that the polyclonal immune response produced upon active immunization with FA-1, was tissue specific which is a major requirement in the development of a contraceptive vaccine in order to minimize side-effects. Sera from immunoinfertile men and women and from vasectomized men reacts strongly with FA-1 whereas sera from fertile men and women does not and there is a good correlation between the presence of these anti-FA-1 antibodies and the failure of *in vitro* fertilization in humans (Naz, 1987b; Naz *et al*, 1989; Bronson *et al*, 1989). Naz *et al* (1987b) demonstrated that it was possible to specifically neutralize the adverse effect of sera from these patients on the hamster egg penetration test by absorption with FA-1. These data suggest that FA-1 is involved in involuntary infertility in humans and that the antigen is tissue specific in man as no adverse side effects are apparent.

M42 is a monoclonal antibody raised against murine spermatozoa which recognizes an antigen located in a restricted region of the plasma membrane overlying the acrosome, called the acrosomal crest (Saling & Lakoski, 1985). It specifically inhibits the induction of the acrosome reaction (Saling, 1986) but discriminates between physiologically- and pharmacologically-induced acrosome reactions, inhibiting only the former (Leyton *et al*, 1989). The antigen is involved in an early step in the acrosome reaction as once initial events have occurred, M42 antibody can not inhibit completion of the acrosome reaction (Leyton *et al*, 1989). This initial step must be bypassed when the acrosome reaction is artificially induced, for example by using the calcium ionophore A23187. In Western blotting experiments, M42 recognizes a doublet at 220/240kD in caput epididymal mouse spermatozoa and at 200/220kD in cauda epididymal mouse spermatozoa suggesting that the M42 antigen is post-translationally modified during epididymal maturation (Lakoski *et al*, 1988). Cross-reactivity is seen

with hamster but not human or rabbit spermatozoa (Saling & Lakoski, 1985) although it must be remembered that monoclonal antibodies only recognize a single epitope on a molecule and this does therefore not preclude a homologue in other species. Passive immunization of female mice with purified M42 inhibits fertilization in a concentration-dependent manner (Saling *et al*, 1986; Saling, 1990) demonstrating that passive transfer of antibodies to affect fertility is a feasible proposition. Using this approach, Saling *et al* (1886) also found that the simultaneous use of two monoclonal antibodies increased the degree of inhibition suggesting that antibodies can work additively and a multivalent vaccine might be the best approach.

HS-63 is a monoclonal antibody raised against human sperm which reacts specifically with a highly conserved sperm antigen (Lee *et al*, 1984). Although it cross-reacts with spermatozoa from different mammalian species where the antigen is localized to the acrosome, no cross-reactivity with an extensive panel of somatic tissues has been identified (Anderson *et al*, 1987). HS-63 inhibits both the zona- and ionophore-induced acrosome reactions of human and mouse spermatozoa thus blocking fertilization (Liu *et al*, 1989b). HS-63 was used in passive immunization studies in male and female mice (Lee *et al*, 1987) and an antifertility effect was seen although this was weaker in the *in vivo* situation than that obtained in the *in vitro* experiments. The antifertility effect was also sex related in that it was lower in passively immunized males than females probably due to the presence of the blood-testis barrier which sequesters much of the male reproductive tract from the immune system. The corresponding mouse sperm antigen, MSA-63, has been purified from mouse testis and elicits a strong isoimmune response in female mice following active immunizations (Liu *et al*, 1989c). This isoimmune serum was shown to have a strong inhibitory effect on the *in vitro* fertilization of mouse oocytes (Liu *et al*, 1989c). The gene encoding MSA-63 was isolated from a mouse testicular cDNA library (Liu *et al*, 1990). This gene was shown to be conserved among different mammalian species and gene expression identified as

tissue specific (Liu *et al*, 1990). Recombinant MSA-63 was produced and specific antisera raised in mice and rabbits. The titre of the antisera raised against the fusion protein was considerably lower than that obtained with the native protein and consequently the antifertility effect of the anti-fusion protein antiserum was also weaker (Liu *et al*, 1990). However an antifertility effect was observed using the recombinant protein, indicating that it is possible to produce suitable immunocontraceptive vaccines using recombinant DNA technology.

2.3.2 Sperm-Zona Pellucida Interaction

Monoclonal antibody 1A1 has recently been reported and was produced using *in vitro* capacitated spermatozoa as the immunogen (Dubova-Mihailova *et al*, 1991). It recognizes a family of proteins of molecular weight 20-34kD in Western blots. The corresponding antigen is located in the acrosome of human spermatozoa and is widely distributed among different mammalian species. The antibody blocks murine, porcine and human *in vitro* fertilization by blocking sperm attachment to and penetration through the zona pellucida. Further studies on this antibody and its corresponding antigen are required to identify their potential for contraceptive vaccine development.

PH20 is an integral membrane protein of 64kD found in guinea pig spermatozoa (Primakoff & Myles, 1983; Myles & Primakoff, 1984; Primakoff *et al*, 1988a). In acrosome intact spermatozoa, PH20 is located exclusively in the posterior head region but after the acrosome reaction, its localization is altered (Myles & Primakoff, 1984). In acrosome reacted spermatozoa, it is found on the inner acrosomal membrane and the number of antigenic sites increases three fold (Cowan *et al*, 1986). PH20 is thought to play a role in the interaction between spermatozoa and the zona pellucida as antibodies raised against PH20 inhibit guinea pig *in vitro* fertilization and the binding of guinea pig spermatozoa to the zona pellucida (Primakoff *et al*, 1985; Myles *et al*, 1987). Active immunization of male and female guinea pigs with PH20 completely blocked fertility

(Primakoff *et al*, 1988b). This effect was long lasting and reversible as fertility was regained within 6-15 months, thus indicating that this approach to the development of a contaceptive vaccine is possible and suitable for use in both males and females. Although a monoclonal antibody raised against PH20 recognizes only guinea pig spermatozoa, polyclonal antibodies cross-react with human spermatozoa (Isojima, 1990) indicating that a human homologue of PH20 may exist.

2.3.3 Sperm-Oolemma Interaction

MHS-10 is a monoclonal antibody produced against human spermatozoa (Herr *et al*, 1985). In Western blotting experiments, MHS-10 recognizes a family of proteins of relative molecular mass 18-34kD (Herr *et al*, 1990a). It is testis- but not species-specific (Anderson *et al*, 1987; Herr *et al*, 1990b) cross-reacting with spermatozoa from baboons, macaques and pigs but not with rabbit, bull, rat, guinea pig and cat spermatozoa (Herr *et al*, 1990b). The corresponding antigen, SP-10, is not found on the surface of acrosome intact spermatozoa but is located within the acrosome. After the acrosome reaction, it remains associated with the inner acrosomal membrane and equatorial segment (Herr *et al*, 1990a). The MHS-10 monoclonal antibody inhibits the sperm-oolemma interaction as assessed using zona-free hamster oocytes (Anderson *et al*, 1987). The gene encoding SP-10 has been isolated from a human testicular cDNA expression library (Wright *et al*, 1990) and no significant homology with other sequences contained in the three sequence databanks searched was identified. The sequence contains a 795bp open reading frame which translates to a 265 amino acid (28.3kD) protein. The deduced amino acid sequence contains several O- and N-linked glycosylation sites and has a very hydrophobic amino terminus which is thought to constitute a signal peptide, required to localize SP-10 to the acrosome. Recombinant fusion protein was produced and polyclonal antisera raised against it. The polyclonal antisera behaved in a similar fashion to the monoclonal antibody when tested in both

Western blots and immunocytochemistry suggesting that the SP-10 fusion protein can act as an effective immunogen. Further experiments using recombinant SP-10 are required to fully evaluate its potential as a candidate for a contraceptive vaccine.

Shaha *et al* (1988) raised a polyclonal antiserum against human spermatozoa which identified a protein band at 40kD in Western blots of human sperm proteins and at 24kD in rat testicular cytosol. These proteins were shown to share immunoreactive epitopes (Shaha *et al*, 1988). The antiserum recognized the acrosome of human, rat, mouse, hamster, rabbit and monkey spermatozoa and blocked attachment of mouse spermatozoa to mouse oocytes *in vitro* (Shaha *et al*, 1988). When it was passively administered to mice, a reduction in fertility resulted (Shaha *et al*, 1988). Monoclonal antibodies were raised against purified 24kD proteins from rat testicular cytosol (Shaha *et al*, 1989). One of these antibodies, HS-D5, was shown to agglutinate human spermatozoa and prevented binding of hamster spermatozoa to zona-free hamster eggs. The 24kD proteins from rat testicular cytosol were then used in active immunization studies in female rats (Shaha *et al*, 1990). High antibody titres were produced and the majority of the animals (83%) became infertile and remained infertile during the three months of the study. Those animals which fell pregnant had the lowest titres indicating that a high titre must be attained in all animals for the contraceptive efficacy to reach 100%.

M29 is a monoclonal antibody of the IgM subclass which was raised against mouse spermatozoa (Saling *et al*, 1983; 1985). It is germ cell-specific but species-cross-reactive recognizing spermatozoa from all of the other three species tested (human, hamster and rabbit; Saling *et al*, 1985). The antibody recognizes a 60kD protein which is extremely sensitive to proteolysis, rapidly breaking down to a 40kD form in the absence of protease inhibitors (Saling, 1990) and is localized to the equatorial segment of the mouse sperm head (Saling *et al*, 1985). M29 inhibits *in vitro* fertilization of mouse eggs in a concentration-dependent manner. The block appears to

be at the level of the oolemma as the inhibition of fertilization by M29 is unaffected by the presence or absence of the cumulus layer or the zona pellucida (Saling *et al*, 1985). Passive immunization studies using M29 in female mice, resulted in a concentration-dependent inhibition of fertilization *in vivo* (Saling & Waibel, 1985). Although fertilization was not completely inhibited at the highest antibody concentration used, further studies have demonstrated that passive administration of two anti-sperm antibodies which interfere at different points in fertilization, has a synergistic effect leading to a complete block to fertilization (Saling *et al*, 1986). Thus if passive immunization was to be used as the method of choice for administering a contraceptive vaccine, an antibody cocktail would be most effective.

PH-30 is an integral membrane protein located on the surface of guinea pig sperm (Primakoff & Myles, 1983; Primakoff *et al*, 1987). It is composed of two tightly associated but immunologically distinct subunits, α and β which have molecular weights of 44 and 60kD respectively and are both synthesized as larger precursor molecules (Primakoff *et al*, 1987; Blobel *et al*, 1990). A monoclonal antibody raised against PH-30 localizes the antigen to the postacrosomal region of the sperm head (Primakoff *et al*, 1987). PH-30 has been implicated in the fusion of the spermatozoon with the oolemma as the anti-PH-30 monoclonal antibody blocks the penetration of zona-free guinea pig eggs by guinea pig spermatozoa but not the binding of the spermatozoa to the oolemma (Primakoff *et al*, 1987). The genes encoding the PH-30 α and β subunits have been cloned and sequenced (Blobel *et al*, 1992). The α subunit nucleotide sequence contains an open reading frame which translates to a 289 amino acid protein which is thought to contain a large extracellular domain, a single membrane spanning domain and a short cytoplasmic tail. It also contains a putative fusion peptide which is similar to viral fusion peptides. The deduced amino acid sequence for the β subunit is 353 amino acids long and it has a similar structure to the α subunit except it lacks the putative fusion peptide in its extracellular domain. Instead the β subunit

contains an integrin binding domain (disintegrin) in this region which is thought to interact with an integrin-like receptor on the oolemma (see Section 2.2.5). Thus PH-30 is thought to mediate sperm-oolemma binding, via the β subunit and integrin receptor, and sperm-oolemma fusion, via the fusion peptide of the α subunit. No human homologue of this protein has yet been identified.

2.4 Summary

Although a great deal of research has been carried out on fertilization, the sequence of events is still poorly understood. With the advent of hybridoma technology, these processes and the molecules involved are now beginning to be elucidated. Monoclonal antibodies raised against spermatozoa from different species are able to identify some of the molecules important for fertilization thus allowing a more detailed insight in the interaction between spermatozoa and the egg.

3. General Materials and Methods

The materials and methods described in the following chapter are common to more than one individual experiment. Those pertaining to a particular experiment are described in the materials and methods section of the relevant chapter.

3.1 Semen Donation

The donors for the experiments described in this thesis were normal healthy males all of whom had undergone a complete physical examination and had been screened for hepatitis and sexually transmitted diseases including HIV. Human spermatozoa were collected by masturbation into sterile plastic containers (Sterilin, Hounslow, Middlesex, UK), from donors exhibiting normal semen profiles (>20 x 10^6 spermatozoa/ml, >40% progressive motility and >40% normal morphology; World Health Organization, 1987). At least 30 minutes were allowed for liquefaction to occur before the spermatozoa were separated from the seminal plasma as detailed below. A conventional semen profile was constructed for each sample using the procedures laid down by the World Health Organization (WHO; World Health Organization, 1987).

3.2 Sperm Preparation

Two different methods of sperm preparation were used. One involved separating the spermatozoa on Percoll gradients and the other used several cycles of washing. These are detailed in Sections 3.2.1 and 3.2.2 respectively. Regardless of the method used to prepare the spermatozoa, the final sperm pellet was resuspended in an appropriate known volume (usually 1ml) of Biggers, Whitten and Whittingham medium (BWW; Biggers *et al*, 1971), analysed to determine the concentration of spermatozoa, using an

improved Neubauer haemocytometer, and finally diluted to a concentration of 20 x 10^6 sperm cells/ml.

3.2.1 Percoll Separation

A slightly modified version of the protocol for the fractionation of semen on Percoll gradients described by Aitken and Clarkson (1988) was used. Liquefied semen was layered on top of a two step discontinuous Percoll gradient containing 3mls 100% Percoll overlayed with 3mls 50% Percoll in a 15 ml conical based sterile centrifuge tube (Falcon; Becton Dickinson Labware, Cowley, Oxford, UK). Isotonic Percoll was created by supplementing 10mls of 10 x concentrated medium 199 (Flow Laboratories, Irvine, Scotland, UK) with 300mg bovine serum albumin (BSA), 3mg sodium pyruvate and 0.37ml sodium lactate syrup (1.3g/ml; final concentration 4.8mg/ml; Sigma, Poole, Dorset, UK) and diluted with 90mls Percoll (Pharmacia LKB, Milton Keynes, UK). This solution was designated 100% isotonic Percoll (Lessley & Garner, 1983) and was subsequently diluted to 50% with BWW medium. The gradients were centrifuged at 500 x g for 20 minutes after which the seminal plasma was discarded and three cellular fractions were collected from the top of the gradient (0% fraction), the 50%/100% interface (50% fraction) and the base of the 100% fraction (100% fraction). The cells from each fraction were then resuspended in 10mls of BWW medium, centrifuged at 500 x g for 5 minutes to remove any residual traces of Percoll and finally resuspended in BWW medium at a concentration of 20 x 10^6 cells/ml.

3.2.2 Washing Preparation

Some semen samples were prepared by a conventional 3 x centrifugation and resuspension technique (Mann, 1945; Aitken *et al*, 1982a&b). Spermatozoa were separated from seminal plasma by 3 cycles of centrifugation (500 x g for 5 minutes)

and resuspension in 10mls BWW medium. Spermatozoa were finally diluted to a concentration of 20 x 10^6 cells/ml.

3.3 Isolation of Sperm Plasma Membrane

Isolation of the plasma membrane fraction from human spermatozoa was achieved by following a protocol previously described by Gillis *et al* (1978) as modified by Aitken *et al* (1987a). Sperm samples were prepared by either method detailed in Section 3.2 and resuspended in 1mM EDTA (pH5.0) containing a protease inhibitor, 1mM phenylmethyl sulphonyl fluoride (PMSF; Sigma). The sperm membranes were detached using an ultrasonicator (Soniprep; MSE, Crawley, Sussex, UK) using 3 x 10 second bursts with a probe amplitude of 30μm. The tubes were kept on ice between bursts to minimize the heating effects. The ultrasonicated suspension was centrifuged at 500 x g to pellet the intact spermatozoa and the supernatant was centrifuged again at 3 000 x g to remove any residual cells. This supernatant was centrifuged at 100 000 x g for 1 hour on an ultracentrifuge (Sorvall OTD-50; Du Pont Instruments, Stevenage, Herts, UK). The crude membrane pellet was resuspended in 0.25M sucrose and layered onto a discontinuous sucrose gradient (1.57M, 1.3M, 1.0M sucrose) and centrifuged at 100 000 x g for 2 hours. The membrane fraction was collected as a discrete layer at the 1.0M/1.3M sucrose interface, diluted in Dulbecco's phosphate buffered saline (PBS; Flow Laboratories) and centrifuged at 100 000 x g to pellet the membranes. The membrane preparations were resuspended in a small known volume of PBS (typically 100μl) and an aliquot (10μl) taken for protein estimation (Section 3.4). The plasma membrane preparations were stored frozen at -20°C until required.

3.4 Protein Estimation

An estimation of the protein content of samples was determined using a bicinchoninic acid (BCA) kit method (Pierce, Chester, Cheshire, UK) which was used according to the manufacturer's instructions. The working solution was prepared by mixing 25mls of BCA reagent with 0.5mls copper sulphate solution. 50µl of the standard or unknown solution (1/100 dilution) was added to 1ml of the working solution and mixed well. Bovine serum albumin diluted in PBS was used as the standard (0.2mg/ml, 0.4mg/ml, 0.6mg/ml, 0.8mg/ml, 1.0mg/ml and 1.2mg/ml) and PBS was used as the blank. The reaction was developed at room temperature for 1 hour and the absorbance at 562nm measured (Pye Unicam, Cambridge, UK). An assay calculation program written by Dr Phil Taylor for the Apple Macintosh Computer (AssayZap; Elsevier Biosoft, Cambridge, UK) was used to calculate the protein concentration of each unknown sample using a plot of the absorbance value at 562nm against the known protein concentrations.

3.5 Animals and Management

All the animals used in the experiments described in this thesis were housed in the Reproductive Biology Unit Animal House in the Centre for Reproductive Biology, Edinburgh. All experiments involving animals were approved by the Home Office under the terms of the Animal (Scientific Procedures) Act 1986; Project Licence PPL 60/00697; Personal Licence PIL 60/02179.

4. Production of Anti-Sperm Monoclonal Antibodies

4.1 Introduction

Since the production of monoclonal antibodies of predefined specificity was first described by Kohler and Milstein in 1975, they have been used for a wide variety of purposes in a host of disciplines. They have been used in areas as diverse as radioimmune assay of hormones and drugs, histocompatability testing, immunotherapy, purification of molecules by affinity chromatography, neurochemistry and microbial and parasitic disease (Goding, 1980). The basic production technique which is summarized in Figure 4.1 and described below, has remained virtually unaltered except for the addition of a few refinements.

A monoclonal antibody is one which is produced by a single clone of cells and structurally it is no different from other antibodies produced under natural conditions. Their production requires that antibody producing B lymphocytes are made immortal in the laboratory and are allowed to continue synthesizing antibody. This is achieved by the fusion of two cells — a B lymphocyte and a myeloma cell. The myeloma cell used is a malignancy of B lymphocyte which has the ability to reproduce itself indefinitely in culture but also has genetic deficiencies which do not allow it to grow under certain conditions. The two cell types are usually fused using polyethylene glycol (PEG; Davison and Gerald, 1976; Kohler and Milstein, 1976; Gefter *et al*, 1977) to promote membrane fusion although Sendai virus or lysolecithin can also be used (Goding, 1980). After the fusion process, the resulting cell is called a hybrid-myeloma or hybridoma but is commonly referred to as a hybrid. Hybrids are immortal antibody producing cell lines and are selected using HAT medium (so called because it contains Hypoxanthine, Aminopterin and Thymidine; Littlefield, 1964). Aminopterin is a

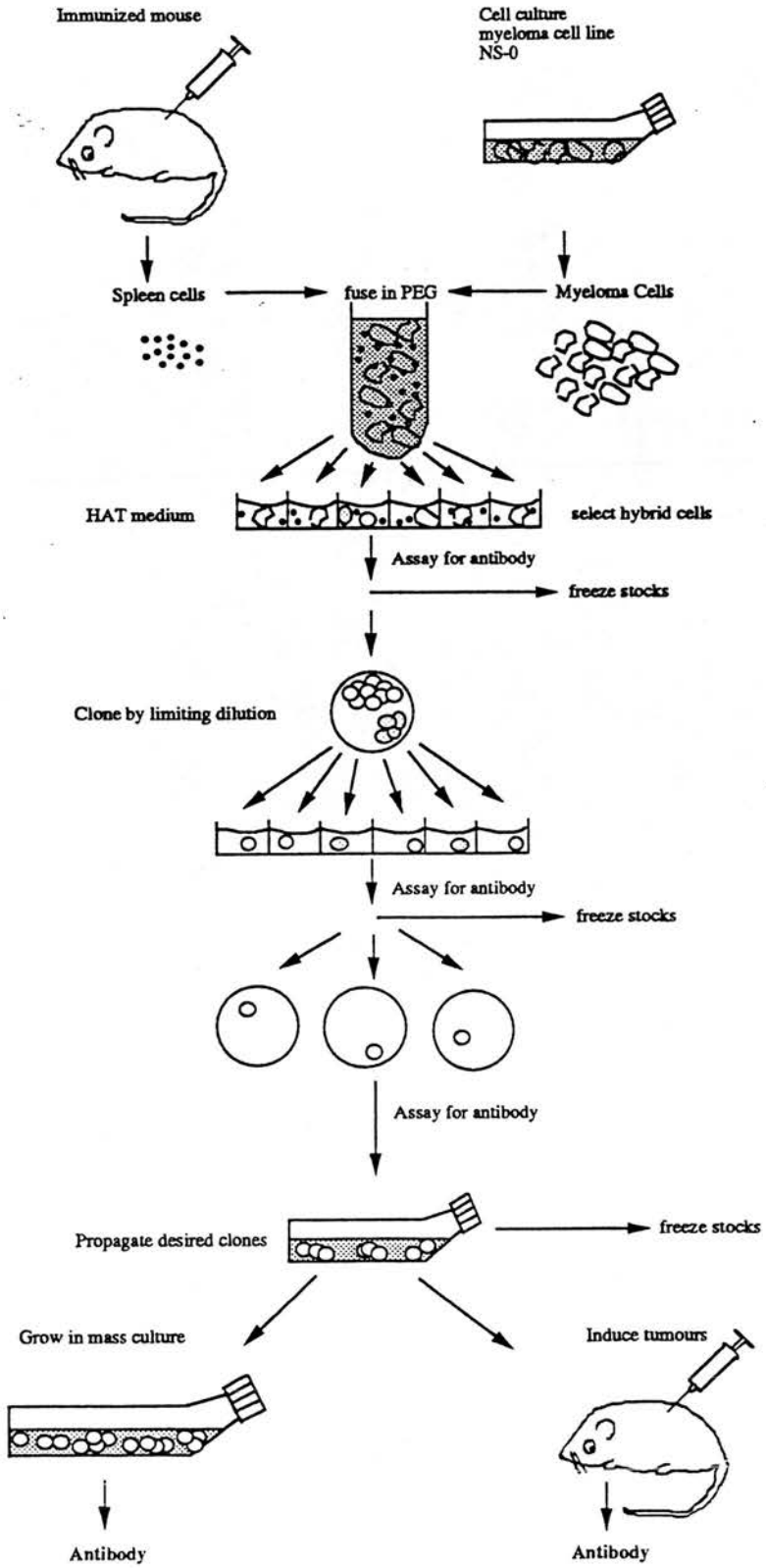

Figure 4.1 Summary of the basic technique of monoclonal antibody production.

powerful toxin which blocks the main pathway for endogenous synthesis of purine and pyrimidine nucleotides which are required for DNA synthesis. This block can be bypassed using the salvage pathway which recycles preformed nucleotides only if the cells are provided with hypoxanthine and thymidine and possess the enzymes hypoxanthine guanine phosphoribosyl transferase (HGPRT) and thymidine kinase (TK). The B lymphocytes used for the fusion have these enzymes and are therefore HAT resistant. The myeloma cell line however lack HGPRT and are therefore HAT sensitive. As a result of the fusion, the only cell type which can survive under HAT selection conditions is the hybrid which obtains HAT resistance (and antibody production capability) from the parental B lymphocyte and immortality from the parental myeloma cell. The other cell types die as a B lymphocyte-B lymphocyte fusion and unfused B lymphocytes are mortal and succumb after 1-2 weeks in culture. Any myeloma-myeloma cell fusions and unfused myeloma cells are sensitive to HAT selection and also die.

In order to increase the chances of obtaining a hybrid which is secreting the antibody of choice, the animal used for the the fusion is primed with antigen. Animals are normally given a primary injection and two boosters to elicit an immune response and a final boost 3-4 days prior to fusion. The immunization of the spleen donor has two main purposes — i) expanding the desired clones and therefore increasing the chances of obtaining a relevant hybrid; and ii) causing the B cells to divide and differentiate into cells which will fuse and form useful hybrids (Goding, 1980). There is evidence that recently activated B cells fuse preferentially and experiments have shown that the best time for fusion is 3-4 days after boosting when antigen induced proliferation is strong (Goding, 1980; French *et al*, 1986).

In order to assign defined biological roles to specific membrane components of the human spermatozoon, monoclonal antibodies have to be produced. Polyclonal

antisera are unsuitable for this task as they contain a complex mixture of antibodies which all have differing biological activities. Monoclonal antibodies are (by definition) specific to one particular epitope and are therefore crucial if this task is to be accomplished. Work using polyclonal antisera has shown that antibodies against the sperm head are responsible for blocking sperm-zona pellucida interaction *in vitro* (Aitken *et al*, 1981). Many workers have used whole spermatozoa as an immunogen when producing monoclonal antibodies and this often results in the production of monoclonal antibodies which localize to cytoskeletal elements of the spermatozoon particularly in the tail (Moore & Hartmann, 1984). This is probably due to the relatively long tail of spermatozoa which provides more antigen and hence provokes a greater immune response. If these antibodies block fertilization, it must be by an indirect method by interfering with sperm transport through the female reproductive tract thus preventing spermatozoa from reaching the site of fertilization (Aitken, 1982; Bronson *et al*, 1984). In order to assign specific biological functions to membrane components, the antibody must be able to interact directly with antigens expressed on the surface of the human spermatozoon. In order to increase the probability of producing a monoclonal antibody directed against a sperm surface antigen involved in fertilization, purified sperm plasma membrane has been prepared and used to preimmunize the mice.

4.2 Materials and Methods

4.2.1 Immunization Protocol

Female Balb/c mice were immunized intraperitoneally (ip) with purifed sperm plasma membrane. Each mouse was given 100µl of immunization mixture at each injection. The primary injection was given when the mice were 12 weeks old and consisted of

antigen emulsified in complete Freund's adjuvant (FCA; Sigma, Poole, Dorset, UK). The ratio of aqueous phase to FCA used was 2:3. Usually two booster injections of antigen adsorbed onto aluminium hydroxide gel (alum; Superfos, Vedbaek, Denmark) at a ratio of 1:1, were given. A final booster injection which contained no alum, was given 3 days prior to fusion. The details of the immunization schedule are shown in Table 4.1.

4.2.2 Bleeding of Mice

Mice were bled periodically to monitor the production of antibodies. They were anaesthetized using ether and a small volume of blood was collected from the tail vein by making a small nick using a sterile scalpel blade. The blood was then allowed to coagulate at room temperature for 30 minutes before centrifuging at 1 250 x g for 10 minutes to obtain serum. The antibody level was determined by ELISA.

4.2.3 Media

RPMI 1640 medium containing 20mM HEPES (Gibco, Paisley, Scotland, UK) was supplemented with 2mM L-glutamine, 10mM sodium pyruvate and 125 000 Units/l penicillin/streptomycin (all Gibco) was used throughout the tissue culture programme. As serum lots differ widely on their ability to support growth of hybrids (Oi & Herzenberg, 1980; Kennett, 1980; Galfre & Milstein, 1981), batches of foetal calf serum (FCS; Gibco) were tested using the parental NS-O cells to ensure that they could support the growth of hybrids. This was kindly carried out by Dr Margaret Paterson. The FCS batch which had optimal cloning efficiency was purchased in sufficient quantities to be used for all the hybridoma work. The concentration of FCS used varied between 5% and 15% (v/v) depending upon the procedure being used and the cell line being cultured. HAT selection media contained 15% (v/v) FCS, 0.1mM

hypoxanthine, 4μM aminopterin and 16μM thymidine in supplemented RPMI media. HT media had the same composition as HAT medium except that no aminopterin was added. Cells were harvested using 0.02% (v/v) EDTA (BDH, Poole, Dorset, UK) in Dulbecco's formula A phosphate buffered saline (PBS; Flow Laboratories, Irvine, Scotland, UK). Mixed thymocyte conditioned medium (MTM) was prepared as described by Micklem *et al* (1987). Briefly the thymus was aseptically removed from two 5-6 week old female rats of different strains (Wistar and Sprague Dawley). A cell suspension was made by pressing the thymus tissue between ground glass slides. The cells were counted and cultured at 5 x 10^6 cells/ml in 15% (v/v) FCS in supplemented RPMI 1640 medium for 48 hours. The culture medium was centrifuged at 1 250 x g for 10 minutes and the supernatant stored at -20°C until required.

4.2.4 Fusion Protocol

A diagram summarizing the fusion procedure is shown in Figure 4.1. Cells were incubated at 90% humidity and 37°C in 5% CO_2/air. A total of four fusions were carried out. The first fusion was kindly performed by Dr Margaret Paterson and her assistance with the other three fusions is also gratefully acknowledged.

Spleen cells The spleen was aseptically removed from the preimmunized mouse and transferred to a petri dish containing 10mls PBS. The spleen was gently teased apart using forceps then homogenized to obtain a cell suspension. The cells were then washed three times in PBS by centrifugation at 200 x g for 10 minutes. The pellet was finally resuspended in 10mls PBS and counted using the fluorescent stain acridine orange/ethidium bromide (each 1 part per million; Parks *et al*, 1962; Lee *et al*, 1975). Approximately 1 x 10^8 cells were typically obtained.

Plasmacytoma Cell Line The Balb/c plasmacytoma cell line, NS-0 (Galfre & Milstein, 1981) was used to produce the monoclonal antibodies. This was a generous

gift from Dr Liesel Micklem. NS-O cells in the log phase of growth were harvested using 0.02% (v/v) EDTA in PBS and washed twice in PBS. The cells were counted using an acridine orange/ethidium bromide vital stain.

Fusion Procedure 5×10^7 spleen cells and an equivalent number of NS-O cells were mixed together and centrifuged at 200 x g for 5 minutes. The supernatant was removed and 1ml 50% (w/v) polyethylene glycol 4000 (PEG; Fisons, Loughborough, Leicestershire, UK) in supplemented RPMI 1640, pH8.5 was slowly added down the side of the tube. The cells were swirled gently for 2 minutes and then centrifuged at 70 x g for 5 minutes. 5mls of supplemented RPMI medium was slowly overlaid over 1-2 minutes without disturbing the pellet. The tube was gently swirled for 3-4 minutes to gradually resuspend the cells. After centrifuging at 150 x g for 5 minutes, the supernatant was discarded and 5mls HAT medium slowly added without disturbing the pellet. This was allowed to stand for 7 minutes before being gently swirled to resuspend the fusion mixture. Finally HAT medium and MTM were added to adjust the cell concentration to 2×10^5 cells/0.2ml in 15% (v/v) MTM/HAT medium. The cells were then plated out (0.2ml/well) into the central 60 wells of a 96 well culture plate (Costar; Northumbria Biologicals, Cramlington, Northumberland, UK). The wells were checked for growth after 7 days and fed with 0.1ml HAT medium. Any well containing bacterial contamination and the 9 wells immediately surrounding it, were treated with sterile 1M NaOH. After a further 7 days and every subsequent 3 days, hybrids were examined. When the hybrid was large enough, the supernatant was screened for antibody production by ELISA. Each hybrid was tested at least twice and those hybrids which had two consecutive positive tests for anti-sperm antibody production were cloned immediately by limiting dilution (Section 4.2.6). Aliquots of each positive hybrid were stored in liquid nitrogen (Section 4.2.8).

4.2.5 Detection of Anti-Sperm Antibodies

Sperm coated plates 96 well microtitre plates were coated with spermatozoa for use in an enzyme linked immunoabsorbant assay (ELISA) to monitor the amount of anti-sperm antibody present. Spermatozoa were isolated by centrifugation through isotonic Percoll (Section 3.2.1) and adjusted to 10 x 10^6 cells/ml in PBS prewarmed to 37°C. 50µl of this solution was added to each well of a microtitre plate (5 x 10^5 cells/well) and the plate spun at 1 000 rpm (Coolspin; MSE, Crawley, Sussex, UK) at 25°C for 5 minutes. After aspirating the supernatant, 100µl of 1% (v/v) para-formaldehyde in PBS was added to each well and the plates incubated at room temperature for 30 minutes. The plates were spun again and washed 3 times in PBS-tween (0.05% v/v, Sigma) to reduce non-specific binding. The plates were dried in an aspirator containing phosphorus pentoxide under vacuum at 4°C. The sperm-coated plates were stored at -20°C prior to use.

ELISA 50µl of antibody solution was added to each well of a sperm-coated plate and incubated either for 1-2 hours at room temperature or overnight at 4°C. Culture supernatants were used neat and solutions to be titrated were added in doubling dilutions from 1/10. The supernatant was aspirated and the wells washed 3 times with PBS-tween (0.05% v/v). A 1/1 000 dilution (in PBS) of second antibody, sheep anti-mouse Ig-alkaline phosphatase conjugate (Sigma) was added and incubated for 1-2 hours at room temperature. The plates were washed again and 50µl of 1mg/ml p-nitrophenylphosphate substrate (Sigma) made up in 10% (v/v) diethanolamine (Sigma), pH9.8 was added to each well. The reaction was allowed to develop for 30-60 minutes at room temperature before the reaction was stopped by adding 50µl 3M NaOH to each well. The absorbance at 405nm was read using a 96 well plate reader (Titertek; Flow Laboratories) using air as the blank. A positive (mouse anti-sperm plasma membrane polyclonal antiserum) and negative (RPMI medium) control were included on each plate. A positive value was defined as any reading which was at least

twice the negative value. The titre was taken as the highest dilution which still gave a positive ELISA result (Voller *et al*, 1976). The assistance of Mr Martin Hulme in screening the hybridomas obtained from fusion number spm 1 is gratefully acknowledged.

4.2.6 Cloning By Limiting Dilution

Positive hybrids were detached from the plate by gentle pipetting with a sterile pasteur pipette. The number of viable cells was calculated using a vital stain (acridine orange/ethidium bromide, 1ppm each). The cells were diluted in HT medium containing 10% (v/v) MTM and plated (0.1ml/well) into the central 60 wells of a 96 well plate at three different cell densities so that 2 rows theoretically contained an average of 5 cells/well, 2 rows an average of 1 cell/well and the last 2 rows an average of 0.5 cells/well. One of these plating concentrations usually yielded wells with monoclonal growth. The plate was incubated undisturbed at 37°C with 5% CO_2. After one week, the wells were examined with an inverted microscope and the wells containing cells noted. It was possible to estimate the actual number of cells originally plated in each well as the cells grow in discrete clusters if the plate is incubated undisturbed. Each well was fed with 0.1ml HT medium containing 10% (v/v) MTM and incubated for a further week. Antibody production was monitored by testing the supernatants by ELISA. Hybrids which had high antibody production and originated from a single progenitor cell (ie showed monoclonal growth) were then chosen to be processed further. Positive hybrids were cloned twice to ensure that a stable cell line had been produced and that a pure clone was obtained. At each stage in the cloning procedure, aliquots of cells were frozen in liquid nitrogen as described in Section 4.2.8.

4.2.7 Bulk Culture

Once a pure cell line had been produced, large quantities (approximately 700mls) of culture supernatant were produced from which the monoclonal antibody was to be purified (Chapter 5). The cells were sequentially grown up in $25cm^2$, $75cm^2$ and $175cm^2$ flasks (Costar; Northumbria Biologicals) in supplemented RPMI medium containing 10% or 5% (v/v) FCS and allowed to overgrow beyond the time taken for the culture to die to allow for the release of all the antibody produced.

4.2.8 Storage of Cell Lines in Liquid Nitrogen

At every point in the production process, several aliqots of hybrid cells were frozen in liquid nitrogen. Cells were grown in $25cm^2$ culture flasks until they were confluent and harvested using 0.02% (v/v) EDTA in PBS. They were centrifuged and gently resuspended in 0.5ml 90% (v/v) FCS, 10% (v/v) dimethylsulphoxide (DMSO; Sigma) before transferring to cryotubes (Nunc; Gibco, Paisley, Scotland, UK). The tubes were stored in the vapour phase of liquid nitrogen for 24 hours before being moved to the main liquid nitrogen cryostat storage.

4.3 Results

A total of four fusions were carried out. The immunization schedules of the mice are detailed in Table 4.1. The mice used for fusions spm 1, spm 2 and spm 3 belonged to a separate group of mice (group 1) to the mouse that was used for fusion number spm 4 (group 2). Group 1 mice received a total of 4 injections each containing 100µg of purified sperm plasma membrane. Mice belonging to group 2 were only given 3 injections as no second booster was administered and varying amounts of antigen were received at each injection. Mice were bled at several stages during the immunization

Fusion number	**spm 1**	**spm 2**	**spm 3**	**spm 4**
Primary injection	100μg day 0	100μg day 0	100μg day 0	40μg day 0
First Boost	100μg day 44	100μg day 44	100μg day 44	50μg day 79
Second Boost	100μg day 130	100μg day 130	100μg day 130	none
Final Boost	100μg day 491	100μg day 749	100μg day 798	100μg day 153
Fusion	day 494	day 752	day 801	day 156

Table 4.1 Immunization schedule for the four fusions numbered spm 1-4. The date of the primary injection was designated as day 0.

procedure and the antibody titre for each mouse determined. Figure 4.2 shows the pooled data for the mice in group 1 and indicates that antibody titre level increases with each immunization although the response of each animal is variable. The data for group 2 are similar although higher levels of antibody titre were achieved. The mean titre for group 2 mice after the first booster injection was 1/11 170 (± 6 702) as compared to 1/272 (± 77) for group 1. This further highlights the individuality of response as these mice received fewer injections containing less antigen. Results of the fusions are summarized in Table 4.2. A total of 388 hybridomas were produced and 12 of these were identified as secreting anti-human sperm antibodies by ELISA. Each positive hybrid was cloned twice before being bulk cultured for antibody purification (Chapter 5). The hybrids were checked at each stage of the cloning procedure to ensure that a stable antibody-secreting cell line was produced and the pooled results are shown in Figure 4.3.

4.4 Discussion

Immunization of the mice to be used for the fusions is crucial to the production of monoclonal antibodies. In an immunological sense, mice will not have seen human spermatozoa before and if the animal does not contain cells expressing the desired antibody then it is not possible to immortalize these cells (Milstein, 1982). Immunization schedules vary from laboratory to laboratory but the most commonly used method consisting of a primary injection followed by two boosters and a final booster three days prior to the fusion, was followed here. The exception to this was the group 2 mouse used for fusion number spm 4 which did not receive a second booster injection. This was not detrimental to the successful production of monoclonal antibodies as this fusion resulted in over half of the monoclonals reported here. Indeed

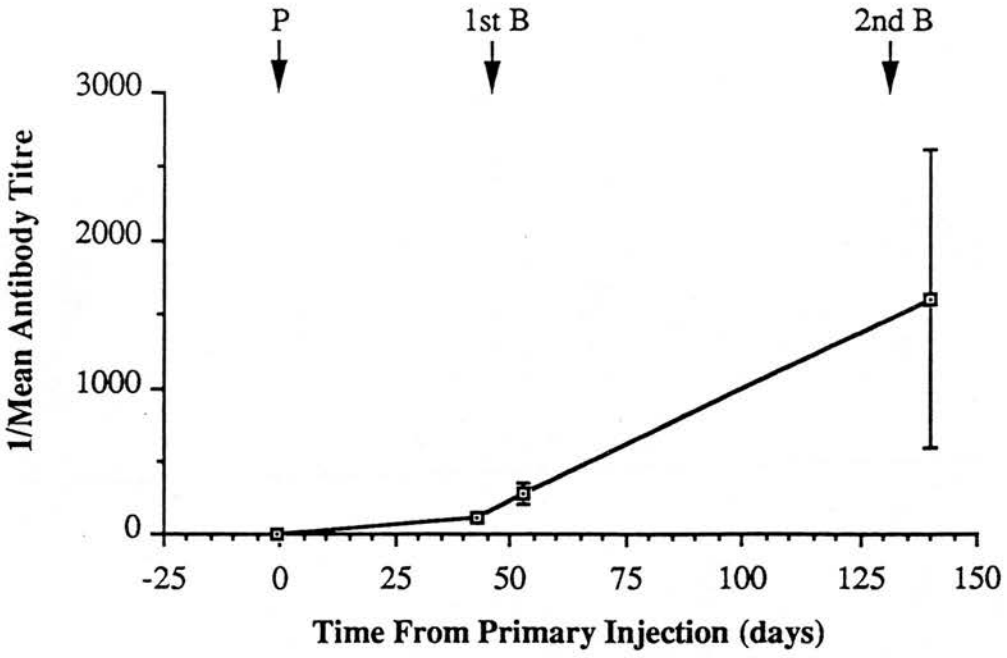

Figure 4.2 Mean antibody titre (± standard deviation) for group 1 mice (n=8) during the immunization procedure. Times of injections are indicated by the arrows — P, primary injection given on day 0; 1st B, first booster injection on day 44; 2nd B, secondbooster injection on day 130.

Fusion number	**spm 1**	**spm 2**	**spm 3**	**spm 4**
Ratio spleen:NS 0	1:1	4:1	5:1	2:1
Number of hybrids	88	123	none	177
Number of +ve hybrids	2	2	none	8

Table 4.2 Summary of the results of the four fusions spm 1-4.

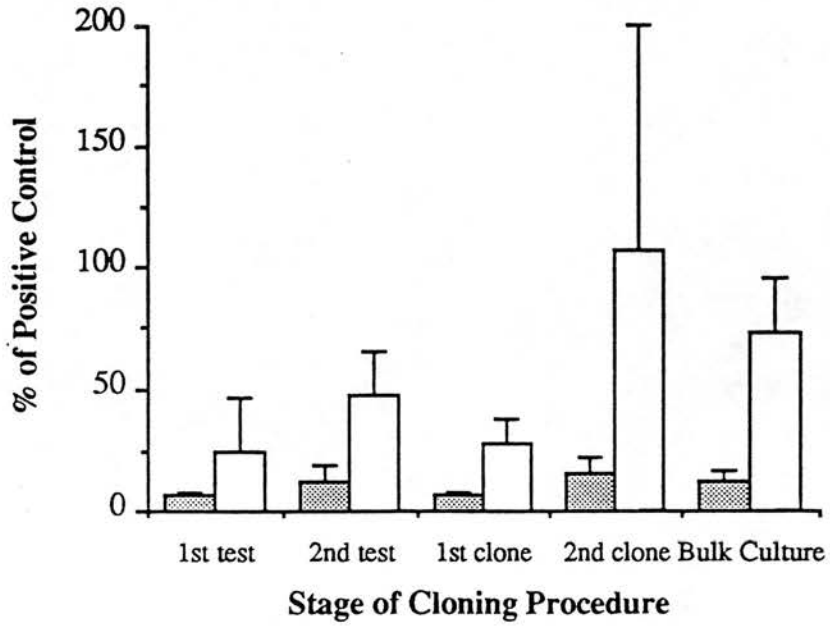

Figure 4.3 Pooled data for the twelve monoclonal antibodies produced showing the ELISA readings (A_{205}) obtained at five different stages during the cloning procedure. Values are expressed as a percentage of the positive control ± standard deviation. ▨, negative control medium; □, hybrid supernatant.

it is possible to do 'blind' fusions where the fusion is carried out 4 days after a single injection of antibody and still produce monoclonals (Trucco *et al*, 1978; Galfre & Milstein, 1981). Kennett *et al* (1978) found that a primary intraperitoneal injection followed by an intravenous injection was sufficient to obtain as many hybrids as could be conveniently screened and processed further and that no significant increase in the number of hybrids produced was seen when several injections over a period of several months were given. On the other hand, French *et al* (1986) reported that the maximum number of hybridomas were obtained if the animals were rested until there was little circulating antibody and then boosted for the fusion.

Monoclonal antibodies directed against biologically active molecules have been produced by using whole washed spermatozoa as the immunogen. For example Hinrichsen-Kohane *et al* (1985) and Kallajoki *et al* (1986a) have both produced anti-acrosin antibodies in this way. However, the immunogen does not have to be particularly pure as the animal will make antibodies against each component of the mixture and the ones directed against the antigen of choice can be selected for. This depends on the ability of the chosen screening procedure to detect only antibodies directed against the antigen of interest. The main drawback of this strategy is that numerous hybrids may have to be screened to find one which is positive. This is not a major problem if the screening method is specific and simple to perform but it can be time consuming and is therefore inefficient. The primary interest of this research is to identify monoclonal antibodies directed against biologically functional molecules on the sperm surface. However the initial screening assay is based on a purely non-functional recognition event so the probability of obtaining monoclonals against biologically active molecules was increased by using purified sperm plasma membrane as the immunogen. This strategy was also followed by Peterson and colleagues who found that several

monoclonal antibodies raised against purified boar sperm plasma membranes were biologically active (Peterson *et al*, 1980a&b, 1981a&b).

The lack of a second booster injection is not the only difference between the immunization schedules of the two groups of mice. Group 1 mice received more antigen over a longer time scale and consequently were older at the time of fusion. The level of antibody titre reached varied a great deal both between the two groups of mice and within each group. It is interesting to note that the mean titre after the first booster injection is greater in the second group of mice (1/11 170 as compared to 1/272) even though they had received less antigen and the immunizations were further apart. The reasons for this are not clear although it is indicative of the variability of the immune response of individual animals which involves suppression as well as induction (Galfre & Milstein, 1981). Herbert (1978) also found that peak antibody titres which were measured following a simple immunization schedule, varied as much as 500 times among individual animals. The immune response is a complex system which is still not fully understood. It is the body's defence system against foreign substances and is thought to have evolved as a protective mechanism against invasion by pathogens such as viruses, bacteria and fungi (Darnell *et al*, 1990). The system works in two ways — by humoral immunity and by cellular immunity. The introduction of a foreign substance into an animal elicits the formation of antibodies (humoral immunity) and immune cells (cellular immunity) both of which can bind to that substance and lead to its elimination from the body. Antibodies are produced by B lymphocytes which are produced with immunoglobulin on their surface in the bone marrow. Binding of antigen to these surface bound antibodies activates the so-called virgin B lymphocyte. This leads to their proliferation and maturation into plasma B cells which are highly specialized for immediate antibody production and secretion, and memory B cells which live for a long time and respond rapidly to further encounters with the antigen.

There are three major types of T cells which are also involved in the immune response — cytotoxic T lymphocytes which directly kill target cells that they recognise; helper T lymphocytes which assist B cells in their reaction to antigen; and suppressor T lymphocytes which dampen the responses of B cells. The response of an individual animal to any foreign substance is therefore variable as it depends on the interaction between these various cell types.

The precise details of fusion procedures vary from laboratory to laboratory (Goding, 1980). The procedure used here was based on the method of Kennett *et al* (1978) using MTM instead of feeder cells (Micklem *et al*, 1987). Ultimately, for the production of monoclonal antibodies, only one hybrid should be produced per culture well when the fusion is plated out. However more than one hybrid is frequently produced in each well and only one of them may be secreting the antibody of interest. The other hybrid may be secreting another irrelevant antibody or not secreting antibody at all. Problems arise when the other hybrid is not secreting any antibody as non-producers tend to overgrow producers and the culture quickly becomes dominated by non-producing variants (Goding, 1980). This is the main reason why positive hybrids should be cloned as soon as possible after their detection. Approaches to this problem have included culturing the cells in a large number of small wells and seeding the culture wells with fewer fused cells (Kennett *et al*, 1978; Oi & Herzenberg, 1980). However de Blas *et al* (1981) reported that the probability of hybridoma survival decreased with post-fusion cell dilution, so feeder cells are widely used to improve culture conditions and increase the ability of cultured cells to grow at very low densities (Galfre & Milstein, 1981). However feeder cells have several disadvantages. The main ones are the increased risk of bacterial contamination and the additional time taken for any procedure. Thus, alternatives to feeder cells in the form of conditioned medium, have been investigated (Micklem *et al*, 1987; Orlik & Altaner, 1988; Bazin &

Lemieux, 1989) and one of these, mixed thymocyte conditioned medium (MTM; Micklem *et al*, 1987), was used in this protocol.

The ratio of spleen cells to NS-O myeloma cells used for each fusion also varied. Ratios of between 1:1 and 5:1 were used but the precise ratio of spleen to myeloma cells has been shown to be non-critical and good results have been achieved using ratios between 1:1 and 10:1 (Goding, 1980). Other variations to the basic fusion protocol have been described by several authors. These include the addition of dimethyl sulphoxide (DMSO) to the PEG (Norwood *et al*, 1976) and changes in the concentration and molecular weight of PEG used (Davidson *et al*, 1976; Gefter *et al*, 1977). However no protocol seems to work better than any other. They have all been used successfully and no one method consistently gives better results (Yelton *et al*, 1980; Galfre & Milstein, 1981; Melamed & Bradley, 1989).

The antibodies produced by the permanently established hybrids from a fusion are generally a representative cross-section of the antibodies produced by the immunized animal which was used for the fusion although there is an enrichment for the antibody secreting phenotype (Milstein, 1982) and routinely more positive hybrids than would be expected are obtained (Yelton *et al*, 1980). Approximately 5% of spleen cells actively secrete immunoglobulin whereas after a fusion as many as 45% of the hybrids from a mouse NS-O myeloma-mouse spleen fusion may secrete antibody (Clark & Milstein, 1981). Thus of the 388 hybrids produced in the fusions reported here, 175 would be expected to be secreting antibody of any description (although this was not determined) and 10% of these (17-18 hybrids) would be expected to secrete specific antibody (Milstein, 1982). This compares well with the twelve hybrids which were actually found to be producing anti-sperm antibodies.

There are two commonly used methods of large scale production of monoclonal antibodies — i) bulk culture of cells *in vitro*; and ii) growing cells as tumours *in vivo*

(Goding, 1980; Galfre & Milstein, 1981). The former method was used and it involves allowing the cultures to overgrow until the cells die, releasing all the antibody into the spent medium. The concentration of antibody produced in this way is typically 10-100μg/ml although the actual concentration attained here was not measured. The alternative method involves reintroducing the cells into an animal inducing the formation of tumours. This results in the production of the antibody in the serum and body fluids of the animal and achieves high concentrations of antibody (3-15mg/ml; Goding, 1980). Usually hybrids are injected into the peritoneum which serves as a growth chamber for the cells. A peritoneal tumour forms and antibody is secreted into the intraperitoneal fluid. This is known as ascitic fluid and is a more concentrated source of antibody than bulk culture supernantant but it also contains more contamination (Galfre & Milstein, 1981). The impurities present in the spent medium can be largely controlled as most are introduced as components of the medium. More importantly the only murine antibody present in the spent medium is the monoclonal of interest unlike the ascitic fluid which is contaminated with naturally occurring immunoglobulin. The monoclonal normally comprises a maximum of 90% of the immunoglobulin component of ascites (Galfre & Milstein, 1981). Thus to obtain pure monoclonal antibody (which is important for the assignation of specific biological properties to the antibody and antigen; de Almeida *et al*, 1991) bulk cultures of the hybrids were prepared. The purification of the monoclonals from this medium and their biochemical characterization are detailed in Chapter 5.

4.5 Summary

A conventional immunization procedure comprising a primary injection, 1 or 2 booster injections and a final booster 3 days prior to fusion, was followed. In order to increase

the probability of obtaining monoclonal antibodies directed against molecules directly involved in biological functions, purified sperm plasma membrane was used as the immunogen. A total of four fusions were performed resulting in the production of 388 hybrids. Twelve of these hybrids were identified as secreting anti-sperm monoclonal antibodies by ELISA and these were cloned twice before being bulk cultured for monoclonal antibody purification.

5. Purification and Biochemical Characterization of Anti-Sperm Monoclonal Antibodies

5.1 Introduction

5.1.1 Purification

As discussed in the previous chapter, purified monoclonal antibodies are required to specifically identify those molecules on the sperm surface that are directly involved in biological events. Bulk culture supernatants contain impurities which may interfere with tests of biological function and thus the monoclonals require to be purified from this medium (de Almeida *et al*, 1991). The usual method of choice is by affinity chromatography in a protein-A column (Goding, 1978). Protein-A is a cell wall protein isolated from the bacterium *Staphlococcus aureus*, which binds to the Fc region of antibodies (Forsgren & Sjoquist, 1966). However not all antibodies bind to protein-A as this is dependent on the species and subclass of antibody (Ey *et al*, 1978; Goding, 1978; Goudswaard *et al*, 1978). Murine IgG_{2a}, IgG_{2b} and IgG_3 bind strongly to protein A but IgG_1 and IgM antibodies bind poorly, if at all (Ey *et al*, 1978; Mackenzie *et al*, 1978). Binding is highly pH dependent (Ey *et al*, 1978) and elution is easily achieved by lowering the pH. For antibodies which do not bind to protein-A, affinity chromatography on anti-immunoglobulin coupled to a support matrix (usually cyanogen bromide-activated sepharose 4B) is the method of choice (Goding, 1980).

Ion exchange on DEAE columns is another method which can be used to purify monoclonal antibodies but this is usually used to purify from ascites or serum as it does not generally provide complete purification from culture fluid (Goding, 1980).

5.1.2 Biochemical Characterization

In 1975, Southern developed a technique for analysing DNA fragments separated on agarose gels by transferring these fragments to a nitrocellulose membrane where they were probed by RNA or cDNA. The method of transfer became known as 'blotting' because the pattern of bands on the nitrocellulose membrane is an exact copy of the pattern on the original gel and thus this technique for the analysis of DNA became known as 'Southern blotting'. A similar technique for the analysis of RNA was developed and this came to be known as 'Northern blotting'. Therefore when another similar method based on proteins was described by Towbin *et al* (1979) and Renart *et al* (1979), this became commonly known as 'Western blotting'. However the scientific community has yet to identify a fourth analogous technique which could be called 'Eastern blotting'.

Western blotting is also known as immunoblotting and has become a widely used technique for the identification of antigens recognized by antibodies (Stott, 1989; Poxton, 1990). Since it was first described, the method has been extensively developed and modified and it has been applied in an enormous variety of studies (Stott, 1989). Although some of the steps in the process are still not fully understood, there is no doubt that it is an extremely powerful technique which has revolutionized the detection of antigens and antibodies (Poxton, 1990).

Prior to its inception, the methods available were severely limited. Although a variety of methods were available for the separation of complex mixtures into their component parts (usually by electrophoresis in an agarose or polyacrylamide gel), some means of identifying the protein of interest was required. Until immunoblotting was developed, the only methods for identification were based on mobility, molecular weight and isoelectric point or some innate activity (eg enzymatic) of the protein in question (Stott, 1989). A technique called immunofixation which involved the direct

overlay of the gel with antibody was developed to identify the antigen of interest. However this method was limited to use with agarose gels which have a large pore size through which an antibody can penetrate. The small pore size of polyacrylamide gels restricts the movement of protein molecules and thus they are not readily penetrated by immunoglobulin molecules. Also the long incubation times involved in immunofixation allow the bands to diffuse resulting in a loss of resolution.

Immunoblotting involves the separation of proteins by electrophoresis (usually on polyacrylamide gels) and their subsequent transfer to a nitrocellulose membrane. The blots are then probed with antibodies and any bound antibody detected. Transfer can be achieved by simple diffusion (Bowen *et al*, 1980), capillary transfer (Renart *et al*, 1979) or electrophoresis (Towbin *et al*, 1979). Diffusion is the simplest method but takes 36-48 hours although transfer can be speeded up by inducing a flow of solvent from the gel through the membrane by capillary action. However transfer from polyacrylamide gels using either the diffusion or capillary action method is very inefficient due to the small pore size of the gel which hinders the movement of large molecules. Thus for transfer from polyacrylamide gels, the electrophoretic method is generally preferred. Thus since Western blotting was first described, it has been extensively used as it permits relatively quick reproducible identification of specific proteins.

5.2 Materials and Methods

5.2.1 Isotyping

Two methods were used to determine the isotype of the monoclonal antibodies, namely Ouchterlony immunodiffusion and red blood cell agglutination.

Ouchterlony Immunodiffusion A kit for determining the isotype of monoclonal antibodies from mouse tissue culture supernatants (The Binding Site, Birmingham, UK) based on the gel double diffusion method of Ouchterlony (1958), was used according to the manufacturer's instructions. Approximately 75µl of the tissue culture supernatant to be tested was pipetted into the central well of one of the rossettes cut in an agarose gel. In a documented fashion, 10µl of each of the six antisera against mouse immunoglobulins (anti-IgG_1, anti-IgG_{2a}, anti-IgG_{2b}, anti-IgG_3, anti-IgA and anti-IgM) was pipetted into the outer six wells of the same rossette. The plate was then covered and incubated at room temperature for 24-48 hours. The result was easily readable as an immunoprecipitate arc formed between the central well and one of the outer wells containing antisera against the appropriate isotype.

Red Cell Agglutination An alternative method used to determine the class of the monoclonal antibodies was a kit (Serotec, Oxford, UK) based on the agglutination of red blood cells coated with anti-mouse immunoglobulin subclass antibodies. The assay was carried out in 96 well round bottomed plates and dilutions of 1/10 and 1/100 in PBS of the supernatant to be tested were used. A 30µl aliquot of the diluted supernatant was pipetted into each of 7 wells across a microtitre plate and then 30µl of each specific isotyping reagent was added. The solutions were mixed and the plate left on a flat surface undisturbed for 1 hour for the results to develop. A part or full carpet of agglutination was taken as a positive result and a small red circle or button of red blood cells in the bottom of the well as a negative result.

5.2.2 Affinity Chromatography

Protein-A Column Protein-A immobilized on a Trisacryl GF-2 000 support (Pierce, Chester, Cheshire, UK) was washed with 0.1M phosphate buffer and left to settle. The supernatant was removed and the resulting slurry poured into a 1 x 10 cm

column (Amicon, Stonehouse, Gloucestershire, UK). The column was equilibrated with 0.1M phosphate buffer, pH8.1 at a flow rate of 20ml/hour for a least 1 hour prior to use. The pH of the antibody containing supernatant was adjusted to 8.1 with 2M Tris before it was loaded overnight onto the column. After washing the column with 0.1M phosphate buffer, pH8.1, the antibody was eluted using 0.1M citrate buffer, pH3.5. The pH of the eluted antibody solution was immediately raised to 7.4 using 2M Tris to prevent denaturation of the antibody. The column was washed thoroughly with 0.1M phosphate buffer, pH8.1 and stored at 4°C in the same buffer containing 1μM azide. All affinity chromatography columns were run at 4°C.

Anti-mouse IgM Column An affinity column was made by coupling a commercially available anti-mouse IgM antibody (Sigma, Poole, Dorset, UK) to cyanogen bromide-activated (CNBr) Sepharose 4B (Pharmacia LKB, Milton Keynes, UK). The CNBr-activated Sepharose 4B was swollen for 2 hours in 1mM HCl and then washed on a sintered glass filter (porosity G 3) with the same solution. Anti-mouse IgM antibody was dissolved in coupling buffer (0.1M $NaHCO_3$, pH 8.3 containing 0.5M NaCl). After washing the gel suspension in coupling buffer, it was immediately mixed with the antibody solution and gently rocked overnight at 4°C. Any remaining active groups were blocked by transferring the gel to buffer with blocking agent (0.2M glycine) and incubating for 4 hours at room temperature. The gel was washed twice to remove any unbound protein using alternating coupling and acetate (0.1M, pH4, containing 0.5M NaCl) buffer, finishing with coupling buffer. The column was then poured and equilibrated with 0.1M phosphate buffer, pH8.1. After adjusting the pH of the antibody containing supernatant to 8.1 with 2M Tris, it was loaded onto the column. The column was washed with 0.1M phosphate buffer, pH8.1 and the monoclonal antibody eluted using glycine/HCl buffer (0.1M adjusted to pH2.5 with 0.2M HCl). The pH of the eluted protein was immediately raised to 7.4 by the addition of 2M Tris.

Finally the column was washed thoroughly with 0.1M phosphate buffer, pH8.1 and then stored at 4°C in the same buffer containing 0.1μM azide.

5.2.3 Concentration

Purified antibody solutions were dialysed against two changes of PBS and then concentrated using a minicon-B125 concentrator (Amicon). After filter sterilization (0.2μm filter; Ministart NML; Sartorius, Belmont, Surrey, UK), aliquots were stored frozen at -70°C until required. The protein content of the concentrated antibody solution was estimated using a BCA protein assay (Pierce) as described in Section 3.4.

5.2.4 Titration

Sperm coated plates Microtitre plates (96 well) were coated with spermatozoa for use in an enzyme linked immunoabsorbant assay (ELISA) to determine the amount of anti-sperm antibody present in the purified solutions. Sperm cells (100% fraction) were prepared (see section 3.2.1) and adjusted to 10×10^6 cells/ml in PBS prewarmed to 37°C. A 50μl aliquot of this solution was added to each well of a microtitre plate and the plate spun at 1 000 rpm (Coolspin; MSE, Crawley, Sussex, UK) at 25°C for 5 minutes. After aspirating the supernatant, 100μl of 1% para-formaldehyde in PBS was added to each well and the plates incubated at room temperature for 30 minutes. The plates were spun again and washed 3 times in PBS-tween (0.05%). The plates were dried in an aspirator containing phosphorus pentoxide under vacuum at 4°C then stored at -20°C until required.

ELISA 50μl of sample was added to each well of a sperm-coated plate. Purified monoclonal antibody solutions were added in doubling dilutions from 1/10 to 1/20 480 and incubated for either 1-2 hours at room temperature or overnight at 4°C. The supernatant was aspirated and the wells washed 3 times with PBS-tween (0.05%;

250µl/well). A 1/1 000 dilution (in PBS; 50µl/well) of second antibody, sheep anti-mouse Ig-alkaline phosphatase conjugate (Sigma), was added and incubated for 1-2 hours at room temperature. The plates were washed again and 50µl of 1mg/ml p-nitrophenylphosphate substrate (Sigma) made up in 10% (v/v) diethanolamine (Sigma), pH9.8 was added to each well. The reaction was allowed to develop for 30-60 minutes, protected from the light, at room temperature before it was stopped with 50µl 3M NaOH per well. The absorbance at 405nm was read using a 96 well plate reader (Titertek; Flow Laboratories, Irvine, Scotland, UK) using air as the blank. A positive (mouse anti-sperm plasma membrane polyclonal antiserum) and negative (PBS or medium) control were included on each plate. A positive value was defined as any reading which was at least twice the negative value. The titre was taken as the dilution at which the ELISA reading changed from positive to negative (Voller *et al*, 1976).

5.2.5 Electrophoresis

One dimensional sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) was performed according to the method of Laemmli (1970) using a Protean electrophoresis cell (BioRad, Hemel Hempstead, Hertfordshire, UK). The stacking gel was composed of 3.6% acrylamide, pH6.8 and the resolving gel contained 10% acrylamide, pH8.8. Samples of purified sperm plasma membrane (80µg protein/track) and seminal plasma (approximately 80µg protein/track) were run under reducing and non-reducing conditions. 2-mercaptoethanol (1%, v/v) was used as the reducing agent and bromophenol blue as the indicator. Molecular weight standards (high range – myosin, 205kD; β-galactosidase, 116kD; phosphorylase b, 97.4kD; bovine albumin, 66kD; egg albumin, 45kD; carbonic anhydrase, 29kD; low range – bovine albumin, 66kD; egg albumin, 45kD, glyceraldehyde-3-phosphate dehydrogenase, 36kD; carbonic anhydrase, 29kD; PMSF treated trypsin, 24kD; soyabean trypsin inhibitor,

20.1kD; α-lactalbumin, 14.2kD; Sigma) were also run. If the gel was to be blotted, prestained molecular weight standards (phosphorylase b, 106kD; bovine serum albumin, 80kD; ovalbumin, 49.5kD; carbonic anhydrase, 32.5kD; soyabean trypsin inhibitor, 27.5kD; lysozyme, 18.5kD; BioRad) were used. Electrophoresis was performed at a constant current of 40mA through the stacking gel, then at 25mA for 2-3 hours through the resolving gel using Tris, glycine, 0.1% SDS as electrode buffer. Some gels were silver stained as described in Section 5.2.6 whereas others were blotted as described in Section 5.2.7.

5.2.6 Silver Staining

Silver staining was performed using a kit (BioRad) used according to the manufacturer's instructions. All solutions were made up using deionized water and the incubations were performed at room temperature on a rocking table. Gels were fixed in 40% methanol/10% acetic acid (v/v) for 1 hour then twice in 10% ethanol/5% acetic acid (v/v) for 30 minutes each. After a 10 minute incubation with oxidizer, the gels were washed three times in deionized water (10 minutes each). After a 30 minute incubation with silver reagent, the gels were washed briefly in deionized water (2 minutes). Developer was added for approximately 30 seconds until the solution turned yellow or a smoky brown precipitate appeared when it was poured off and fresh developer added. Development was stopped when the bands reached the desired intensity in relation to the background, by adding 5% acetic acid (v/v).

5.2.7 Immunoblotting

Dot Blotting Preliminary dot blotting experiments were carried out by spotting approximately 12μg of purified sperm plasma membrane and seminal plasma under

both reducing and non-reducing conditions onto nitrocellulose and then developed as described below.

Western Blotting The Western blotting experiments were carried out using a modified version of the method of Towbin *et al* (1979). A dry blotting procedure using an LKB Multiphor II electrophoresis system (Pharmacia LKB) was followed. A continuous buffer system (39mM glycine, 48mM Tris, 0.0375% SDS, 20% methanol) was used and the nitrocellulose paper was placed anodal to the gels. The transfers were typically carried out at 250mA constant current for 1 hour.

Development of Blots The blots were blocked in 5% dried milk (Marvel, Premier Brands, Knighton, Adbaston, Stafford, UK) in TBS-tween (0.05%) for 1-2 hours and incubated with the first antibody diluted 1/200 in the same solution overnight at 4°C. The blots were washed 3 times in TBS-tween and incubated with the second antibody, sheep anti-mouse Ig-horseradish peroxidase (HRP) conjugate (Amersham, Aylesbury, Bucks, UK), diluted 1/2000 in TBS-tween-milk for 2-3 hours at room temperature. The blots were washed again and developed using either the classical colour development method using 4-chloro-1-naphthol as the substrate (BioRad) or enhanced chemiluminescence (ECL; Amersham).

Nitrocellulose Two different types of nitrocellulose were used for the immunoblotting experiments. If the blots were to be colour developed, Immobilon PVDF (Millipore, Watford, Hertfordshire, UK) was used. However if they were to be developed using the ECL system, Hybond C-Super (Amersham) was used.

5.3 Results

The twelve monoclonal antibodies were named according to the fusion from which they were produced. Thus the two monoclonals from fusion spm 1 were named spm 1/1

and spm 1/2, the two from fusion spm 2 were named spm 2/1 and spm 2/2 and the eight from fusion spm 4 were named spm 4/1 to spm 4/8.

In order to determine the chosen method for purification, the antibodies were isotyped to indicate what immunoglobulin subclass they belonged to. Two methods of isotyping were used — Ouchterlony immunodiffusion and red cell agglutination. A photograph of a representative Ouchterlony gel is shown in Figure 5.1 and details of the isotyping results are shown in Table 5.1. These experiments indicated that nine of the antibodies belonged to the IgM subclass while the remaining three were IgG_{2a} antibodies.

The antibodies were purified from bulk cultured supernatants (Chapter 4). They were purified by affinity chromatography on either a protein-A column (those belonging to IgG_{2a} subclass) or an anti-IgM column (those belonging to IgM subclass). A representative trace showing the absorbance at 280nm (A_{280}) of the fluid eluting from a protein-A column during a run is shown in Figure 5.2. The column eluates were dialysed against two changes of PBS and concentrated before being filter sterilized. The titre and protein concentration of each antibody solution were determined and the results are summarized in Table 5.1.

The ability of the monoclonals to recognize sperm plasma membrane proteins under reducing and non-reducing conditions, was checked using a dot blot technique. As seminal plasma proteins are known to coat the entire sperm surface (Russell *et al*, 1983), the antibodies were also blotted against seminal plasma proteins to identify any directed against accessory gland products that coat spermatozoa. The dot blotting results are summarized in Table 5.2. Eleven of the twelve antibodies recognized the sperm proteins and of this eleven, four recognized seminal plasma proteins with the same intensity, six had a reduced intensity and one did not recognize any seminal plasma proteins. One monoclonal did not dot blot nor Western blot.

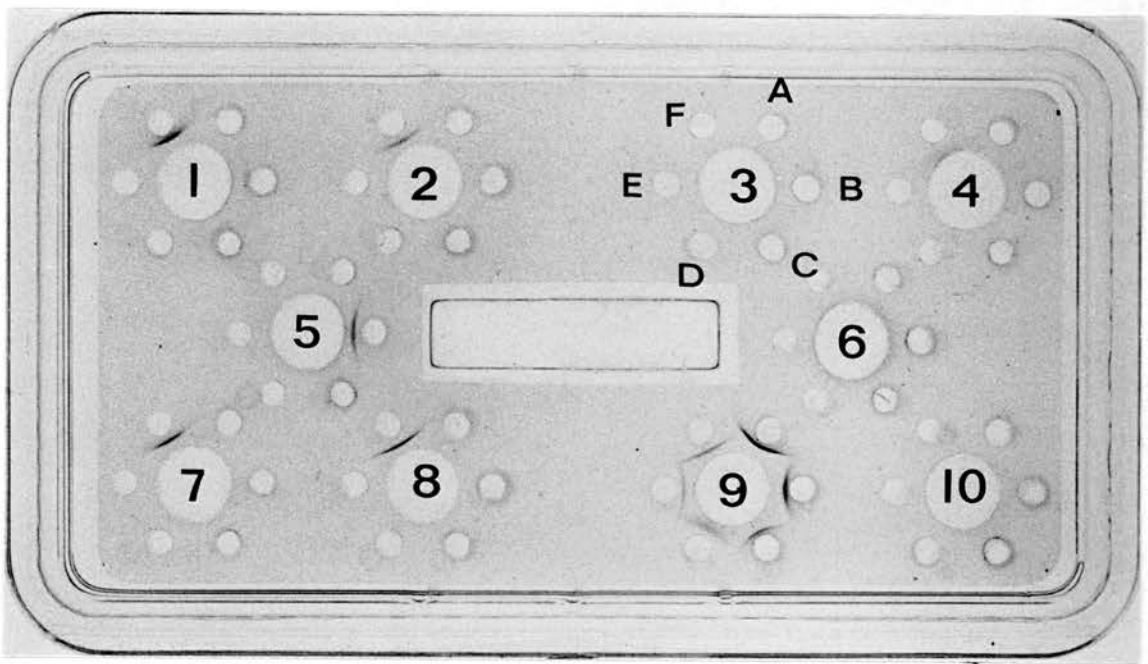

Figure 5.1 A representative Ouchterlony immunodiffusion isotyping gel. The large central wells contained culture supernatant — well 1, spm 4/1 supernatant; well 2, spm 4/2; well 5, spm 4/5; well 7, spm 4/7; well 8, spm 4/8; and well 9, polyclonal positive control serum. Six antisera against mouse immunoglobulin were pipetted into the outer six wells of each rosette as shown on rosette 3 — A, anti-IgG1; B, anti-IgG2a; C, anti-IgG2b; D, anti-IgG3; E, anti-IgA; and F, anti-IgM. As the antibodies diffused through the agarose, an immunoprecipitate arc formed between the central well containing the antibody to be isotyped and one of the outer wells of the rosette containing antisera against the appropriate isotype. Thus spm 4/1 (well 1), spm 4/2 (well 2), spm 4/7 (well 7) and spm 4/8 (well 8) were all identified as IgM antibodies and spm 4/5 (well 5) as an IgG_{2a} antibody.

Antibody	Isotype	1/Titre	Protein Concentration
spm 1/1	IgM	2 560	2.16
spm 1/2	IgM	320	1.01
spm 2/1	IgM	320	0.79
spm 2/2	IgM	320	0.73
spm 4/1	IgM	20 480	0.9
spm 4/2	IgM	20 480	0.5
spm 4/3	IgG2a	10	0.25
spm 4/4	IgM	40	0.2
spm 4/5	IgG2a	20 480	3.1
spm 4/6	IgG2a	1 280	1.77
spm 4/7	IgM	10 240	1.36
spm 4/8	IgM	10 240	1.67

Table 5.1 Details of the isotypes, titres and protein concentrations of the twelve purified monoclonal antibody solutions. Protein concentrations are given in mg/ml.

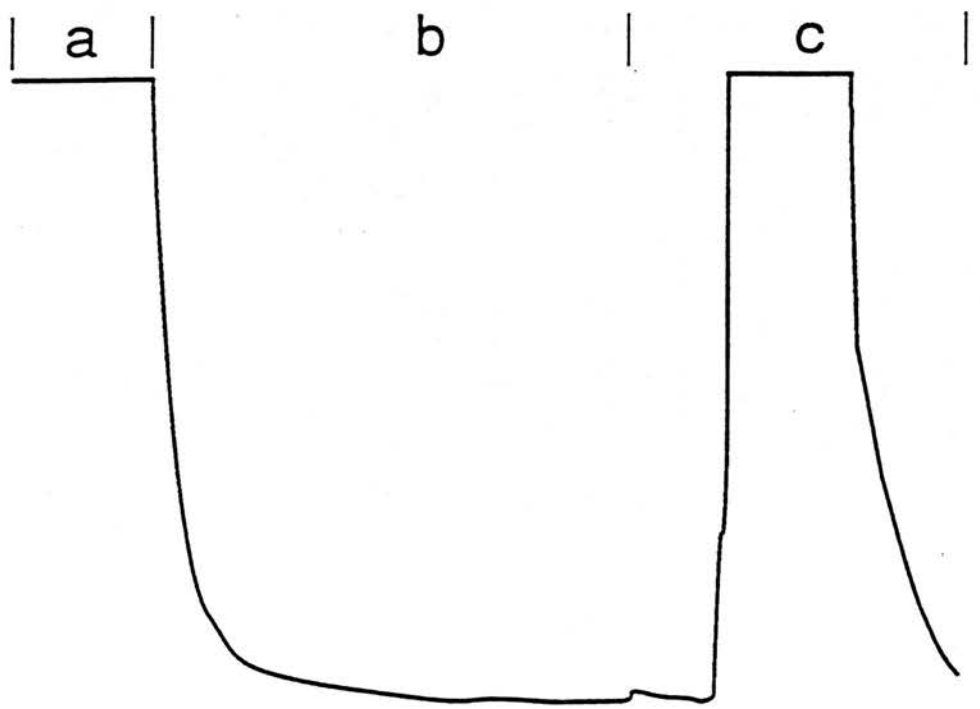

Figure 5.2 A_{280} profile of eluate from a protein-A affinity chromatography column during purification of spm 4/5 from bulk culture supernatant. a, last of antibody solution passing through column; b, washing of column; c, elution of monoclonal antibody from the column.

Antibody	spm R	spm NR	sem pl R	sem pl NR
spm 1/1	++	++	+	+
spm 1/2	+	+	+	+
spm 2/1	+	+	(+)	(+)
spm 2/2	+	+	+	+
spm 4/1	+	+	+	+
spm 4/2	+	+	+	+
spm 4/3	++	++	-	-
spm 4/4	-	-	-	-
spm 4/5	++	++	+	+
spm 4/6	++	++	+	+
spm 4/7	+	+	(+)	(+)
spm 4/8	+	+	(+)	(+)

Table 5.2 Summary of dot blot results against sperm plasma membrane (spm) and seminal plasma proteins (sem pl) under reducing (R) and non-reducing (NR) conditions. ++, strong positive reaction; +, positive reaction; (+), weak positive reaction; -, negative reaction.

In order to characterize the antigens recognized by the antibodies in more detail, purified sperm plasma membrane and seminal plasma proteins were electrophoresed under reducing and non-reducing conditions on one dimensional SDS polyacrylamide gels. After transferring to nitrocellulose, these blots were then probed with the monoclonals. Figure 5.3 shows silver stained gels before and after blotting indicating that little residual protein is left in the gel after blotting. More residual protein is visible in the high molecular weight region of the gel and this is not unexpected as high weight bands transfer less efficiently than low weight bands due to the small pore size of acrylamide gels which restricts the movement of large molecules (Stott, 1989). Positive control blots probed with polyclonal anti-sperm plasma membrane antibodies were developed using enhanced chemiluminescence (ECL) and a colour development system using 4-chloro-1-naphthol as substrate. Photographs of representative blots developed using these methods are shown in Figure 5.4. It can be seen that the ECL system (Figure 5.4a) is more sensitive than the colour development system (Figure 5.4b). This can particularly be seen in the seminal plasma protein lanes (lanes 3 and 4 in Figure 5.4a and b) where more minor bands are visualized. Thus the ECL system was used as the method of choice for the development of blots probed with these monoclonals.

Preimmune mouse serum recognized two proteins (28kD and 12kD) in spermatozoa and at least seven proteins in seminal plasma (115kD, 81kD, 20kD, 18kD, 16kD, 11kD and 10kD) in addition to a high molecular weight band in each lane which did not enter the gel. However as all the monoclonals were tested as purified antibody solutions, other monoclonal antibodies were used as controls to identify the level of non-specific binding. Thus a monoclonal raised against human growth hormone (Sigma) was used (Figure 5.5). It recognized a high molecular weight band which did not enter the gel, in sperm preparations particularly under reducing conditions and four

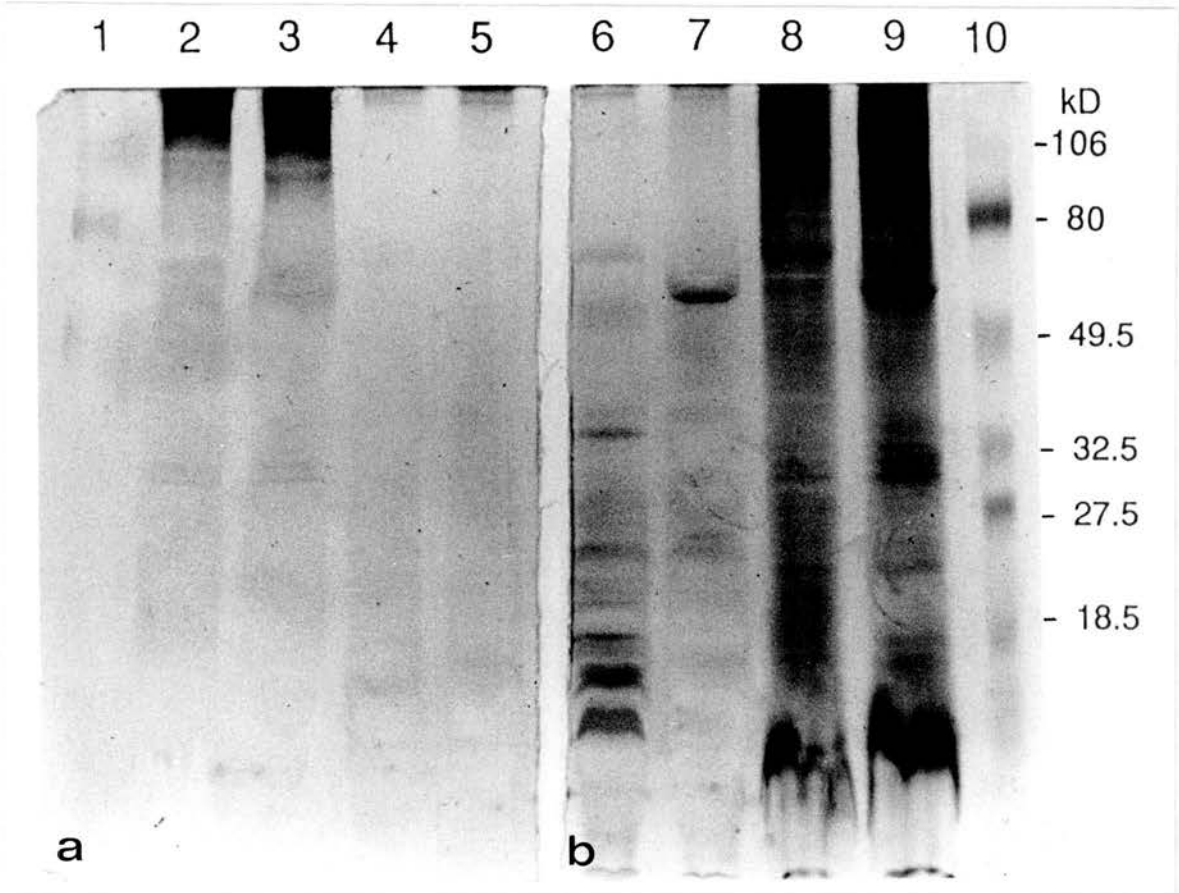

Figure 5.3 Silver stained gels before (lanes 6-10) and after (lanes 1-5) blotting. Lanes 1 & 10 contain molecular weight standards, lanes 2 & 8 and 3 & 9 contain purified sperm plasma membrane under reducing and non-reducing conditions respectively and lanes 4 & 6 and 5 & 7 contain seminal plasma under reducing and non-reducing conditions respectively. Approximately 80µg protein was loaded into each sperm plasma membrane and seminal plasma lane. Molecular weight markers as indicated on right hand side of the gels.

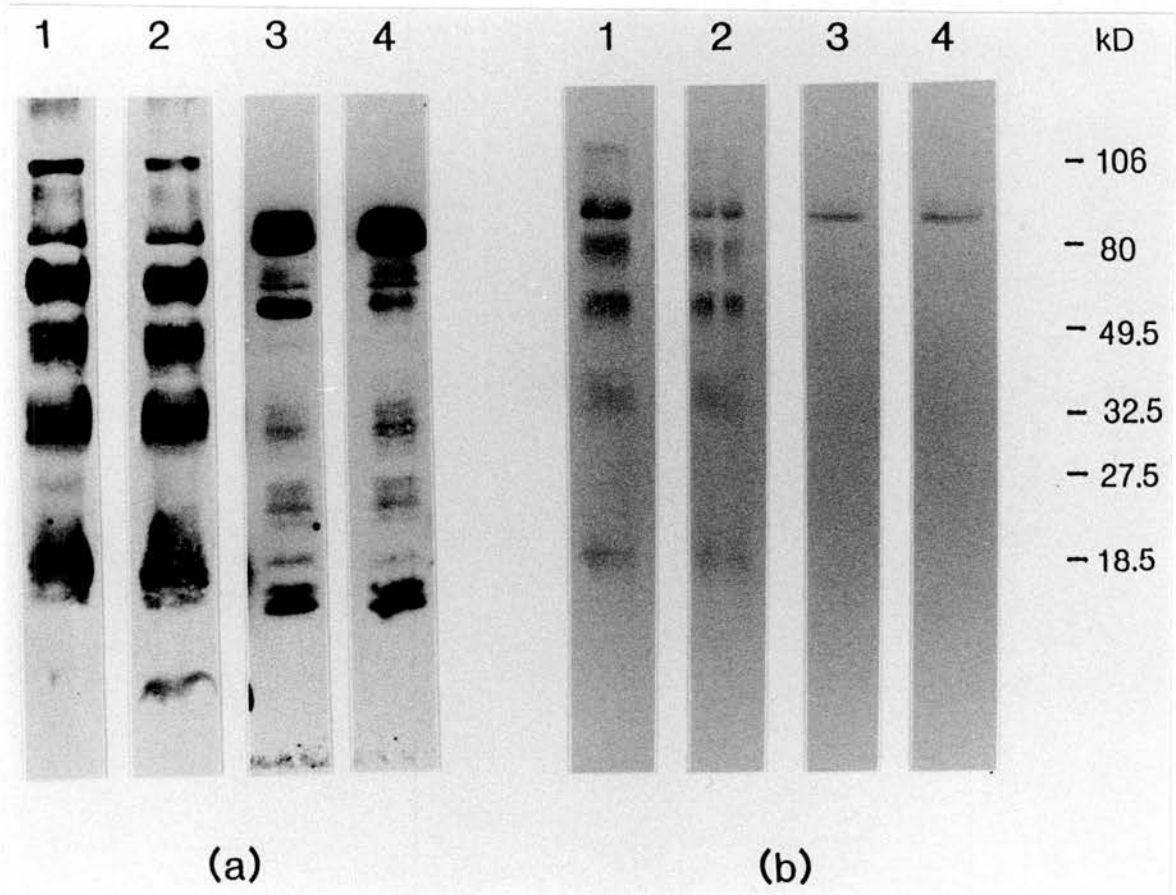

Figure 5.4 Blots of sperm plasma membrane (lanes 1 and 2) and seminal plasma proteins (lanes 3 and 4) under reducing and non-reducing conditions respectively, probed with polyclonal anti-sperm plasma membrane serum. Blot (a) developed using the ECL system, blot (b) using colour development.

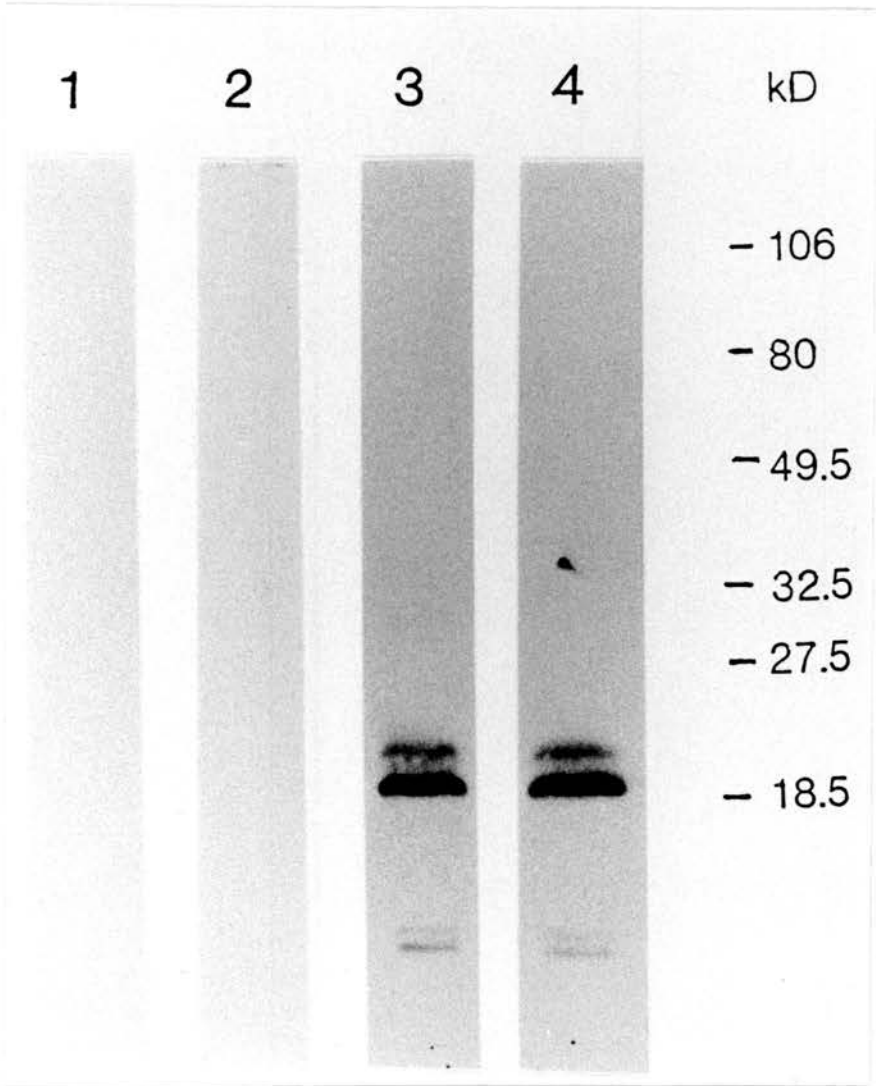

Figure 5.5 Blot of sperm plasma membrane (lanes 1 & 2) and seminal plasma proteins (lanes 3 & 4) under reducing and non-reducing conditions respectively, probed with a monoclonal antibody raised against human growth hormone. A high molecular weight band was identified in sperm plasma membrane under reducing conditions in addition to four other bands of relative molecular mass 20kD, 18kD, 11kD and 10kD in the seminal plasma lanes.

further proteins present only in seminal plasma with relative molecular masses of 20kD, 18kD, 11kD and 10kD. Another control antibody raised against leucocyte common antigen (Scottish Antibody Production Unit, Carluke, Scotland, UK) was also used (Figure 5.6). In addition to the high molecular weight proteins and the four proteins in the seminal plasma lanes recognized by anti-human growth hormone, anti-leucocyte common antigen antibody also recognized proteins with relative molecular masses of 127kD and 63kD in sperm plasma membrane preparations. This may give an indication of the level of leucocyte contamination in the sperm plasma membrane preparations. Photographs of representative blots probed with each of the monoclonals are shown in Figures 5.7 to 5.17 and details of the major protein bands recognized are detailed in the legend of the corresponding figure. The antibodies bound to a number of proteins of varying molecular weight. Several antibodies recognized more than one sperm protein thus indicating that these molecules contain shared epitopes. Omission of the primary or secondary antibody or use of the development reagents alone, produced no immunostaining on the blot. The majority of non-specific binding appears to be restricted to four proteins present in seminal plasma which have relative molecular masses of 20kD, 18kD, 11kD and 10kD. Also a high molecular weight band is often present which is thought to consist of aggregated proteins and thus it does not enter the gel.

5.4 Discussion

When an animal encounters a foreign substance, the body mounts an immune response to eliminate it. Part of the response is to produce antibodies against the foreign substance. This is called the humoral response and is composed of four distinct phases – i) a lag phase where no antibody is detected; ii) a log phase in which the

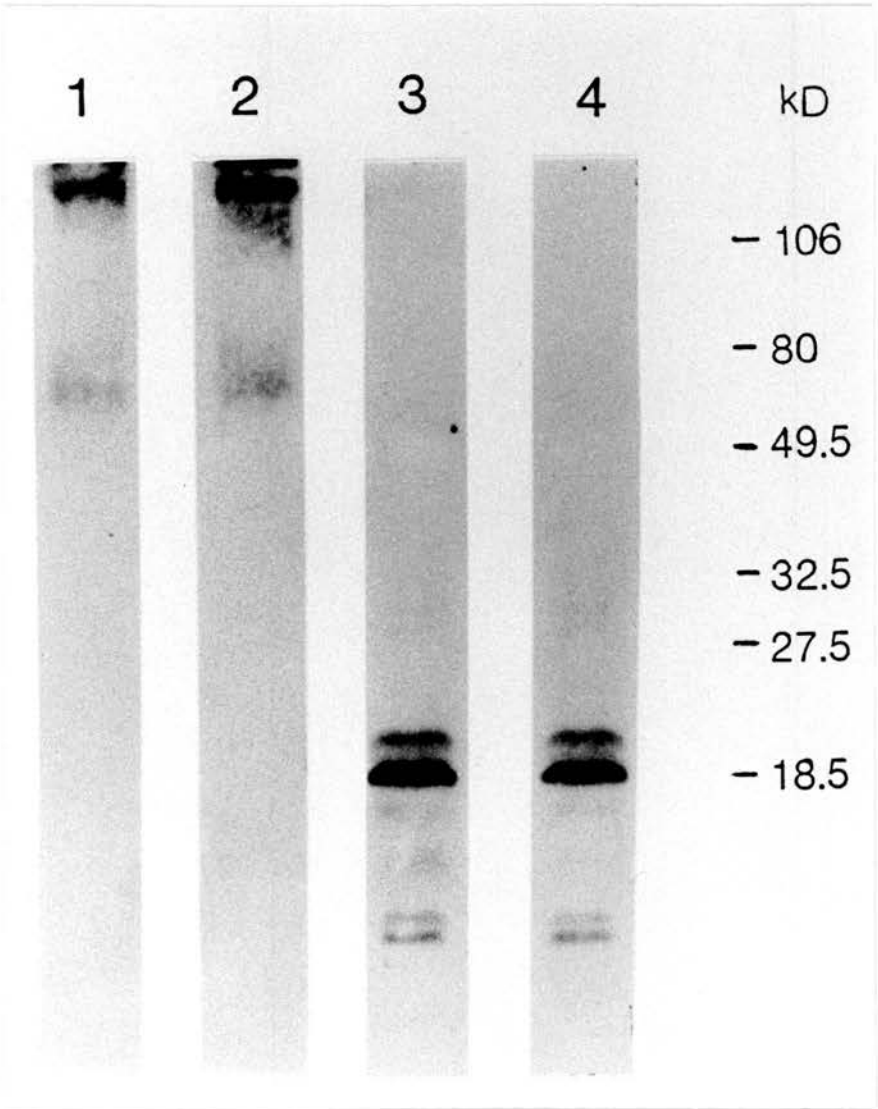

Figure 5.6 Blot of sperm plasma membrane (lanes 1 & 2) and seminal plasma proteins (lanes 3 & 4) under reducing and non-reducing conditions respectively, probed with a monoclonal antibody raised against leucocyte common antigen. In addition to a high molecular weight band which did not enter the gel, the other major bands recognized had molecular masses of 127kD and 63kD in sperm plasma membrane preparations and 20kD, 18kD, 11kD and 10kD in seminal plasma.

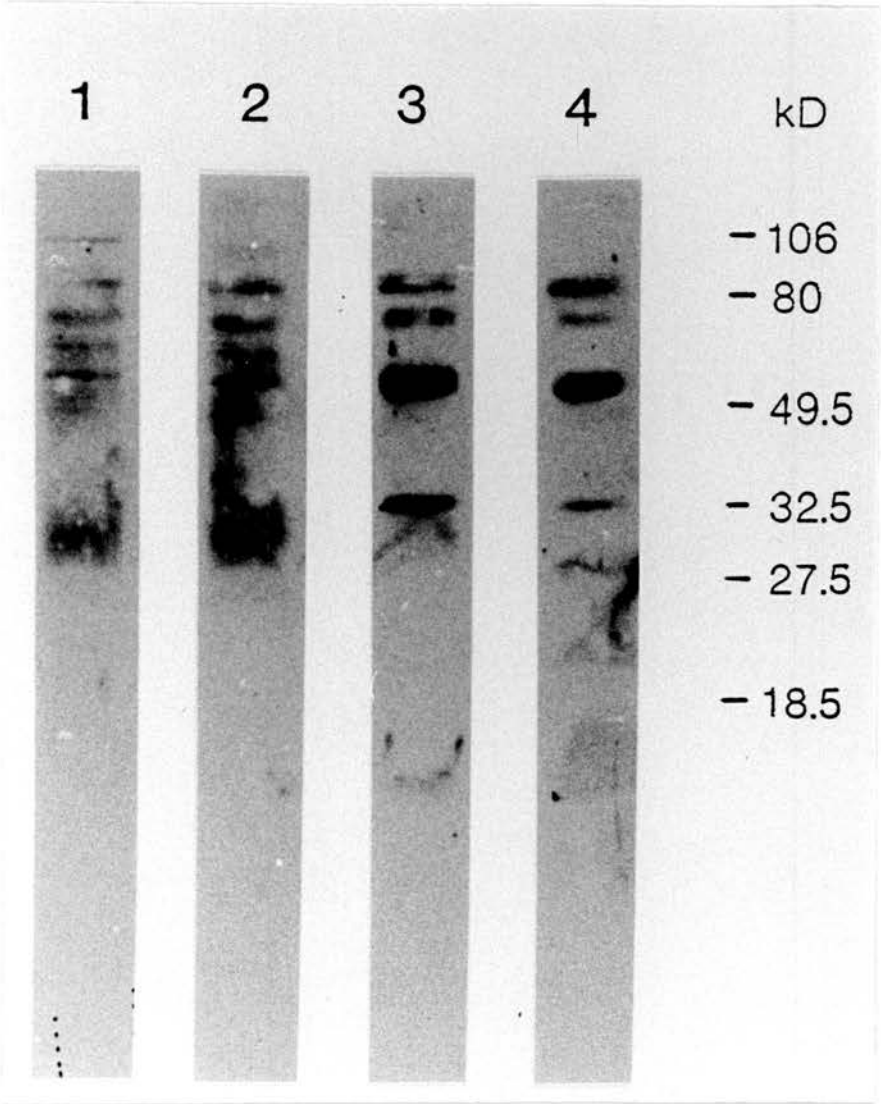

Figure 5.7 Blot of sperm plasma membrane (lanes 1 & 2) and seminal plasma proteins (lanes 3 & 4) under reducing and non-reducing conditions respectively, probed with monoclonal antibody spm 1/1. Three proteins of 82kD, 72kD and 56kD were recognized in all four lanes. Two proteins of 94kD and 63kD and a broad band centred around 30kD were identified in sperm plasma membrane only and one of 36kD in seminal plasma only.

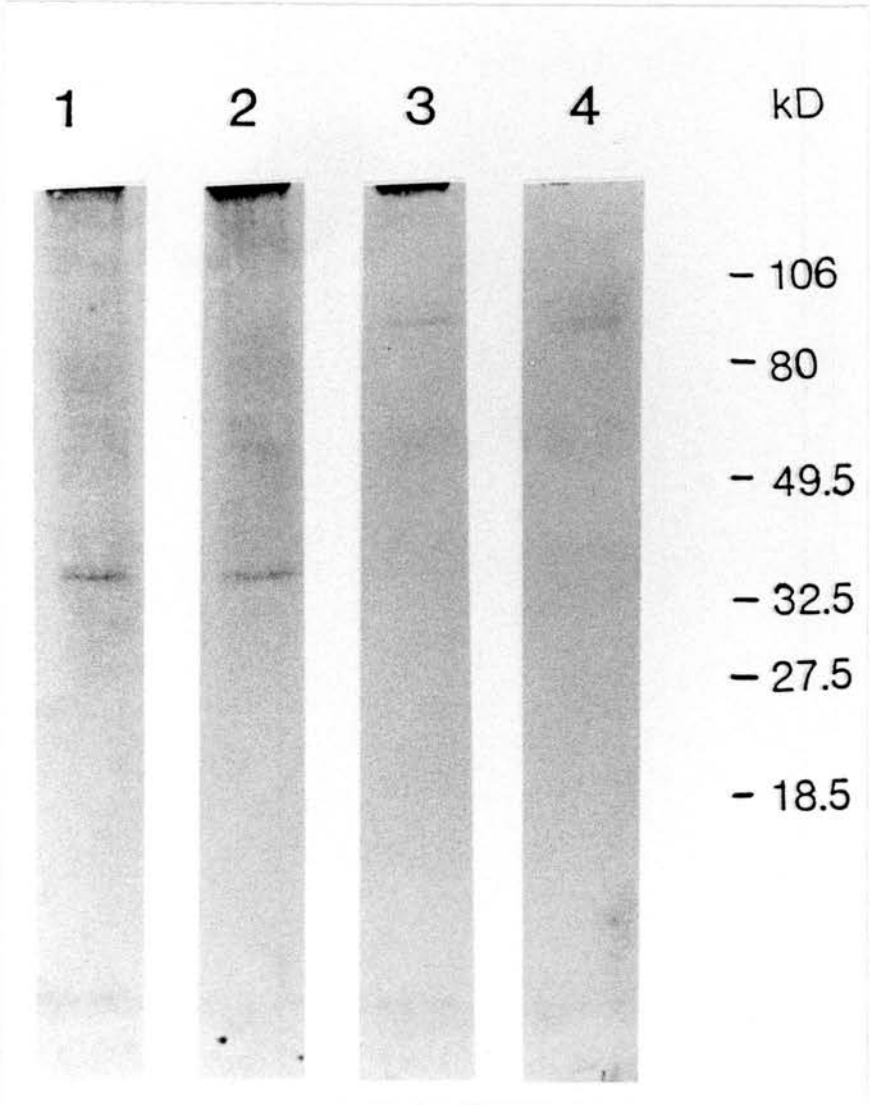

Figure 5.8 Blot of sperm plasma membrane (lanes 1 & 2) and seminal plasma proteins (lanes 3 & 4) under reducing and non-reducing conditions respectively, probed with monoclonal antibody spm 1/2. In addition to a high molecular weight band visualized in all four lanes, spm 1/2 recognized a protein of 36kD in sperm plasma membrane and one of 86kD in seminal plasma.

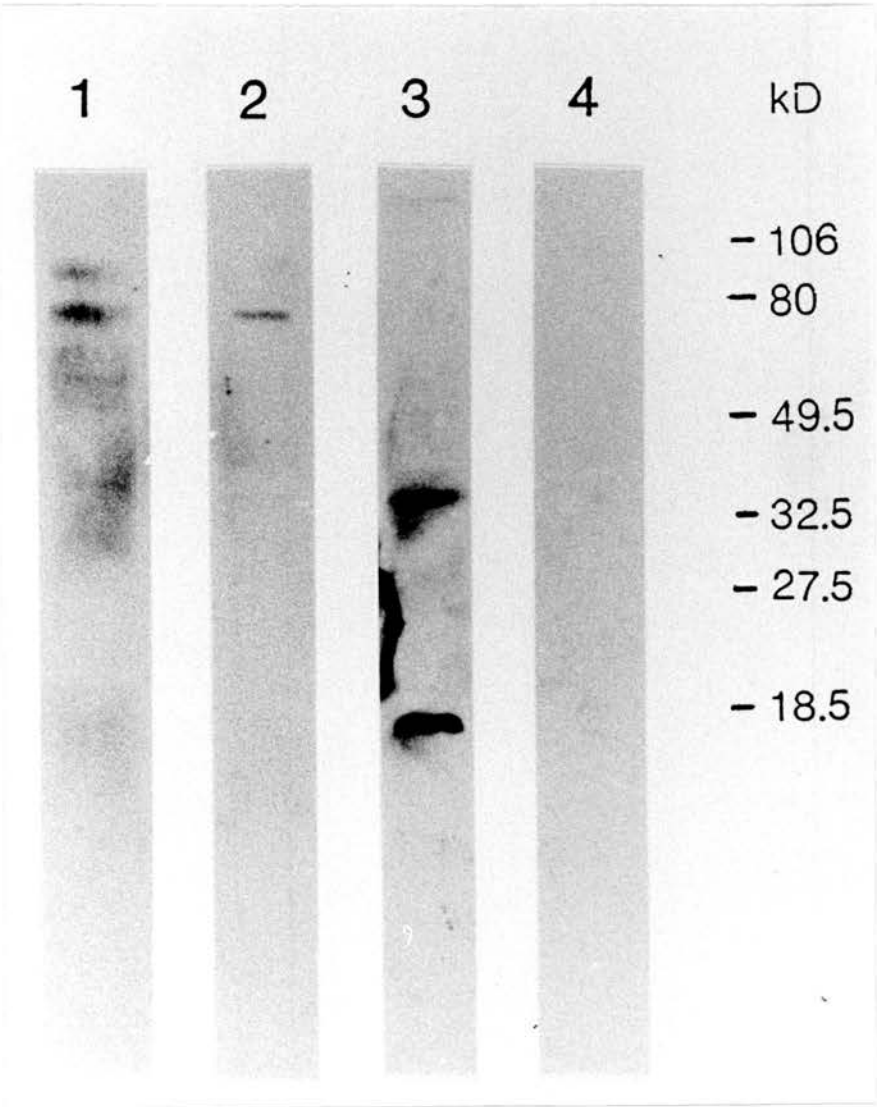

Figure 5.9 Blot of sperm plasma membrane (lanes 1 & 2) and seminal plasma proteins (lanes 3 & 4) under reducing and non-reducing conditions respectively, probed with monoclonal antibody spm 2/1. A band at 77kD was identified in sperm plasma membrane under both reducing and non-reducing conditions and one at 40kD was identified in sperm plasma membrane and seminal plasma under reducing conditions. Further bands at 88kD and 60kD were seen in sperm plasma membrane under reducing conditions and at 120kD and 16kD in seminal plasma also under reducing conditions. The two bands at 40kD and 16kD seen in seminal plasma under reducing conditions were also present under non-reducing conditions although they were faint.

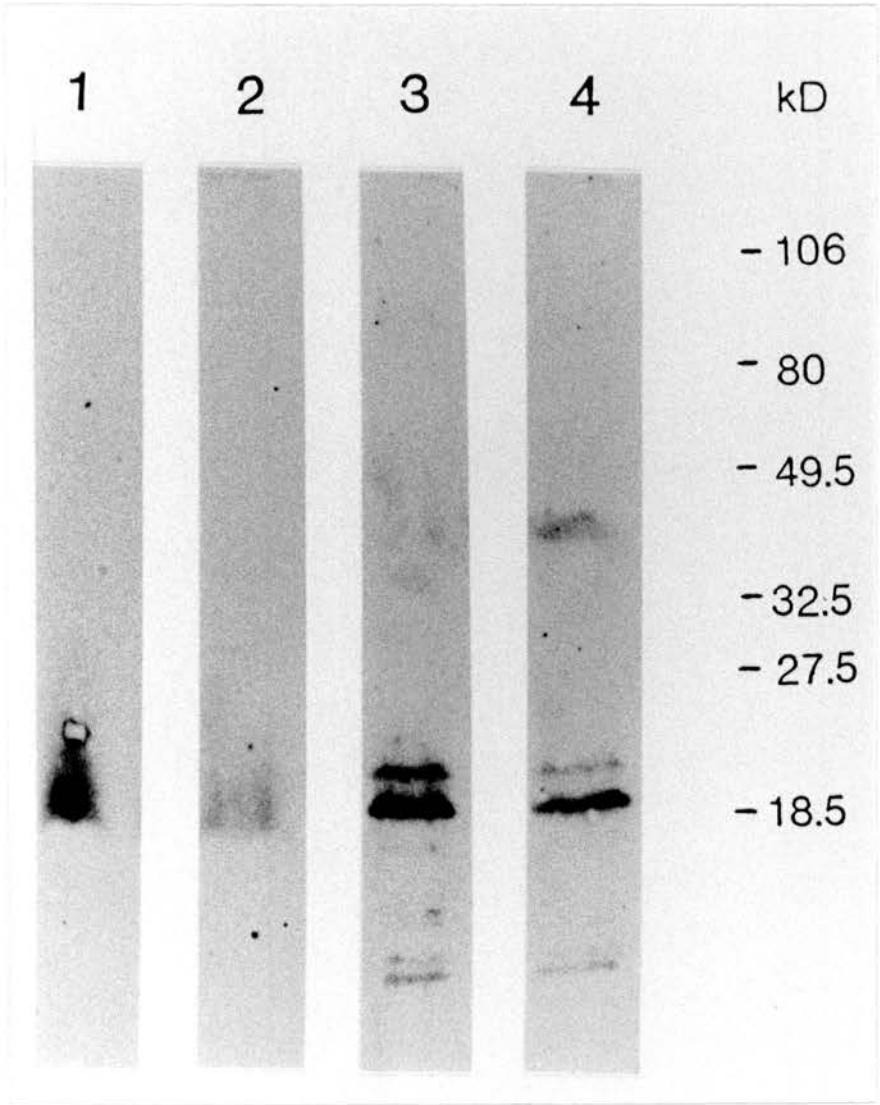

Figure 5.10 Blot of sperm plasma membrane (lanes 1 & 2) and seminal plasma proteins (lanes 3 & 4) under reducing and non-reducing conditions respectively, probed with monoclonal antibody spm 2/2. The antibody recognized a high molecular weight band in sperm plasma membrane under reducing conditions, a protein smear of 21kD to 17kD in both lanes containing sperm plasma membrane and five proteins of 44kD, 20kD, 18kD, 11kD and 10kD in seminal plasma.

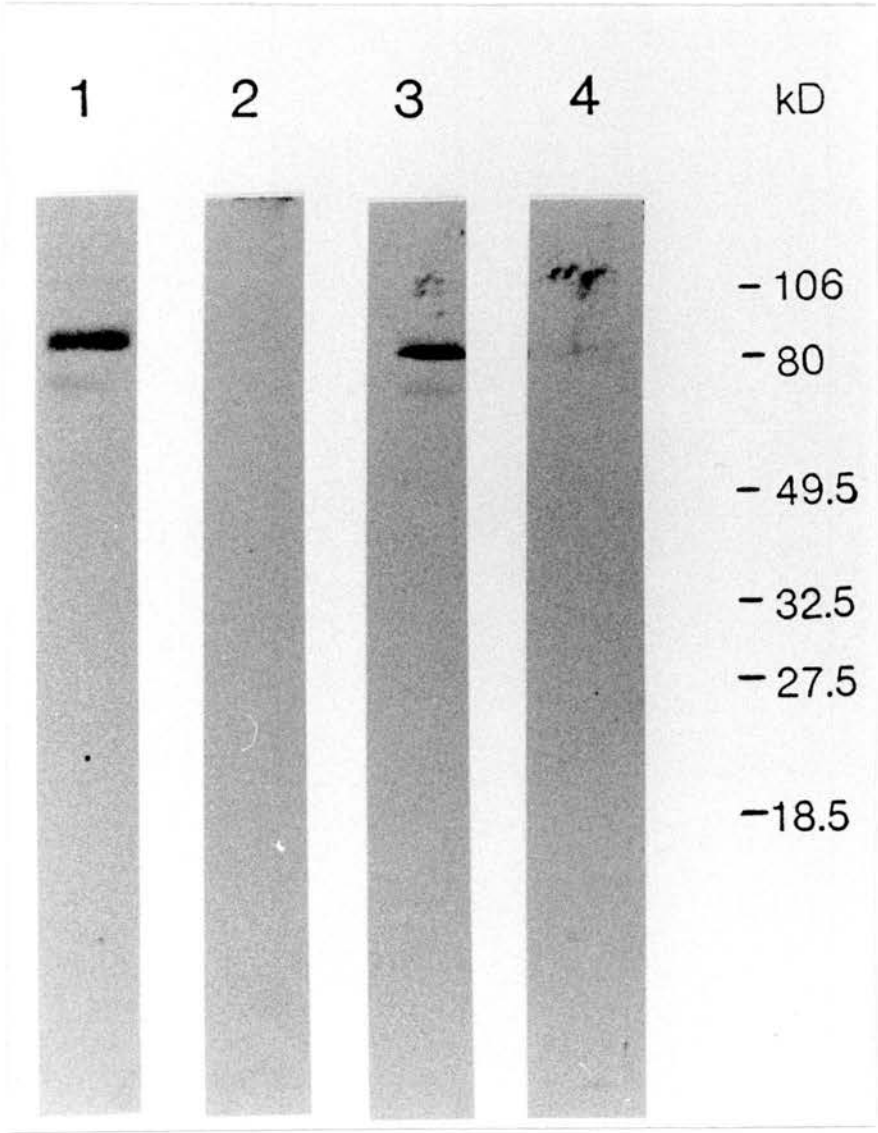

Figure 5.11 Blot of sperm plasma membrane (lanes 1 & 2) and seminal plasma proteins (lanes 3 & 4) under reducing and non-reducing conditions respectively, probed with monoclonal antibody spm 4/1. Two bands of relative molecular mass 82kD and 72kD were recognized in both sperm plasma membrane and seminal plasma but only under reducing conditions. Under non-reducing conditions only a very high molecular weight band which did not enter the gel, was visualized.

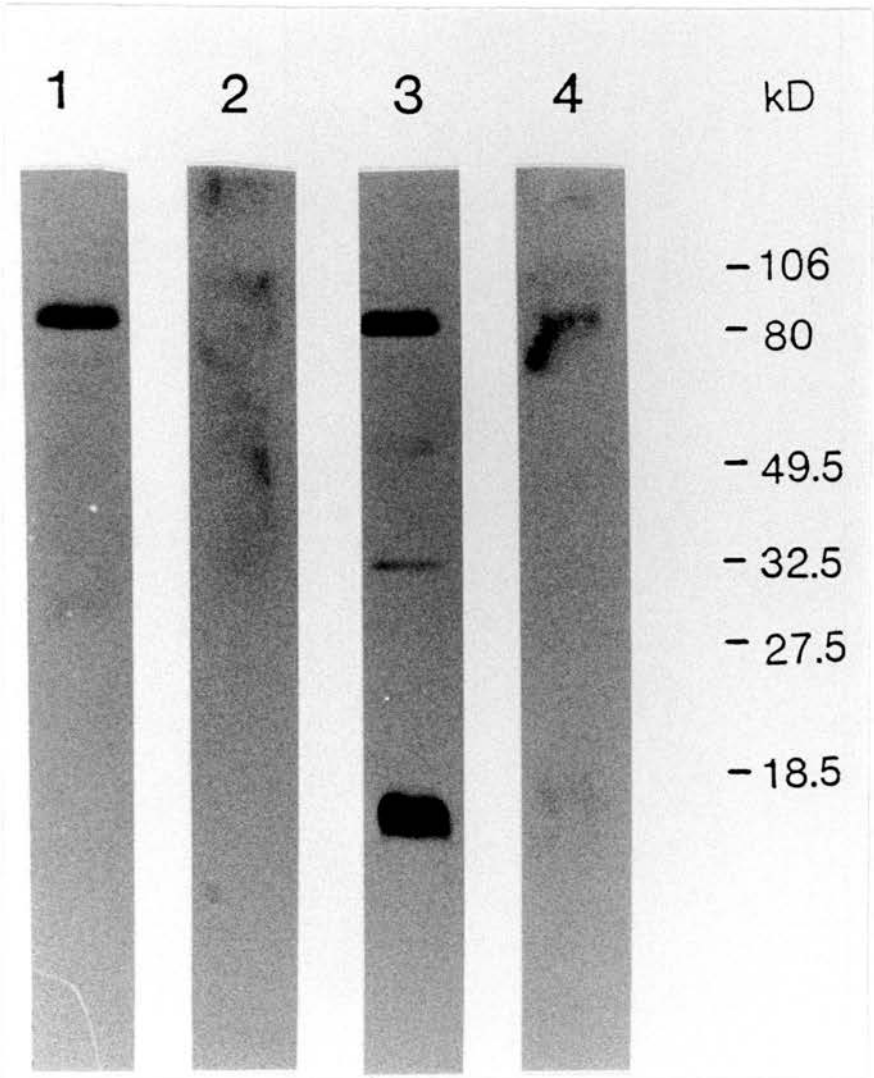

Figure 5.12 Blot of sperm plasma membrane (lanes 1 & 2) and seminal plasma proteins (lanes 3 & 4) under reducing and non-reducing conditions respectively, probed with monoclonal antibody spm 4/2. Under reducing conditions one protein of 89kD was identified in sperm plasma membrane and was also seen in seminal plasma. Two weakly staining bands of 58kD and 38kD and a heavily staining doublet of 16kD and 15kD were also visible in seminal plasma under reducing conditions. Under non-reducing conditions, a faint band at 97kD was seen in sperm plasma membrane and another at 86kD was seen in seminal plasma.

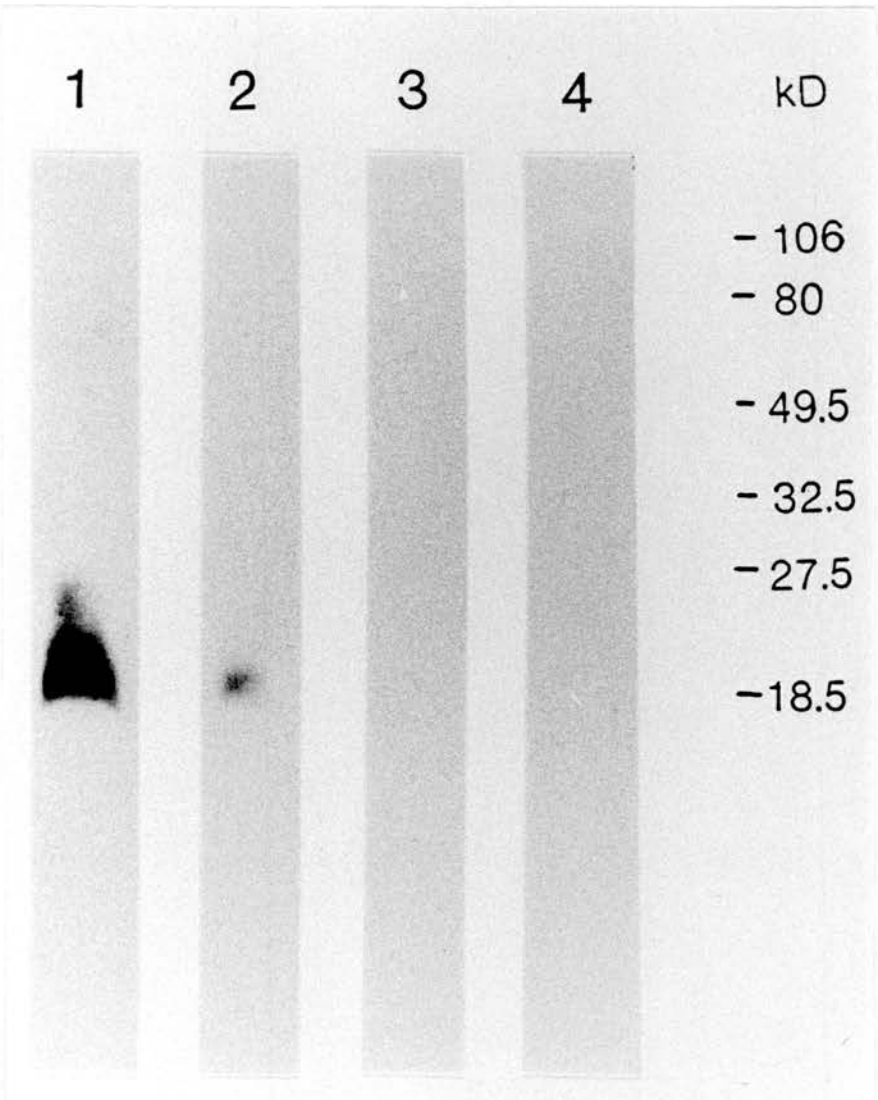

Figure 5.13 Blot of sperm plasma membrane (lanes 1 & 2) and seminal plasma proteins (lanes 3 & 4) under reducing and non-reducing conditions respectively, probed with monoclonal antibody spm 4/3. A protein smear from 22kD to 17kD was recognized in both lanes containing sperm plasma membrane although the reaction was stronger under reducing conditions. No reactivity with seminal plasma proteins was detected.

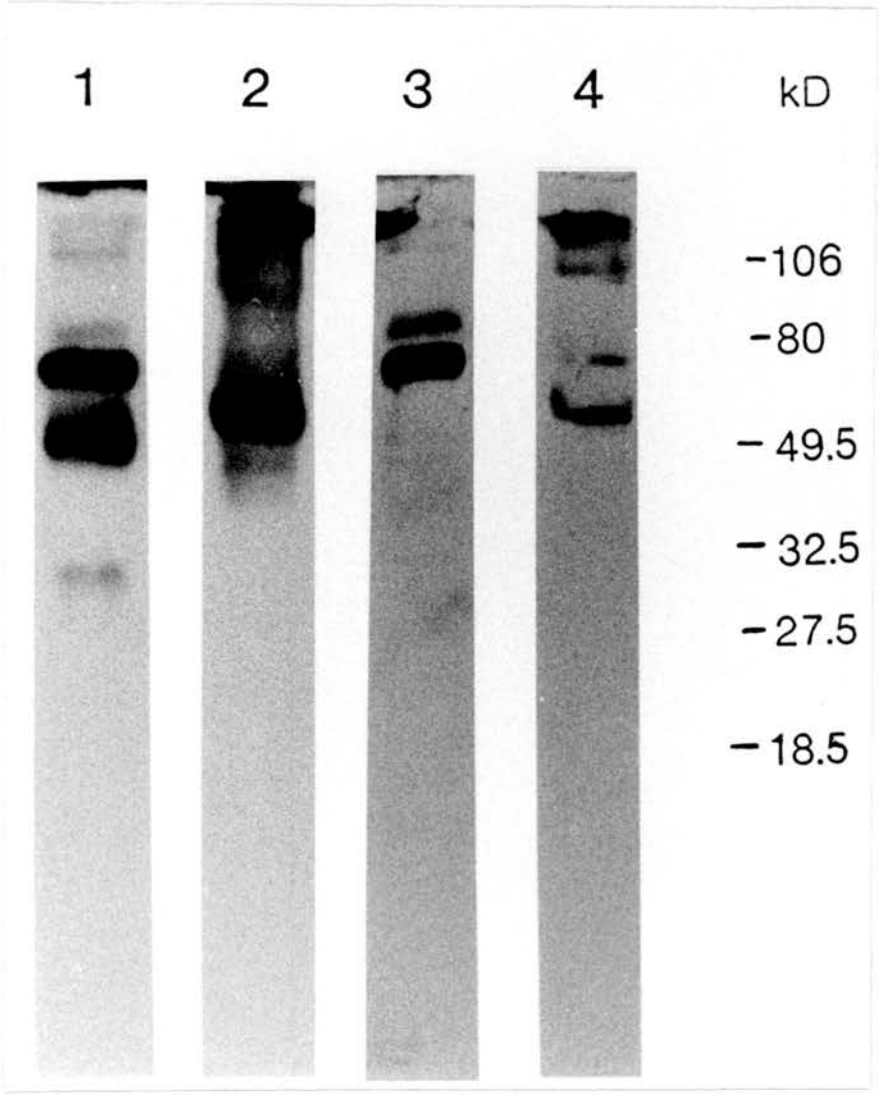

Figure 5.14 Blot of sperm plasma membrane (lanes 1 & 2) and seminal plasma proteins (lanes 3 & 4) under reducing and non-reducing conditions respectively, probed with monoclonal antibody spm 4/5. A high molecular weight band which did not enter the gel is seen in all four lanes. A protein of 71kD was heavily stained in both sperm plasma membrane and seminal plasma under reducing conditions and was also present under non-reducing conditions although it was not so heavily stained. Another heavily staining protein of 55kD was seen in sperm plasma membrane under reducing conditions but was not present in any other lane and a protein of 58kD which stained heavily in sperm plasma membrane under non-reducing conditions was also present in seminal plasma under non-reducing conditions.

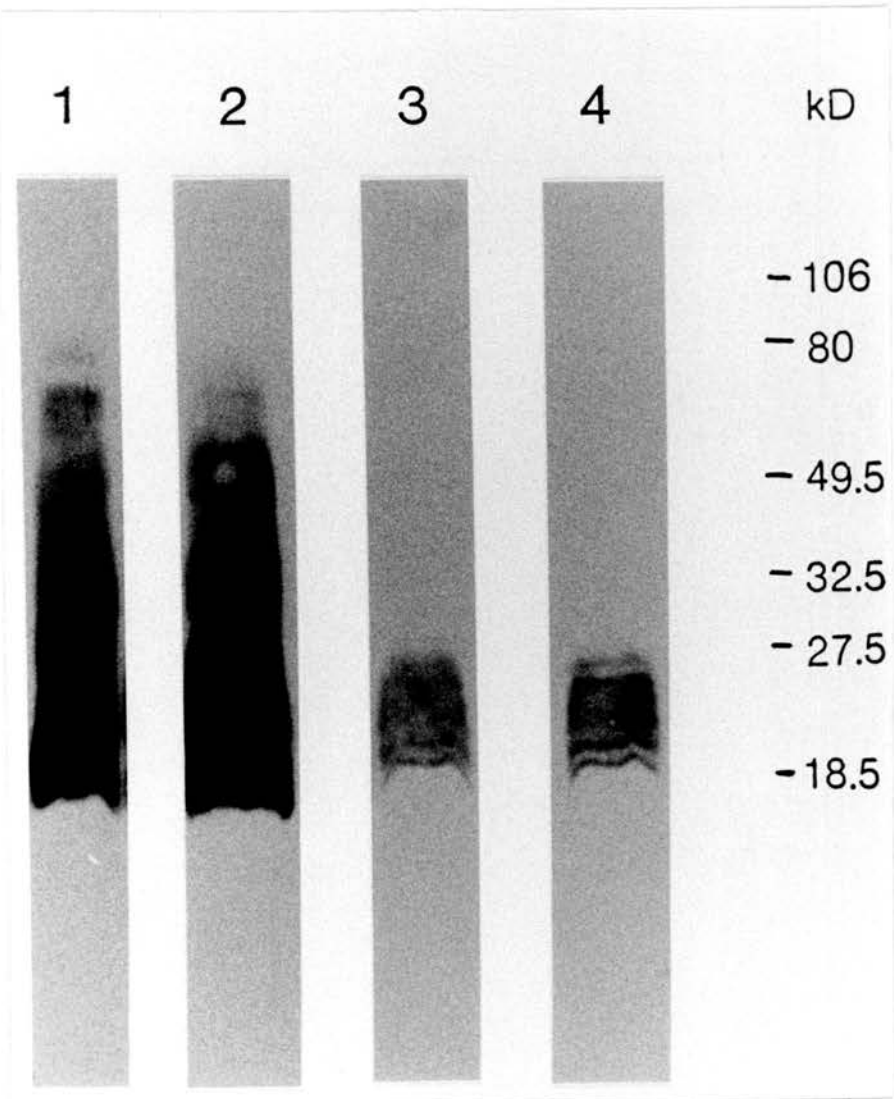

Figure 5.15 Blot of sperm plasma membrane (lanes 1 & 2) and seminal plasma proteins (lanes 3 & 4) under reducing and non-reducing conditions respectively, probed with monoclonal antibody spm 4/6. A large protein smear from 90kD to 16kD was visualized in sperm plasma membrane under both reducing and non-reducing conditions. In seminal plasma, spm 4/6 recognized a smaller smear which was composed of at least six protein bands of 26kD, 25kD, 23kD, 21kD, 19kD and 18kD under reducing and non-reducing conditions.

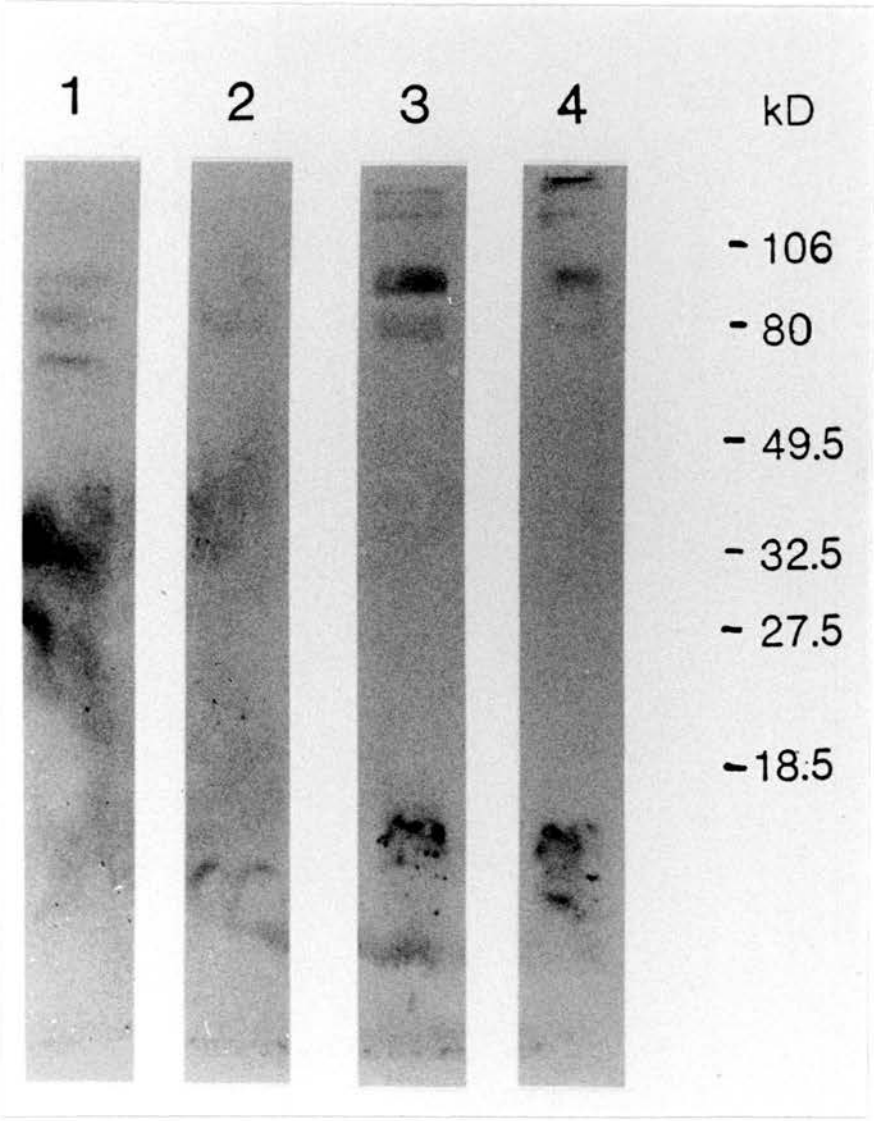

Figure 5.16 Blot of sperm plasma membrane (lanes 1 & 2) and seminal plasma proteins (lanes 3 & 4) under reducing and non-reducing conditions respectively, probed with monoclonal antibody spm 4/7. Two proteins of 87kD and 74kD and a distorted band which migrated behind the ion front were recognized in all four lanes. In addition a protein of 65kD was identified only in sperm plasma membrane under reducing conditions. In seminal plasma, two further bands of 119kD and 110kD were observed in both lanes and an additional band of 123kD was present under non-reducing conditions.

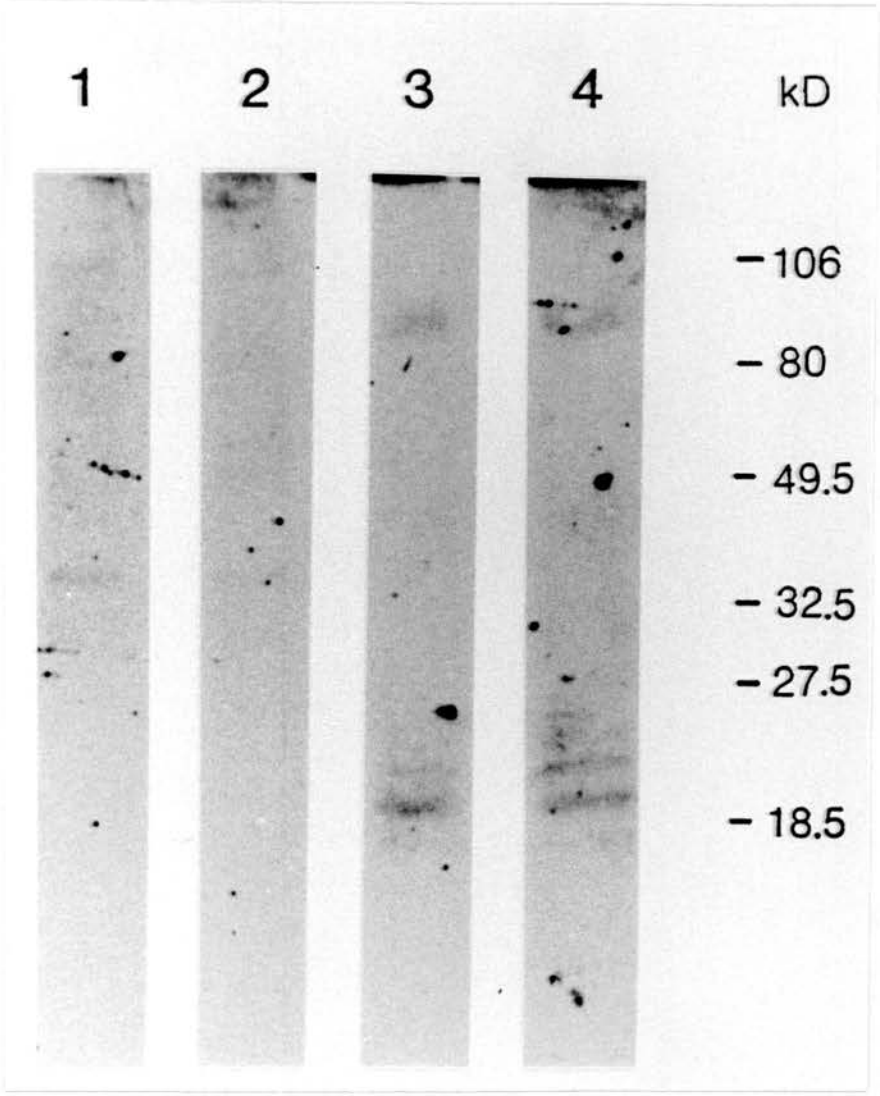

Figure 5.17 Blot of sperm plasma membrane (lanes 1 & 2) and seminal plasma proteins (lanes 3 & 4) under reducing and non-reducing conditions respectively, probed with monoclonal antibody spm 4/8. A high molecular weight band which did not enter the gel was visualized in all four lanes. Two proteins of 100kD and 37kD were recognized in both lanes containing sperm plasma membrane and three proteins of 84kD, 20kD and 17kD were identified in both seminal plasma lanes.

antibody titre rises logarithmically; iii) a plateau phase in which the antibody titre stabilizes; and iv) a decline phase during which the antibody is cleared or catabolized (Roitt *et al*, 1985). The first encounter produces a primary antibody response which is initially dominated by IgM and subsequently by IgG. A second encounter with the same antigen leads to a secondary response which comprises a prompt and massive IgG response with a smaller IgM component (Darnell *et al*, 1990). As all the fusions were carried out after an immunization schedule which included a primary injection and at least one booster injection before the final boost, each animal would contain a large number of cells producing antibodies of the IgG class due to the production of secondary immune responses. It is therefore surprising that such a large proportion of the monoclonals produced were of the IgM class. Seventy five percent of the antibodies belonged to this class and this may be compared with the average of 33% reported at two workshops on anti-sperm antibodies organized by the World Health Organization (Anderson *et al*, 1987). One possible reason for the production of such a high number of IgM antibodies could be related to the immunogen. The sperm plasma membrane preparations used for the immunizations are particulate and particulate antigens have been found to stimulate good antibody responses that have a large IgM component (Herbert, 1978).

Large scale production of monoclonal antibodies by bulk culture *in vitro* typically produces antibody concentrations of 10-100µg/ml (Goding, 1980). As approximately 700mls of bulk culture supernatant were produced for each antibody, the solution would be expected to contain between 7 and 70mgs of antibody. After purification and concentration, the antibodies were finally resuspended in 5mls PBS and aliquotted for storage. The protein content of each of these concentrated antibody solutions was determined and found to range from 0.2 to 3.1mg/ml (Table 5.1). Thus the final amount of antibody purified from the culture supernatants varied between 1

and 15.5mg. This is less than would be predicted but can probably be accounted for by loss of antibody during the purification and concentration procedure, for example by non-specific binding to dialysis membranes.

Antisera can be compared semi-quantitatively by determining the end point of their titration curves (Hudson & Hay, 1980). The method usually involves making doubling dilutions of the antibody solution and testing these for the presence of antibody (Dausset & Colombani, 1964). The highest dilution still showing a positive reaction in the test for antibody is taken as the end point and termed the titre (Humphrey & White, 1963). It is a relative measure of the antigen binding capacity of a solution and thus gives an indication of the avidity of antibodies in the original solution for the antigen of interest (Humphrey & White, 1963; Hudson & Hay, 1980). Affinity is a measure of the strength of binding between an antigenic determinant (epitope) and an antibody combining site (paratope; Roitt *et al*, 1985). When multivalent antigens and antibodies are involved, the interaction is more complex and the term avidity is used (Hudson & Hay, 1980). The avidity is therefore a measure of the functional combining strength of an antibody with its antigen and is related to both the affinity of the reaction between the epitopes and paratopes and the valencies of the antibody and antigen (Roitt *et al*, 1985). The titre of an antibody solution therefore gives an indication of the avidity of the antibody for the antigen of interest. It does not give an accurate measurement of the antibody concentration (Dausset & Colombani, 1964) as can be seen by comparing the titres and protein concentrations given in Table 5.1. Antibody solutions with similar protein concentrations can have vastly different titres and vice versa (eg spm 1/2 and spm 4/7 which have protein concentrations of 1.01mg/ml and 1.36mg/ml and titres of 1/320 and 1/10 240 respectively). It gives a relative measure of the antigen binding capacity of a solution and is therefore only sufficient to allow a

comparison to be made between different antibody solutions when they are tested in parallel with the same antigen (Hudson & Hay, 1980).

The level of contamination of the purified antibody solutions with other immunoglobulin molecules has been minimized. The use of FCS in the culture medium means that a small amount of bovine antibodies will be present in the culture supernatant. However these should not be copurified with the monoclonals as the anti-IgM column used to purify the IgM antibodies, was made with antibodies targetted specifically against mouse IgM molecules and the monoclonals are the only murine antibodies present in the culture supernatants. Little bovine IgG is bound by protein A and thus the purity of the IgG monoclonals purified in this manner is high (Oi *et al*, 1978; Goding *et al*, 1979). The level of contamination of the purified antibody solutions with other immunoglobulin molecules is therefore negligible if any are present at all.

Semen is a heterogeneous mixture composed of various fluid secretions, somatic cells, cellular fragments, and particulate matter in addition to spermatozoa (Lessley & Garner, 1983). Sperm populations are also very heterogeneous. There is a large degree of variability of spermatozoa both within one man (in one ejaculate and between different ejaculates) and between different men (Katz *et al*, 1989a; Mallidis *et al*, 1991) and thus sperm samples prepared in different ways can have differing protein compositions. For example, Gupta *et al* (1990) found that different extraction procedures from the same pooled sample can have different gel electrophoresis profiles and that different three times washed pools varied in their protein pattern. However they also showed that swim-up sperm extracts had yet another protein pattern but this was more consistent than those of the thrice washed spermatozoa. Naaby-Hansen (1990) also prepared spermatozoa using a three times washing protocol and found that

the overall protein profile was highly reproducible although minor variations between different protein preparations were observed even though the method of sperm preparation had been standardized. This can be explained by the fact that a significant number of leucocytes are present in the ejaculates of normal men although the actual number present varies (Olsen & Shields, 1984; Wolff & Anderson, 1988; Aitken & West, 1990). The swim-up method of sperm preparation is a selective method in that only motile spermatozoa are isolated. Spermatozoa prepared by the three times washing protocol, a non-selective method, will thus comprise a more heterogeneous mixture of motile and non-motile spermatozoa, and round cells including germ cells and leucocytes. It is interesting to note that the SDS PAGE profiles of each sperm plasma membrane preparation made here were very similar even though they were prepared from a mixture of Percoll prepared spermatozoa and three times washed spermatozoa. The separation of spermatozoa on Percoll gradients is a selective method of preparation and spermatozoa prepared in this way have been shown to be functionally eqivalent to those prepared using the swim-up technique (Morales *et al*, 1991). Spermatozoa prepared on Percoll gradients have been shown to contain very little leucocyte contamination (Lessley & Garner, 1983; Aitken & West, 1990) and as the bulk of the starting material for each sperm plasma membrane preparation was prepared on Percoll gradients, the contribution of leucocyte contamination will be minor. Peterson *et al* (1983) used a similar method of sperm plasma membrane preparation to the one used here, also based on the method of Gillis *et al* (1978) although they used boar spermatozoa. They also found that the protein pattern, albeit on two dimensional gels, was consistent.

A large number of bands are visible in the silver stained gels before blotting (Figure 5.3b). This is indicative of the vast array of proteins present in spermatozoa and confirms the findings of other research workers. Studies on boar spermatozoa by

Peterson *et al* (1983) have shown the presence of more than 100 different polypeptides in the plasma membrane. Russell *et al* (1983) identified an even greater number (more than 250) also on boar spermatozoa. Using two dimensional electrophoresis, Naaby-Hansen (1990) has shown the presence of more than 260 proteins in human spermatozoal extracts and at least 60 of these have been identified as intrinsic membrane proteins.

Polyclonal murine anti-human sperm plasma membrane antibodies were used as a positive control for the blotting procedure (Figure 5.4a) and a large number of the bands were stained. This confirms the findings of Lee *et al* (1982a) who showed that anti-human sperm antisera raised in the mouse or rabbit reacted with almost all the protein bands of blots of human sperm proteins. Preimmune mouse serum reacted with several proteins in human seminal plasma and spermatozoa. This is a reflection of the xenogeneic status of human spermatozoa in mice and thus the proteins detected are likely to be primarily 'human' antigens rather than sperm-specific antigens. This is further supported by the work of Lee *et al* (1982a) who found that in a homologous situation, preimmune rabbit serum did not detect any protein bands on blots of rabbit sperm proteins. Alternatively some of the proteins recognized by the preimmune serum in the heterologous situation may represent cross-reacting anti-microbial antibodies which are present in the immunoglobulin repertoire of the mice (World Health Organization Reference Bank, 1977; Naaby-Hansen & Bjerrum, 1985).

However as all the monoclonals were tested as purified antibody solutions from culture supernatants, other monoclonal antibodies were used as controls to identify the level of non-specific binding. The majority of non-specific binding was confined to four protein bands of 20kD, 18kD, 11kD and 10kD in seminal plasma and to high molecular weight bands which did not enter the gels. These latter bands are thought to comprise aggregates of proteins and their presence is sporadic depending on whether

this part of the gel was removed with the stacking gel before blotting or not. This high molecular weight material has been reported previously by Young & Goodman (1980) and Naaby-Hansen & Bjerrum (1985). They also found that the bands were more frequently present under non-reducing conditions which is consistent with the hypothesis that they comprise aggregates of proteins which precipitate at the origin of the gel.

Several studies have investigated the surface labelling of human spermatozoa in order to identify proteins which are externally orientated (Young & Goodman, 1980; Naaby-Hansen & Bjerrum, 1985; Aitken *et al*, 1987a). Naaby-Hansen & Bjerrum (1985) identified several major bands of 112kD, 64kD, 41kD and 32kD and a lesser band of 78kD, although under non-reducing conditions the 64kD and 32kD bands were not seen. The protein of 64kD is similar to the one of 66kD identified by Aitken *et al* (1987a) although they do not report on its behaviour under non-reducing conditions. This protein may correspond to the antigen recognized by monoclonal antibody spm 4/7 which identified a protein of 65kD only present under reducing conditions. Other proteins which may correspond include the 41kD and 78kD proteins (Naaby-Hansen & Bjerrum, 1985) with the 40kD and 77kD antigens recognized by spm 2/1.

There is now a substantial body of evidence implicating antibodies present in the sera of some infertile people as the cause of their infertility (Jones, 1980; Bronson, 1988; Alexander, 1989). Several studies have been carried out using human sera from patients exhibiting idiopathic autoimmunity to identify the major antigens involved (Poulsen & Hjort, 1981; Lee *et al*, 1983; Naaby-Hansen & Bjerrum, 1985; Aitken *et al*, 1987a). The most heavily staining band in two of these studies (Lee *et al*, 1983; Aitken *et al*, 1987a) had a molecular weight of 90kD and could correspond to the antigens recognized by several of the monoclonals namely spm 1/1 (94kD), spm 2/1 (88kD), spm 4/2 (89kD) and spm 4/7 (87kD). A further protein of 35kD identified by

Aitken *et al* (1987a) may correspond to the 36kD antigen recognized by spm 1/2 or the 37kD antigen recognized by spm 4/8. Interestingly the 64/66kD protein identified as a surface protein, which may correspond to the 65kD antigen recognized by spm 4/7 discussed earlier, was also recognized by autoimmune sera (Naaby-Hansen & Bjerrum, 1985; Aitken *et al*, 1987a) as were the 40/41kD and 77/78kD antigens recognized by spm 2/1.

Several monoclonals exhibited binding to bands which were present in both spermatozoa and seminal plasma and are thus thought to recognize epitopes on sperm coating proteins (Weil *et al*, 1956; Weil, 1965; Mancini *et al*, 1971). Examples include spm 1/1 which recognized three antigens of 82kD, 72kD and 55kD and spm 4/7 which recognized two proteins of 87kD and 74kD. Other monoclonals recognized epitopes on putative sperm coating proteins but only under reducing conditions (eg spm 4/1 which identified two proteins of 82kD and 72kD and spm 4/2 which bound to a protein of 89kD) or non-reducing conditions (eg spm 4/5 which recognized a protein of 58kD). Alternatively such antibodies may recognize epitopes which are shared antigenic determinants between spermatozoa and seminal plasma proteins. It is interesting to note that Young & Goodman (1980) identified by lactoperoxidase catalysed $Na^{125}I$ radiolabelling, three major components of the human sperm surface which were proteins adsorbed from seminal secretions. One of these had a molecular weight of approximately 72-74kD and may correspond to the 72-74kD antigen recognized by spm 1/1, spm 4/1 and spm 4/7. They also identified two other major proteins present in seminal plasma which were not found to be labelled on spermatozoa. One of these proteins, HP-3, had a molecular weight of 44kD and may correspond to the 44kD antigen recognized in seminal plasma by spm 2/2.

Carbohydrates have been shown to be important in many cell-cell recognition events and fertilization is no exception (Shur, 1989; Wassarman, 1989; Miller & Ax,

1990). Many of the proteins on the surface of spermatozoa have been identified as glycoproteins (Koehler, 1981; Kumar *et al*, 1990) and the carbohydrate is thought to play a fundamental role in the species specificity of fertilization (Wassarman, 1989). It is therefore not surprising that at least one of the antibodies may recognize an epitope on the carbohydrate component of the sperm proteins. Antibody spm 4/6 recognized a large protein smear from 90 to 16kD in spermatozoa and a smaller smear of low molecular weight in seminal plasma. This antibody is thought to recognize a frequently occurring carbohydrate epitope which is common to many sperm proteins. Two other monoclonals, spm 2/2 and spm 4/3, also recognized protein smears. These were most heavily stained at 17kD and had a tapering tail of staining to 21kD. This is indicative of molecular weight heterogeneity which is characteristic of differential glycosylation of a protein (Hamilton, 1981).

The monoclonal spm 4/7 recognized a distorted band which migrated just behind the ion front. This was also visible as a large heavily staining component of the purified sperm plasma membrane in silver stained gels (lanes 8 and 9, Figure 5.3b) and is thought to have a lipid-like composition as Aitken *et al* (1987a) found that it was readily removed by organic solvents.

Many of the antibodies recognize more than one sperm protein band. This is concordant with the work of Peterson *et al* (1981b) and Lee *et al* (1984) who also found that some antibodies exhibited binding to more than one protein band indicating that the same antigenic determinants are frequently shared by more than one sperm protein. Alternatively protein degradation could be occurring but as the protease inhibitor PMSF was present during the plasma membrane purification procedure, this is unlikely.

Some antibodies do not Western blot and this is particularly true for monoclonal antibodies which recognize only one epitope (Stott, 1989). The monoclonal spm 4/4

described here is one example. This is usually due to denaturation of the antigen with subsequent loss of the epitope during the blotting procedure which involves such harsh treatment as heating the protein to 100°C in an SDS solubilization buffer (Poxton, 1990). Thus conformationally dependent epitopes are lost. However many antibodies still recognize their corresponding antigen after blotting from denaturing gels as the SDS is stripped off the proteins during transfer thus permitting renaturation to occur (Lee *et al*, 1982b; Stott, 1989).

5.5 Summary

The twelve monoclonal antibodies were isotyped and nine were identified as belonging to the IgM class and the remaining three belonged to the IgG_{2a} subclass. They were purified from bulk culture supernatants by affinity chromatography on either a protein-A column (IgG_{2a} antibodies) or an anti-IgM column (IgM antibodies). The titre and protein concentration of each purified antibody solution were determined and found to range from 1/10 to 1/20 480 and from 0.2mg/ml to 3.1mg/ml respectively.

The ability of the monoclonals to recognize sperm plasma membrane proteins and seminal plasma proteins under reducing and non-reducing conditions was investigated using a dot blotting technique. Eleven of the twelve antibodies were found to recognize purified sperm proteins and ten also recognized seminal plasma proteins. One antibody did not blot at all. The antigens recognized by the antibodies were further characterized by blotting against one-dimensional SDS-polyacrylamide gels of seminal plasma and purifed sperm plasma membrane proteins. Non-specific binding was found to be directed against a high molecular weight band composed of aggregated proteins which did not enter the gels, and four seminal plasma proteins of relative molecular masses 20kD, 18kD, 11kD and 10kD. The antibodies exhibited binding to a number of

proteins of varying molecular weight. Several were tentatively identified as sperm coating proteins due to their presence in both seminal plasma and spermatozoa. Some antibodies recognized proteins which may correspond to antigens which have previously been implicated as playing a role in autoimmune infertility. One antibody recognized an epitope on the carbohydrate component of many of the sperm proteins and two other antibodies identified a protein which may exhibit differential glycosylation. Most of the monoclonals recognized more than one protein band indicating that the same antigenic determinant is present on several sperm proteins.

6 Immunocytochemical Localization of Sperm Surface Antigens

6.1 Introduction

Despite the fact that spermatozoa are covered by a continuous plasma membrane, it exhibits a mosaic structure within which there are boundaries limiting the lateral diffusion of particles to discrete domains on the sperm surface, delineating such regions as the acrosome, post-acrosomal region, midpiece and tail (Cardullo & Wolf, 1990). Using antibodies or lectins as probes, the localization of molecular entities to specific regions of the sperm surface has been repeatedly demonstrated (Koehler, 1975; O'Rand & Romrell, 1981; Feuchter *et al*, 1981). The sperm cell surface is therefore highly differentiated (Primakoff & Myles, 1984) with the result that different sperm-specific antibodies bind to different regions of the cell (Bellve & Moss, 1983; Holt, 1984; Primakoff & Myles, 1983). These regions may have differing functions, their structural composition reflecting the presence of functional domains involved in the fertilization process (Jones *et al*, 1983; Mortillo & Wassarman, 1991). Indeed several molecules, the function of which have been putatively identified with fertilization and the acrosome reaction, have been localized to the anterior region of the head where the acrosome reaction occurs (Moore & Hartman, 1984; Cardullo & Wolf, 1990). In addition anti-sperm antibodies directed against antigens associated with the sperm head have been shown to alter the ability of spermatozoa to penetrate zona-free hamster eggs (Bronson *et al*, 1981, 1983; Alexander, 1984; Haas *et al*, 1985). Components of the zona pellucida have also been shown to bind preferentially to different sperm membrane compartments, ZP3 binding to acrosome intact spermatozoa and ZP2 to acrosome reacted spermatozoa (Mortillo & Wassarman, 1991).

It is unlikely that antibodies against intracellular antigens (with the possible exception of acrosomal antigens) will affect the process of fertilization (Haas *et al*, 1988; Cross & Moore, 1990) and therefore a variety of techniques have been developed to reveal whether anti-sperm antibodies are directed against surface antigens and to which region of the sperm surface they bind (Bronson *et al*, 1986; Smithwick & Young, 1990). These methods include agglutination (Hargreave & Hjort, 1983), immunobead binding (Bronson *et al*, 1981; Clarke *et al*, 1985; Shulman *et al*, 1985; Adeghe *et al*, 1986; Clarke, 1987), immunofluorescence (Isahakia & Alexander, 1984; Cross & Moore, 1990) and immunoenzymatic methods (Paul *et al*, 1982; Cordell *et al*, 1984; Holcberg *et al*, 1986). Immunobead assays have poor spatial resolution because the beads are relatively large in comparison to spermatozoa and much higher resolution can be achieved using indirect immunofluorescence (Cross & Moore, 1990). However immunofluorescent staining of surface antigens is often of low intensity (Jones *et al*, 1983; Cross & Moore, 1990) and therefore an enzymatic method which permits amplification of the signal was used in these studies (Cordell *et al*, 1984). This procedure which is depicted diagrammatically in Figure 6.1, utilizes an unlabelled 'bridge' antibody (rabbit anti-mouse immunoglobulins; RAM) which links the primary antibody with the labelled antibody complex and an alkaline phosphatase anti-alkaline phosphatase complex (APAAP). Simple repetition of these two incubation steps substantially increases the intensity of staining allowing this enhanced APAAP procedure to reveal antigens at concentrations below the threshold of sensitivity of other immunohistologic labelling procedures (Cordell *et al*, 1984).

One prerequisite for a contraceptive vaccine based on sperm surface antigens is that the molecule should be gamete specific (Shaha *et al*, 1991; Talwar *et al*, 1989) in order that intervention can be made specifically on reproductive processes without

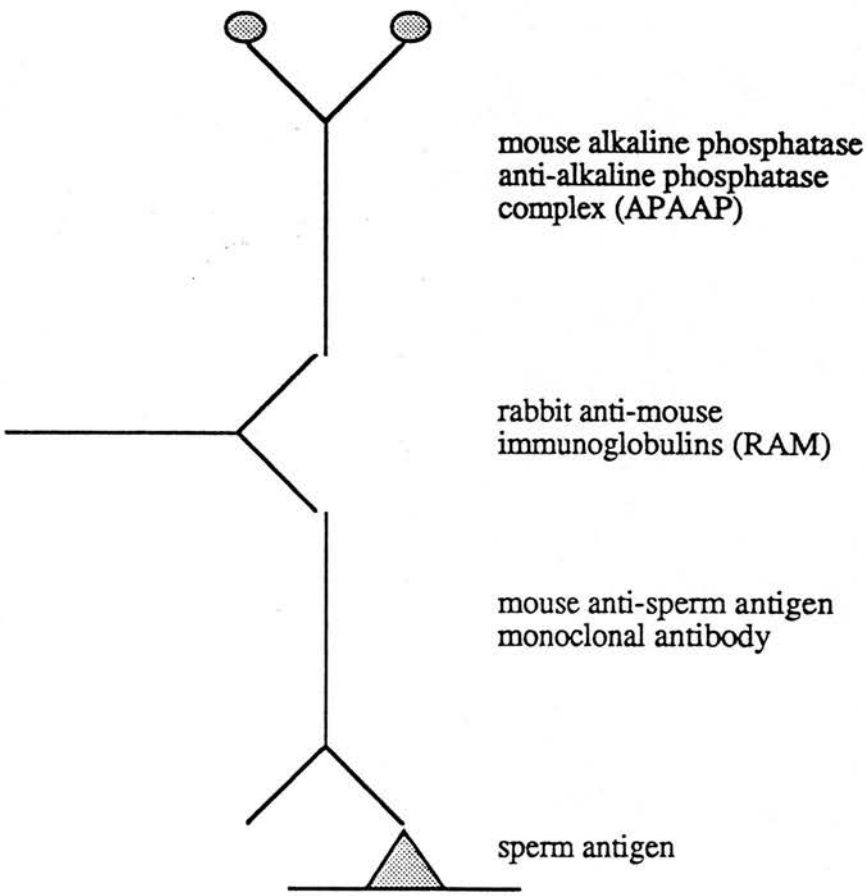

Figure 6.1 A schematic representation of RAM APAAP staining.

generalized effects on other tissues or functions of the body (Talwar, 1980). Sperm antigens share many antigens with somatic cells (Freund *et al*, 1955; Kerek, 1974; Mathur *et al*, 1981; Chaffee & Schachner, 1978). In light of this requirement, the same technique used to identify the regional binding specificity of the antibodies, was also used to determine the extent of cross-reactivity of the monoclonal antibodies with a variety of antigens.

Antigenic similarities between mammalian spermatozoa were first described by von Moxter as early as 1900 (Isahakia & Alexander, 1984) and although cell specificity is a prerequisite for a contraceptive vaccine candidate, species specificity is not. Indeed cross-reactivity with spermatozoa from other species may be advantageous permitting the use of animal models for further investigation and testing of promising candidate vaccines *in vivo* (Anderson & Alexander, 1983). Perhaps the most dramatic demonstration that common antigenic determinants exist on the surface of all animal spermatozoa has been obtained in a study in which a polyclonal antiserum raised against sea urchin sperm plasma membranes was shown to cross-react with spermatozoa from at least 28 species representing 7 phyla of the animal kingdom (Lopo & Vacquier, 1980).

6.2 Materials and Methods

6.2.1 RAM APAAP Staining

A schematic diagram of this staining procedure is provided in Figure 6.1. Prepared spermatozoa were washed and resuspended in PBS at a concentration of 20 x 10^6/ml. Aliquots of this sperm suspension (5µl) were spotted onto each well of a 12 well Hendley slide (CA Hendley, Essex, UK) and air dried overnight at room temperature. The slides were then wrapped individually in aluminium foil and stored in a dessicator

at -70°C. Immediately prior to use, the slides were defrosted for at least 30 minutes and subsequently unwrapped. They were fixed in 1% para-formaldehyde in PBS for 30 minutes and then washed twice in TBS (0.05M Tris, 0.15M NaCl, pH7.6). Neat antibody solution (5µl) was added to each well and the slides were incubated for 1 hour in a humid chamber at room temperature. The slides were again washed twice in TBS before 10µl of rabbit anti-mouse immunoglobulins (RAM; Dako, High Wycombe, Bucks, UK) at a 1/25 dilution in TBS were added to each well and incubated for 30 minutes in a humid box. The slides were washed twice in TBS and then 10µl of alkaline phosphatase/anti-alkaline phosphatase complex (APAAP; Dako) were added to each well. After incubation for 1 hour, the slides were once again washed twice in TBS. Two further rounds of RAM APAAP staining (15 minutes each) were performed before the slides were developed. Alkaline phosphatase substrate (10µl; 0.5mM napthol AS-MX phosphate, 2% dimethylformamide, 0.1M Tris, pH8.2, 0.01M levamisole, 3.9mM Fast Red TR salt (Sigma) was added per well and incubated for 18 minutes. The slides were first washed in TBS then in gently running tap water before being mounted in Apathy's aqueous mounting medium (BDH, Poole, Dorset, UK).

6.2.2 Boar Spermatozoa

Boar spermatozoa were kindly provided by The Meat and Livestock Commission Pig Breeding Centre (Selby, North Yorkshire, UK). The spermatozoa were washed three times in PBS and resuspended at 20 x 10^6 cells/ml. Slides were prepared and stained using the enhanced RAM APAAP protocol described in Section 6.2.1.

6.2.3 Marmoset Spermatozoa

Marmoset spermatozoa were collected by the staff at the Edinburgh Primate Colony. After copulation, spermatozoa were collected from ovariectomized female marmosets by

vaginal lavage. The spermatozoa were washed twice in BWW and the concentration adjusted to 20 x 10^6 cells/ml. Slides were prepared and stained using the enhanced RAM APAAP protocol described in Section 6.2.1.

6.2.4 Leucocyte Preparation

Leucocytes were prepared from whole blood using Polyprep (Nycomed, Birmingham, UK). Blood was collected from healthy volunteers who were all regular blood donors and immediately placed into a 7ml polystyrene tube (Sarstedt, Leicester, UK) containing heparinized beads (Lithium Heparin Barrier Beads; Sarstedt) to prevent coagulation. Anticoagulated whole blood (5mls) was layered over 3.5mls of Polyprep and centrifuged at 500 x g for 30 minutes. After centrifugation, two leucocyte bands were visible. The top band at the sample/medium interface consisted of mononucleated cells (lymphocytes) and the lower band of polymorphonucleated cells (neutrophils and macrophages) while the erythrocytes were pelleted. The two leucocyte bands were harvested using a Pasteur pipette and washed in BWW medium. The pellet was resuspended in a known volume of BWW (normally 1ml) and the cells counted. The percentage of white blood cells (WBC) was determined using Testsimplet slides (Boehringer Mannheim, Lewes, East Sussex, UK). The number of WBC/ml was calculated and the cell concentration adjusted to 1 x 10^6 WBC/ml PBS. Slides were made and the leucocytes stained using the enhanced RAM APAAP protocol described in Section 6.2.1.

6.3 Results

The regional binding of the twelve monoclonal antibodies to human spermatozoa was investigated using enhanced RAM APAAP staining of para-formaldehyde fixed cells.

Although all twelve monoclonals bound to the entire surface of human spermatozoa, staining was more intense in certain regions particularly the midpiece and the acrosome. A representative example of this staining is shown in Figure 6.2. No staining was observed when no primary antibody was used nor when a monoclonal antibody raised against leucocyte common antigen (CD45; Scottish Antibody Production Unit, Carluke, Scotland, UK) was used as the primary antibody however intense red staining of the whole sperm surface was achieved when polyclonal anti-human sperm plasma membrane antiserum was employed (Figure 6.2).

A preliminary investigation into the species specificity of the monoclonals was performed using spermatozoa from the common marmoset (*Callithrix jacchus*) and from White Yorkshire pigs. The results are summarized in Table 6.1 and representative photographs of the staining are shown in Figures 6.3 (marmoset) and 6.4 (boar). Four monoclonals, namely spm 1/2, spm 2/1, spm 4/3 and spm 4/4, did not cross-react with spermatozoa from either species. The other eight antibodies cross-reacted with both marmoset and boar spermatozoa, each binding to the entire cell surface. Once again, staining was not evenly distributed over the sperm surface. The acrosomal region and the midpiece of some marmoset spermatozoa were more intensely stained (Figure 6.3) and in boar samples, staining of the cytoplasmic droplet and midpiece were particularly strong (Figure 6.4). As with human spermatozoa, no staining was achieved using no primary antibody nor with anti-leucocyte common antigen antibody and the entire sperm surface from both species was stained when polyclonal anti-human sperm plasma membrane antiserum was used (Figures 6.3 and 6.4).

The ability of the antibodies to recognize somatic cells was assessed using cells isolated from human blood by density centrifugation. The results are shown in Table 6.2 and representative examples of this staining are provided in Figure 6.5. Four monoclonals (spm 1/2, spm 2/1, spm 4/3 and spm 4/4) did not recognize any

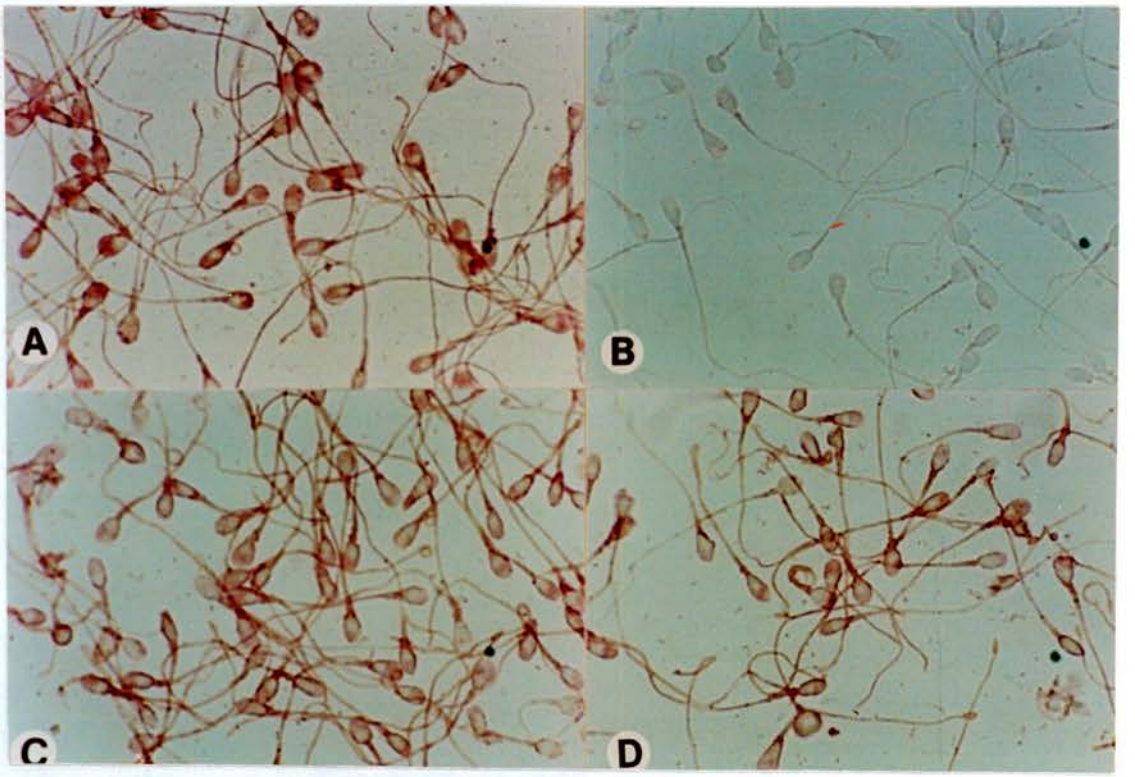

Figure 6.2 Representative RAM APAAP staining of human sperm cells — (A) polyclonal anti-human sperm plasma membrane antiserum, (B) no first antibody, (C) monoclonal antibody spm 4/3, (D) monoclonal antibody spm 4/6.

Antibody	Human	Marmoset	Boar
spm 1/1	+	+	+
spm 1/2	+	-	-
spm 2/1	+	-	-
spm 2/2	+	+	+
spm 4/1	+	+	+
spm 4/2	+	+	+
spm 4/3	+	-	-
spm 4/4	+	-	-
spm 4/5	+	+	+
spm 4/6	+	+	+
spm 4/7	+	+	+
spm 4/8	+	+	+

Table 6.1 Summary of the results of cross reactivity tests assessed by immunostaining of spermatozoa from different species. Positive staining is indicated, + and no staining, -.

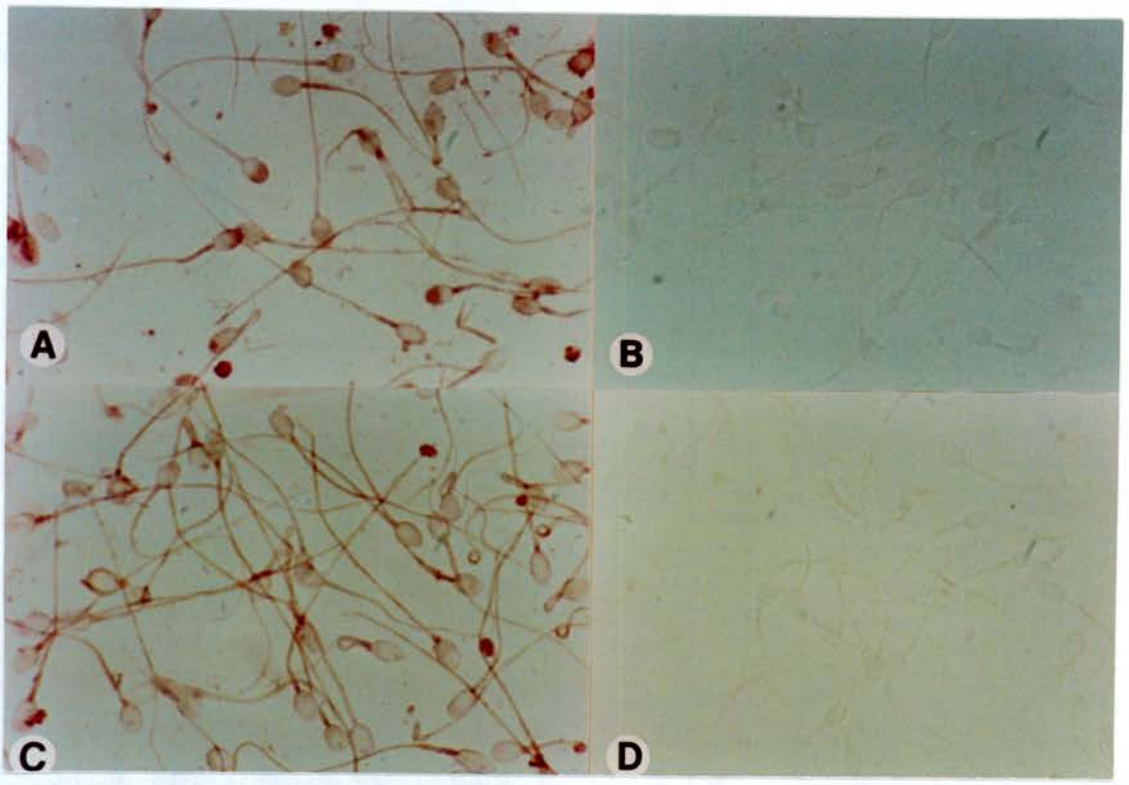

Figure 6.3 Representative RAM APAAP staining of marmoset spermatozoa — (A) polyclonal anti-human sperm plasma membrane antiserum, (B) anti-leucocyte common antigen monoclonal antibody, (C) monoclonal antibody spm 1/1, (D) monoclonal antibody spm 1/2.

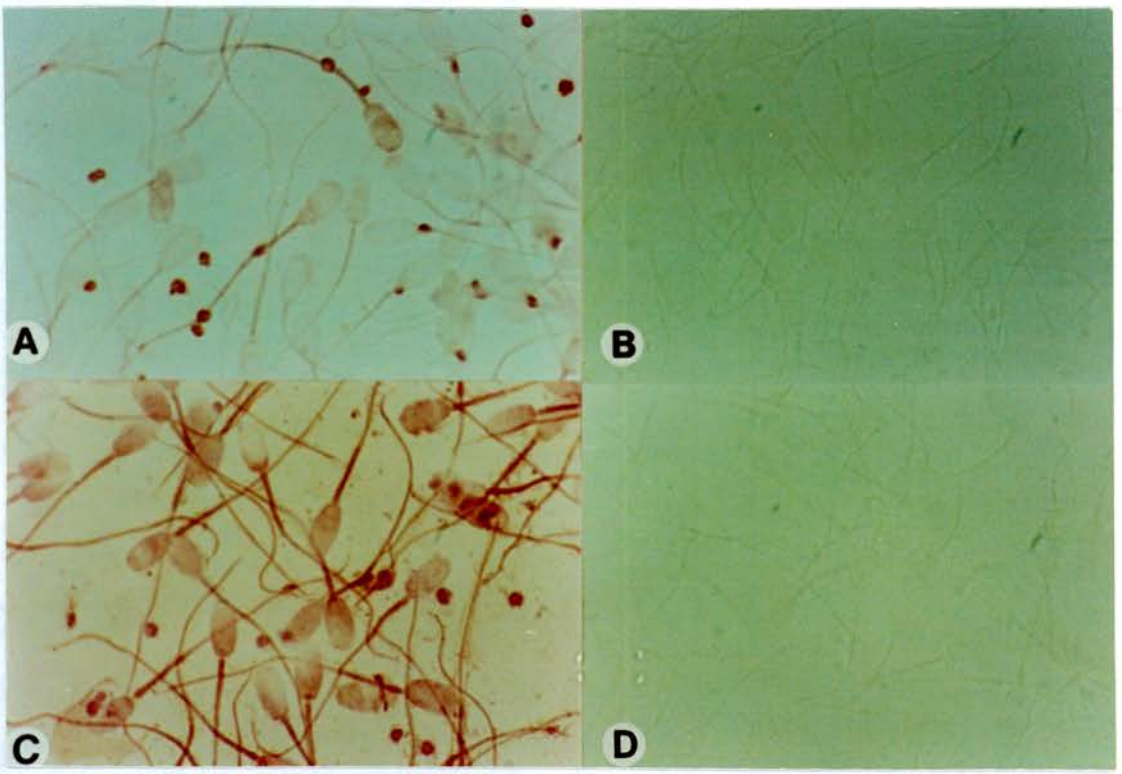

Figure 6.4 Representative RAM APAAP staining of boar spermatozoa — (A) polyclonal anti-human sperm plasma membrane antiserum, (B) anti-leucocyte common antigen monoclonal antibody, (C) monoclonal antibody spm 4/5, (D) monoclonal antibody spm 4/3.

Antibody	Lymphocytes	Neutrophils & Macrophages
spm 1/1	+	+
spm 1/2	-	-
spm 2/1	-	-
spm 2/2	+	+
spm 4/1	+	+
spm 4/2	+	+
spm 4/3	-	-
spm 4/4	-	-
spm 4/5	+	+
spm 4/6	+	+
spm 4/7	+	+
spm 4/8	+	+

Table 6.2 Results of cross-reactivity tests assessed by immunostaining of different cells found in human blood. Positive staining is indicated, + and no staining, -.

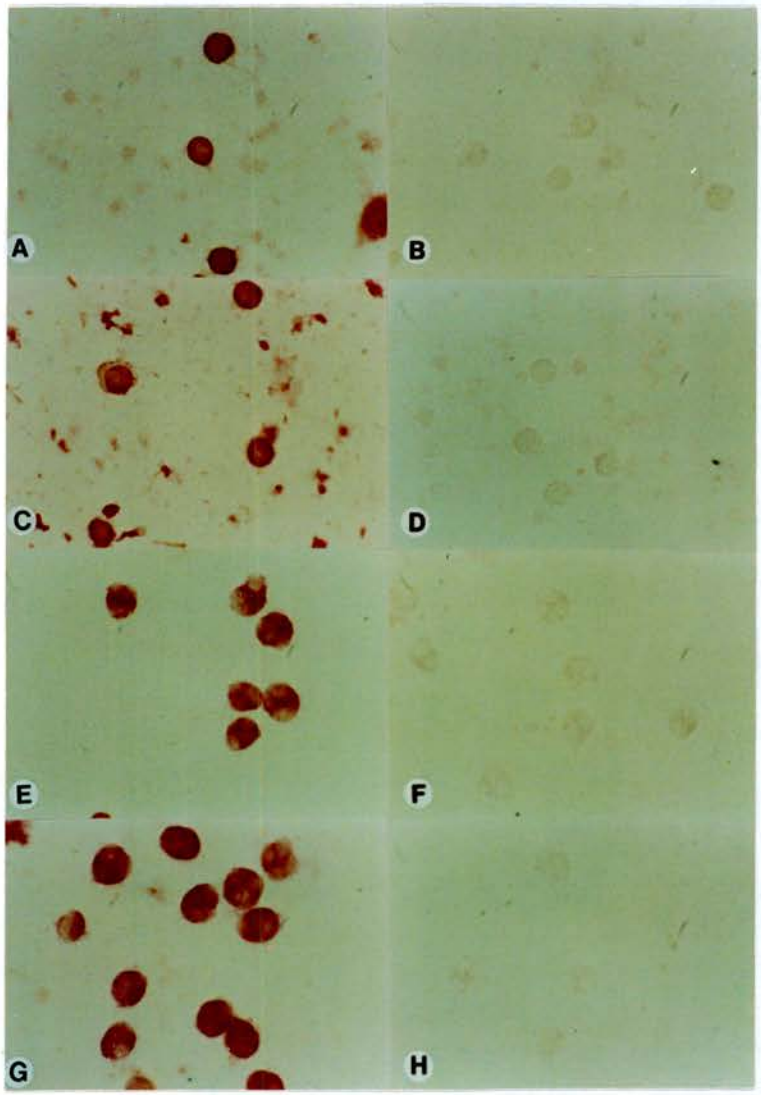

Figure 6.5 Representative RAM APAAP staining of cells isolated from human blood by density centrifugation. Panels A-D show mononucleated cells (lymphocytes) and panels E-H show polynucleated cells (neutrophils and macrophages) — (A) and (E) positive staining of leucocytes with anti-leucocyte common antigen monoclonal antibody, (B) and (F) background staining using no primary antibody, (C) monoclonal antibody spm 4/6, (D) monoclonal antibody spm 4/4, (G) monoclonal antibody spm 2/2, (H) monoclonal antibody spm 4/3.

of the other cells whereas the other eight immunoglobulins cross-reacted with lymphocytes, neutophils and macrophages. No staining was seen when no primary antibody was used (figure 6.5) nor when control antibody (anti-growth hormone) was used (data not shown).

6.4 Discussion

It is remarkable that the distribution of 'surface' molecules often corresponds closely to well-defined intracellular structures such as the acrosome, equitorial segment, post-acrosomal region, midpiece and tail. Some researchers have suggested that the surface localization of some restricted antigens may be an artifact and that these molecules are in fact intracellular (Hjort & Hansen, 1971; Jones *et al*, 1983). Contradictory results from different assays are sometimes obtained (Boettcher *et al*, 1977; Hancock & Faruki, 1985; Bronson *et al*, 1986) but these can be attributed to variations in the sensitivity and specificity of the method used (Smithwick & Young, 1990). Results may be markedly affected by a change in method as this often alters or destroys sperm surface antigens (Villarroya & Scholler, 1986; Saxena *et al*, 1986). It is therefore important to maintain both the morphologic and antigenic integrity of cells in order to accurately identify the antibody binding region (Haas *et al*, 1988) and the importance of standardizing the conditions of fixation and staining used in immunocytochemical analysis has been stressed by several investigators (Hancock *et al*, 1982; van Ewijk *et al*, 1984; Walker *et al*, 1984; Haas *et al*, 1988). Methanol fixation permeabilizes the cells thus exposing internal antigens (Hjort & Hansen, 1971; Haas *et al*, 1988). However fixation in gluteraldehyde or para-formaldehyde is much less harsh and neither reagent appears to alter the sperm plasma membrane morphologically, although they slightly increase the level of non-specific binding of

immunoglobulin (Haas *et al*, 1988). Thus the cells used for immunocytochemical analysis were all fixed in para-formaldehyde.

All twelve monoclonal antibodies bound to the entire human sperm plasma membrane thus indicating that common membrane components exist throughout the plasmalemma. However, the intensity of staining did vary over different regions of the sperm surface and was particularly strong over the midpiece and acrosome. This uneven distribution of staining was not peculiar to the monoclonal antibodies, as it was also seen when polyclonal antiserum was used and may reflect the varying depth of the sperm cell in different regions.

Antigens recognized by other antibodies have similarly been shown to be located over the entire cell surface (Jones *et al*, 1983; Moore & Hartman, 1984). The production of such a high proportion of monoclonal antibodies not localized to a specific domain may be partly due to the immunogen used for antibody production, which was a purified sperm plasma membrane preparation (Aitken *et al*, 1987a). During the membrane preparation production process, two sorts of membrane vesicle are made — (1) those that are electron dense and are composed of outer acrosomal membrane and (2) those which are distended or collapsed consisting of plasma membrane. After sucrose density centrifugation, the membrane preparation is solely composed of the latter type of vesicle (Aitken *et al*, 1987a). Boar sperm plasma membrane preparations isolated by a similar technique have also been shown to be of a high purity (Gillis *et al*, 1978; Peterson *et al*, 1980a) and apparently free of acrosomal membranes (Peterson *et al*, 1980a). Thus as no acrosomal membranes are present in the immunogen, the monoclonal antibodies produced from this fusion would not be expected to localize specifically to the acrosome. An ELISA was the primary screening assay used to monitor the production of antibodies directed against spermatozoa by the hybridomas and this may also be a contributory factor in the high incidence of

monoclonal antibodies not localized to a specific domain. It has been previously shown that radioligand or enzyme-linked binding assays tend to select antibodies directed towards antigens distributed on the sperm tail or over the entire cell as this type of localization generally produces the largest signal (Moore & Hartman, 1984; Saling *et al*, 1985).

Even though post-testicular spermatozoa have little or no ability to synthesize plasma membrane lipids or proteins (Lardy & Phillips, 1941; Scott *et al*, 1967; Bragg & Handel, 1979), the lipid and protein composition of the sperm plasma membrane changes drammatically during maturation and capacitation (Cardullo & Wolf, 1990; Fusi & Bronson, 1990; Berger, 1990). Fusi & Bronson (1990) demonstrated that different antibody binding patterns could be obtained using the same sera and spermatozoa from the same donor but in different functional states (capacitated versus uncapacitated). Using monoclonal antibodies, Myles & Primakoff (1984) showed that guinea pig sperm surface antigens were redistributed during capacitation as did Saxena *et al* (1986) for boar spermatozoa and Okabe *et al* (1986a&b) for mouse spermatozoa. O'Rand (1977) demonstrated that some rabbit sperm surface antigens maintained a certain fluidity over the sperm surface prior to exposure to capacitating conditions but following capacitation their domain became restricted. Similar changes were shown to occur in human spermatozoa during capacitation by Margalioth *et al* (1992). Thus the ability of some monoclonal antibodies to bind to spermatozoa or the regionality of this binding, alters with the functional state of the spermatozoon (Anderson & Alexander, 1983). The effect of capacitation state on the binding of the monoclonal antibodies reported here was not investigated.

Eight of the antibodies cross-reacted with spermatozoa from the other two species tested (marmoset and boar). As was found with human spermatozoa, although the intensity of staining varied over different regions, all eight antibodies recognized

molecules which were present over the entire cell surface. The conservation of regionality of binding between species has been previously noted. Isahakia (1991) reported a monoclonal raised against baboon spermatozoa which localized to the acrosome, BSA 4. This antibody cross-reacted with human, rat and mouse spermatozoa where it was also restricted to the acrosomal domain. Monoclonals, however, recognize only a small region of a particular molecule and it is therefore possible that other parts of the molecule may differ between species. Thus the structure and function of the antigens recognized in different species by cross-reacting antibodies may also differ (Isahakia & Alexander, 1984) and cross-reacting monoclonals which have an effect on sperm function in one species will not necessarily have the same effect on the function of spermatozoa from other species (Anderson & Alexander, 1983).

It is interesting to note that the same four monoclonal antibodies which did not cross-react with either boar or marmoset spermatozoa, also did not cross-react with leucocytes suggesting that these four monoclonals recognized molecules on human spermatozoa which are both species- and gamete cell-specific. Although the antibodies did not recognize any molecules on the surface of leucocytes, it does not necessarily follow that the epitopes recognized will not be present on other cells in the body. Similarly the antigens may be present on spermatozoa from other species which were not tested. Testing the monoclonal antibodies against a more comprehensive panel of cells and spermatozoa from other species would resolve this question.

6.5 Summary

All twelve monoclonal antibodies displayed no regionality of antigen localization when assessed by enhanced RAM APAAP staining of para-formaldehyde fixed cells.

Although they bound to the entire surface of human spermatozoa, the intensity of staining over different regions did vary and was particularly strong over the midpiece and acrosome. The species specificity of the monoclonals was assessed using spermatozoa from marmoset and boar. Eight immunoglobulins (spm 1/1, spm 2/2, spm 4/1, spm 4/2, spm 4/5, spm 4/6, spm 4/7 and spm 4/8) cross-reacted with spermatozoa from both species and the other four did not bind to spermatozoa from either species. Cross-reactivity with somatic cells was assessed using cells isolated from human blood by density centrifugation. Eight antibodies cross-reacted with lymphocytes, neutrophils and macrophages whereas the other four monoclonals (spm 1/2, spm 2/1, spm 4/3 and spm 4/4) did not elicit staining of any of the other cells tested. These four monoclonals (spm 1/2, spm 2/1, spm 4/3 and spm 4/4) did not recognize spermatozoa from either marmoset or boar nor did they cross-react with leucocytes. The antigens recognized by these four antibodies may therefore be highly specific to human spermatozoa.

7 Effect of Monoclonal Antibodies on *in vitro* Tests of Sperm Function

7.1 Introduction

The prevention of gamete interaction and thus fertilization by anti-sperm antibodies can occur directly by blocking the sperm-egg receptors, or indirectly by preventing spermatozoa from reaching the site of fertilization by impeding sperm transport mechanisms (Aitken, 1982; Bronson *et al*, 1984). The research reported here is concerned with the former type of mechanism namely those antibodies which directly interfere with fertilization and thus the latter type will not be considered further.

The process of fertilization is complex and involves many steps. On copulation, spermatozoa are ejaculated into the vagina of the female and they must undergo a long journey through the female reproductive tract to reach the ampulla of the fallopian tube which is the site of fertilization. A series of biochemical and morphological changes collectively known as 'capacitation' (Austin, 1952) occur in spermatozoa during this journey. This process is known to involve alterations to the sperm plasma membrane and the exhibition of hyperactivated motility (Alexander, 1989; Sidhu & Guraya, 1989). On reaching the site of fertilization, spermatozoa traverse through the cumulus cells surrounding the egg and bind to the zona pellucida where interaction with one of the zona proteins, ZP3, causes the spermatozoon to acrosome react (Leyton & Saling, 1989). The acrosome reaction (AR) involves a series of fusions of between the plasma and outer acrosomal membranes culminating in the release of hydrolytic enzymes contained within the acrosome (Alexander, 1989). Bound acrosome-reacted spermatozoa can penetrate the zona pellucida to reach the perivitelline space where they bind to and fuse with the egg plasma membrane (Wassarman, 1988a). Anti-sperm antibodies can block fertilization at any one of these

points and thus there is not one single test which can measure all aspects of sperm function and a variety of tests have been developed (Bronson *et al*, 1981; Katz *et al*, 1989a; Aitken, 1990).

7.1.1 Acrosome Reaction

The acrosome reaction does not result in any morphological changes in human spermatozoa which are visible under the light microscope and thus tests which involve the use of a stain to specifically label the acrosomal contents or outer acrosomal membrane have been developed (Cross & Meizel, 1989; Aitken, 1990). Labels conjugated to either monoclonal antibodies (Wolf *et al*, 1985; Aitken, 1988a) or plant lectins (Talbot & Chacon, 1980; Mortimer *et al*, 1987) have been used successfully. One of the major problems with these stains is their inability to distinguish between a true physiological acrosome reaction and degenerative acrosomal loss (Aitken, 1989a; Tesarik & Testart, 1989). This problem can be overcome by combining the stain monitoring the acrosome with a vital stain to indicate the viability of the spermatozoa such as the supravital stain Hoechst 33258. This is a fluorescent DNA-binding dye that has limited membrane permeability and thus stains only non-viable cells (Cross *et al*, 1986; Mortimer *et al*, 1990; Holden *et al*, 1990). Alternatively the hypo-osmotic swelling test (HOS) may be used. This method identifies living cells that have an intact plasma membrane by virtue of the coiled configuration of the sperm tail which occurs when the spermatozoa are forced to swell by immersion in a hypo-osmotic medium (Drevius & Eriksson, 1966; Jeyendran *et al*, 1984; Schrader *et al*, 1986). A second problem with these tests is the low level of acrosome reactions (AR) which occur spontaneously (Mallet *et al*, 1985; Morales *et al*, 1988). Ideally AR tests should determine the ability of capacitated spermatozoa to undergo the AR in response to its physiological stimulus, the zona pellucida. However this is seldom possible and an

alternative stimulus such as the divalent cation ionophore, A23187 (Wolf *et al*, 1985) is used. A23187 induces an influx of exogenous calcium (Irvine & Aitken, 1986b) and a progressive increase in intracellular pH which consequently induce a sequence of membrane changes leading to the AR (Aitken *et al*, 1984). Thus the addition of A23187 replaces the stimulus normally delivered by the zona pellucida and induces the AR in all competent cells (Aitken *et al*, 1984; Tesarik & Testart, 1989).

7.1.2 Sperm-Zona Pellucida Interaction

Some sperm samples can show a normal capacity to acrosome react but their poor motility renders the spermatozoa incapable of penetrating the zona pellucida (Tesarik & Testart, 1989). This is the main source of the inconsistencies which exist between the results of *in vitro* tests of the AR and the actual fertilizing ability of spermatozoa *in vivo*. Tests have therefore been developed to evaluate the ability of human spermatozoa to penetrate the human zona pellucida *in vitro*. The only technique available to study this interaction utilizes intact or salt-stored human ova (Aitken, 1989a). The first penetration test reported used non-living immature oocytes recovered from the ovaries of cadavers 8-45 hours *post mortem* (Overstreet & Hembree, 1976) but nowadays ova that have failed to fertilize in an IVF programme are used. They are fixed in a high salt solution which destroys the oocyte but leaves the zona pellucida intact (Yanagimachi *et al*, 1979). Once the salt-stored zonae are washed free of salt, they readily interact with human spermatozoa and permit assessment of the capacity of the spermatozoon to bind to and penetrate the zona pellucida (Aitken, 1989a; Tesarik & Testart, 1989). As the oocyte is destroyed during the fixation process, many complex legal and ethical problems are avoided. A second consequence of destroying the oocyte is that the block to polyspermy is not produced and many spermatozoa can penetrate each zona (Tesarik & Testart, 1989). As the ova are obtained from IVF programmes, it is possible that

they have already been bound by spermatozoa and thus several ova must be run with each sample tested and the appropriate controls performed (Aitken, 1989a). Alternatively the spermatozoa being tested can be labelled (Liu *et al*, 1991). Recently the hemizona assay has been developed which involves the microsurgical bisection of human zonae to produce matched halves which can be used for comparison of treated and control spermatozoa (Burkman *et al*, 1988; Hodgen *et al*, 1988; Oehninger *et al*, 1990). The use of viable living oocytes would give a more accurate picture of the fertilizing capacity of the spermatozoa tested although there are many ethical and practical reasons why this is not routinely performed.

7.1.3 Sperm-Oocyte Fusion

Concomitant with the acrosome reaction, a specialized area of plasma membrane overlying the equatorial segment acquires the ability to fuse with the vitelline membrane of the oocyte and it is this area which initiates sperm-egg fusion (Bedford *et al*, 1979; Yanagimachi *et al*, 1988a). Ideally this fusion process should be assessed using a homologous IVF system with mature human ova however this has both ethical and logistical problems (Aitken, 1989a). The alternative approach is a heterologous IVF system employing cross-species fertilization of zona-free hamster oocytes, the hamster egg penetration test (HEPT; Yanagimachi *et al*, 1976). Hamster ova are unique in that the block to interspecies fertilization resides entirely in the zona pellucida and thus when the zona is enzymatically removed, the naked oocyte will fuse with acrosome-reacted spermatozoa from a wide variety of species (Yanagimachi *et al*, 1976; Aitken, 1989a). Human spermatozoa are no exception and once the acrosome reaction is complete they can fuse with the hamster vitelline membrane in a similar manner to the homologous system where fusion is initiated by the plasma membrane overlying the equatorial segment (Koehler *et al*, 1982). The HEPT therefore provides a measure of the ability

of the human sperm plasma membrane to undergo the sequence of membrane associated changes involved in capacitation, the completion of the acrosome reaction and the genesis of a fusogenic equatorial segment which is capable of interacting with the vitelline membrane of the oocyte (Aitken, 1989a) and thus gives an indication of the fertilizing ability of spermatozoa. Although not every aspect of sperm biology involved in the fertilization of human ova is measured, HEPT results generally reflect those obtained using the homologous IVF system particularly when the ionophore A23187 is used to induce the AR (Gould *et al*, 1983; Aitken *et al*, 1984).

7.2 Materials and Methods

7.2.1 Agglutination

Spermatozoa from the 100% Percoll fraction (at a concentration of 20 x 10^6/ml; Section 3.2.1) were mixed with an equal volume (usually 100µl) of the antibody solution to be tested in the wells of a microtitre plate. The plate was covered and incubated for 20 minutes at 37°C and then examined for agglutination.

7.2.2 Acrosome Reaction

The effect of the antibodies on the acrosome reaction (AR) was studied using fluorescently labelled peanut agglutinin. Spermatozoa isolated from isotonic Percoll (at a concentration of 20 x 10^6/ml) were diluted with an equal volume of antibody solution. For each antibody tested, a second tube was prepared which also contained the ionophore A23187 (free acid; Calbiochem, Nottingham, UK) at a final concentration of 1.25µM. After a 3 hour incubation at 37°C with 5% CO_2, a small aliquot (10µl) was removed and used to determine the motility of the spermatozoa. The rest of the sample was centrifuged at 500 x g for 5 minutes and the pellet resuspended

at a concentration of 20 x 10^6 spermatozoa/ml in medium BWW. Hypo-osmotic swelling medium (HOS; 25mM sodium citrate, 75mM fructose) was added and the sample incubated for a further hour at 37°C in 5% CO_2. The solution was centrifuged at 500 x g for 5 minutes and the sperm pellet resuspended in ice cold methanol at a concentration of 20 x 10^6/ml. The spermatozoa were applied to each spot of a 4-well Hendley slide (CA Hendley, Essex, UK) and air dried at 37°C. Each spot was overlaid with 2mg lectin-fluorescein isothiocyanate/ml PBS (from *Arachis hypogaea* [peanut]; Sigma, Poole, Dorset, UK) and incubated at 4°C for 15 minutes. The excess lectin was washed off using PBS and the slides mounted in Citifluor (Citifluor Ltd, London, UK). The slides were examined using a fluorescent microscope. Scoring criteria were as follows. The spermatozoon was classified as acrosome reacted if only the equatorial segment was fluorescent or no fluorescence was visible on the sperm head. However if the equatorial segment was fluorescent and the acrosomal region had either patchy or complete fluorescence, the spermatozoon was classified as not acrosome reacted. Only spermatozoa with curly tails were classified (those which were alive and therefore represented 'true' acrosome reactions). At least 200 spermatozoa were counted and categorized for each treatment.

7.2.3 Zona Binding Assay

The effect of the antibody solutions on the interaction between spermatozoa and the zona pellucida was assessed using salt-stored human eggs (Yanagimachi *et al*, 1979). The assistance of Professor John Aitken and Ms Donna Buckingham in performing this assay is gratefully acknowledged.

Sperm Preparation Prepared 100% Percoll fraction spermatozoa (at 20 x 10^6/ml; Section 3.2.1) were diluted by adding an equal volume of antibody solution or control and incubated at 37°C with 5% CO_2 for 1-2 hours. They were then washed by centrifugation at

500 x g for 5 minutes and resuspended in BWW at a concentration of 10 x 10^6/ml before being placed as discrete drops under liquid paraffin at 37°C in small petri dishes.

Oocyte Preparation Unfertilized human oocytes from *in vitro* fertilization (IVF) cycles were obtained from the IVF programme at Kings College Hospital, London; the Department of Obstetrics and Gynaecology, University of Cambridge and the Bourn Hall Clinic, Bourn, Cambridge. They were stored in 1.5M $MgCl_2$ in 0.1% dextran. Salt stored human ova were isolated and washed thoroughly in BWW medium to ensure that contamination with spermatozoa from the IVF procedures was minimal. Three or four ova were added to each drop of antibody/spermatozoa mixture and incubated at 37°C for 2-3 hours. The eggs were washed thoroughly in BWW to remove any spermatozoa not bound to the zona pellucida before being fixed and stained in 0.2mg/ml H33258, 0.5% gluteraldehyde for 15 minutes. After washing the oocytes in BWW, they were mounted on a slide under a coverslip supported at the corners by a mixture of paraffin, vaseline and beeswax, (1:10:0.5) and examined under ultraviolet light. The number of spermatozoa bound to the zona pellucida were counted using a Hamilton-Thorn Motility Analyzer (Hamilton-Thorn Research, Danvers, MA, USA).

7.2.4 Hamster Egg Penetration Test

The effect of the antibody solutions on the interaction between spermatozoa and the oolemma was assessed using the hamster egg penetration test (HEPT; Yanagimachi *et al*, 1976). The assistance of Professor John Aitken and Ms Donna Buckingham in performing this test is gratefully acknowledged.

Sperm Preparation Spermatozoa from the 100% Percoll fraction (Section 3.2.1) were used and for each antibody solution or control, two tubes were prepared. Both contained a 1:1 dilution of spermatozoa and antibody but only one contained the ionophore A23187 (free acid; Calbiochem) at a final concentration of 1.25μM. The tubes were incubated at 37°C

with 5% CO_2 for 3 hours. A small aliquot was removed and used to assess the state of the acrosome reaction (Section 7.2.2) and the motility of the sperm population. The remaining spermatozoa were washed by centrifugation at 500 x g for 5 minutes and resuspended in BWW at a concentration of 10 x 10^6/ml. The sperm suspension was placed in discrete 50µl droplets under liquid paraffin ready for the addition of zona free hamster oocytes.

Egg Preparation Zona free hamster oocytes were prepared using conventional procedures as described by Yanagimachi *et al* (1976) and Aitken *et al* (1982a). Female Syrian hamsters (*Mesocricetus auratus*) were injected in the morning of day one of the oestrous cycle with 80 International Units (IU) pregnant mare's serum (PMS; Folligon; Intervet Laboratories, Cambridge, UK), in the evening of day three (56 hours later) with 80IU of human chorionic gonadotrophin (hCG; Gonoadotrophon LH; Paines and Byrne Ltd, Greenford, UK) and the eggs recovered the following day in the early afternoon. The hamsters were sacrificed, the fallopian tubes were dissected out and the cumulus mass containing ova was freed into BWW. Cumulus cells were removed by incubation in hyaluronidase (0.1% in BWW) for 5 minutes. The eggs were washed three times in BWW and then transferred to trypsin (0.1% in BWW) for 1-2 minutes to remove the zona pellucida. The oocytes were washed three times in BWW to ensure efficient removal of the granulosa cells, then placed in the droplets of spermatozoa under liquid paraffin. The dishes containing the zona free hamster eggs and the human spermatozoa were incubated at 37°C in 5% CO_2 for 3 hours. The eggs were washed free of any loosely attached spermatozoa and then compressed gently on slides to a depth of about 30µm under 22 x 22mm coverslips supported at the corners by a paraffin, vaseline and beeswax mixture. The slides were examined by phase contrast microscopy for the presence of any decondensed sperm heads with adjacent or closely associated sperm tails. The percentage of eggs which had spermatozoa within

their cytoplasm and the mean number of incorporated spermatozoa per oocyte were calculated.

7.2.5 Data Analysis

The percentage of cells acrosome reacted in each treated population as compared to the control was analysed using the chi-square test (Lewis & Burke, 1949; Cochran, 1952, 1954). The percentage of eggs penetrated in the HEPT was also analysed using the chi-square test whereas the actual number of spermatozoa penetrating each egg was analysed using the Mann-Whitney *U* test (Mann & Whitney, 1947). The number of spermatozoa bound per mm^2 of zona pellucida was also analysed using the Mann-Whitney *U* test. The significance level was set at $p \leq 0.05$ (Siegel, 1956).

7.3 Results

7.3.1 Agglutination

The ability of the purified monoclonal antibody solutions to cause sperm samples to agglutinate was assessed and the results are presented in Table 7.1. All twelve antibodies caused some degree of mixed agglutination causing spermatozoa to clump together via their heads and tails. Five antibodies in particular (spm 4/2, spm 4/4, spm 4/6, spm 4/7 and spm 4/8) were strong agglutinators.

7.3.2 Acrosome Reaction

The effect of the monoclonal antibodies on the acrosome reaction was tested on three separate occasions in a blind manner and the results from all three experiments pooled before data analysis was performed. The results of the tests performed without ionophore are shown in Figure 7.1a and those performed in the presence of ionophore

Antibody	Agglutination
spm 1/1	+
spm 1/2	+
spm 2/1	+
spm 2/2	(+)
spm 4/1	+
spm 4/2	++
spm 4/3	(+)
spm 4/4	++
spm 4/5	+
spm 4/6	++
spm 4/7	++
spm 4/8	++

Table 7.1 Summary of effect of monoclonal antibodies on the agglutination of spermatozoa. Different degrees of agglutination are indicated (+), weak agglutination; +, agglutination; ++, strong agglutination.

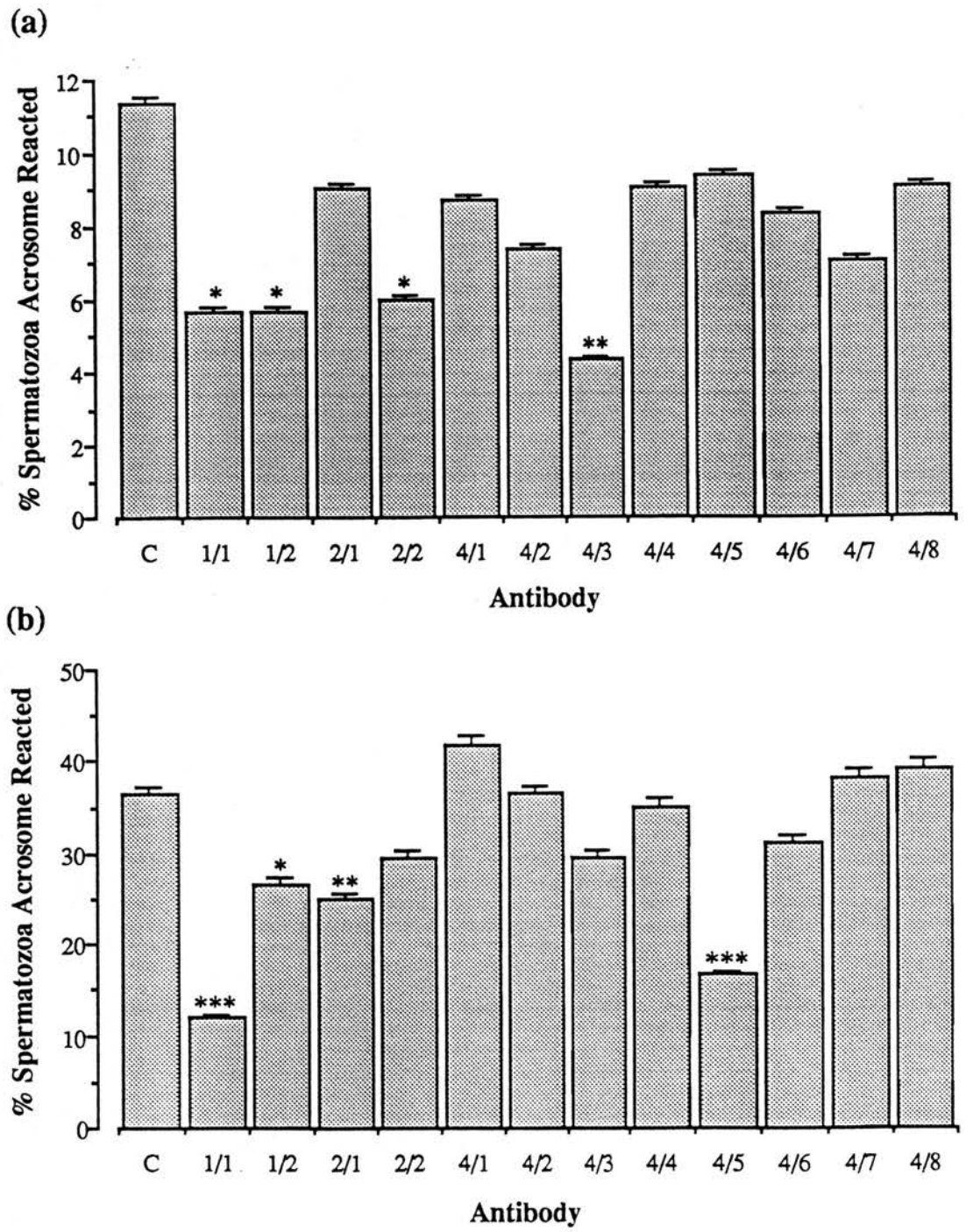

Figure 7.1 Effect of monoclonal antibodies on the percentage of acrosome reacted spermatozoa in a population (± standard error). The test was performed in the absence (a) or presence (b) of the ionophore A23187. Significance levels were determined by chi-square analysis — *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

are shown in Figure 7.1b. Two of the antibodies, namely spm 1/1 and spm 1/2, caused significant decreases in the number of acrosome-reacted spermatozoa both in the absence and presence of A23187. Four further antibodies caused significant decreases in the number of spermatozoa acrosome reacted only in the absence (spm 2/2 and spm 4/3) or presence (spm 2/1 and spm 4/5) of ionophore.

7.3.3 Zona Binding Assay

The influence of the monoclonal antibodies on the interaction between spermatozoa and the zona pellucida was assessed using salt-stored human oocytes. The results are detailed in Table 7.2 and depicted graphically in Figure 7.2. Two of the antibodies, spm 1/2 and spm 4/6, caused significant decreases in the mean number of spermatozoa binding per mm^2 of zona resulting in binding of only 35-40% of the number of spermatozoa bound to control zonae. The other ten antibodies had no effect on the sperm-zona interaction.

7.3.4 Hamster Egg Penetration Test

The effect of the different monoclonal antibodies on the sperm-oocyte interaction was assessed using the hamster egg penetration test (HEPT) and the results are summarized in Tables 7.3 (percentage of eggs penetrated) and 7.4 (mean number of spermatozoa penetrating each egg). The tests were carried out in the presence and absence of the ionophore A23187 in order to provide conditions under which both inhibitory and stimulatory antibodies could be identified.

Antibody spm 4/4 caused a marked decrease in the number of spermatozoa penetrating each egg in the presence of A23187 but a corresponding decrease in the penetration rate was not seen. This is indicative of the fact that only one spermatozoon is required to penetrate each egg for a maximal penetration rate of 100% to be achieved.

Antibody	Control	Treated
spm 1/1	29.7±3.4 (10)	23.2±5.8 (7)
spm 1/2	26.8±3.9 (11)	10.9±3.7 (8)[a]
spm 2/1	29.7±3.4 (10)	28.9±1.3 (8)
spm 2/2	29.7±3.4 (10)	28.7±3.2 (9)
spm 4/1	29.7±3.4 (10)	31.4±1.8 (9)
spm 4/2	29.7±3.4 (10)	28.5±2.5 (8)
spm 4/3	11.6±5.4 (8)	19.4±3.2 (8)
spm 4/4	11.6±5.4 (8)	13.1±5.1 (7)
spm 4/5	11.6±5.4 (8)	16.6±5.7 (9)
spm 4/6	26.8±3.9 (11)	9.4±3.4 (7)[a]
spm 4/7	11.6±5.4 (8)	8.6±3.9 (8)
spm 4/8	11.6±5.4 (8)	8.9±3.1 (8)

Table 7.2 Pooled results of the effect of monoclonal antibodies on the interaction between spermatozoa and the zona pellucida. Values are expressed as the mean number of spermatozoa bound x 10^3 per mm^2 of zona ± standard deviation and the number of zonae tested is shown in brackets. Significance levels were determined using the Mann-Whitney *U* test and indicated a, $p < 0.001$.

The area of the zona pelludica shown on the screen of the Hamilton Thorn Motility Analyser (HTMA) was estimated using the formula for the area of a circle, πr^2. The microscope diaphragm was reduced to produce a circle which had a diameter of 80mm on the HTMA screen. Using a micrometer, it was found that 100μm on the microscope stage corresponded to 65mm on the screen of the HTMA. Thus the zona pellucida viewed down the microscope corresponded to a circle of diameter 80 x 0.1/65 = 0.12mm with an area of $0.011mm^2$. The number of spermatozoa counted by the HTMA was therefore equivalent to the number bound per $0.011mm^2$ of zona pellucida. The results were expressed as the number of spermatozoa bound x 10^3 per mm^2 of zona pellucida.

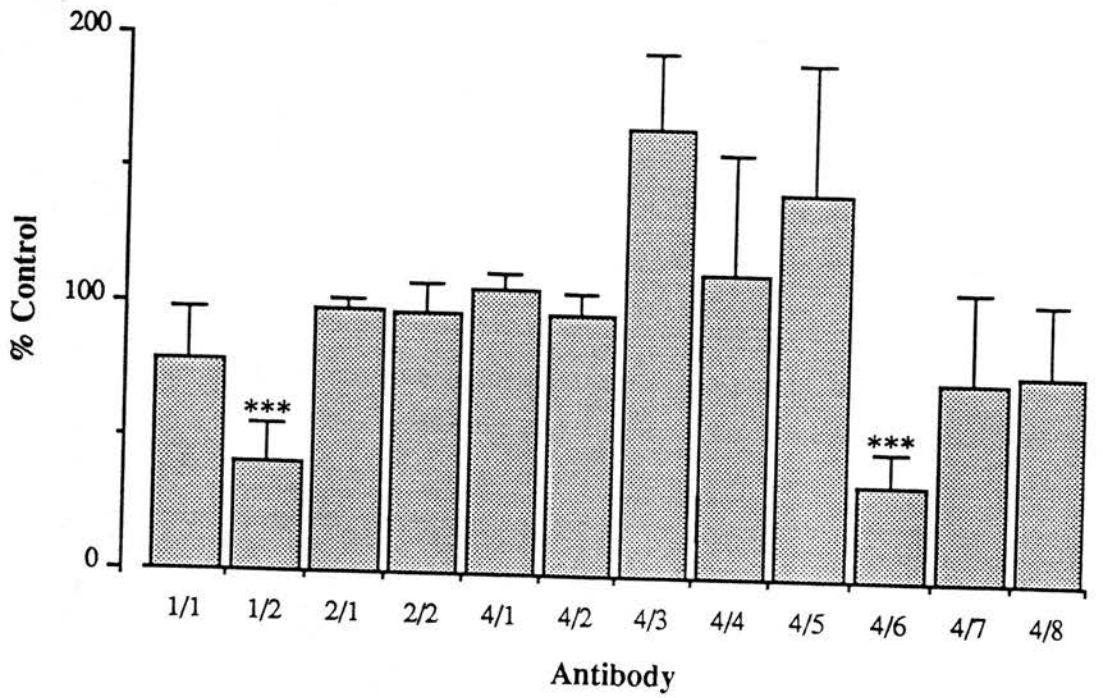

Figure 7.2 Pooled results of the effect of monoclonal antibodies on the mean number of spermatozoa bound to the zona pellucida. Values are expressed as a percentage of the control value (± standard deviation). Significance levels were determined using the Mann-Whitney *U* test and indicated ***, $p < 0.001$.

	- A23187		+ A23187	
	Control	**Treated**	**Control**	**Treated**
spm 1/1	17.9% (28)	69.2% (13)[b]	100.0% (8)	100.0% (8)
spm 1/2	20.7% (116)	30.4% (69)	100.0% (63)	41.0% (83)[c]
spm 2/1	0.0% (26)	5.6% (18)	100.0% (6)	100.0% (6)
spm 2/2	0.0% (26)	13.3% (15)	100.0% (6)	100.0% (6)
spm 4/1	0.0% (26)	0.0% (22)	100.0% (6)	100.0% (6)
spm 4/2	42.1% (19)	38.9% (18)	100.0% (6)	100.0% (6)
spm 4/3	42.1% (19)	35.0% (20)	100.0% (6)	100.0% (6)
spm 4/4	0.0% (24)	7.1% (14)	100.0% (6)	100.0% (6)
spm 4/5	0.0% (24)	4.8% (21)	100.0% (6)	100.0% (4)
spm 4/6	9.8% (82)	17.6% (51)	100.0% (59)	36.1% (72)[c]
spm 4/7	14.0% (50)	33.3% (51)[a]	100.0% (35)	100.0% (32)
spm 4/8	14.0% (50)	34.4% (32)	100.0% (35)	91.2% (34)

Table 7.3 Pooled data for the percentage of eggs penetrated in HEPT. The number of eggs analysed for each antibody is shown in brackets. Significance levels were determined by chi-square analysis — a, $p < 0.05$; b, $p < 0.01$; c, $p < 0.001$.

	- A23187		+ A23187	
	Control	**Treated**	**Control**	**Treated**
spm 1/1	0.21±0.50 (28)	1.31±1.44 (13)[a]	12.50±2.56 (8)	9.50±1.31 (8)[b]
spm 1/2	0.47±1.29 (116)	0.99±1.98 (69)	19.32±7.49 (63)	5.73±9.73 (83)[c]
spm 2/1	0.00±0.00 (26)	0.06±0.24 (18)	16.67±3.44 (6)	19.17±4.31 (6)
spm 2/2	0.00±0.00 (26)	0.13±0.35 (15)	16.67±3.44 (6)	16.50±1.87 (6)
spm 4/1	0.00±0.00 (26)	0.00±0.00 (22)	16.67±3.44 (6)	14.33±2.88 (6)
spm 4/2	0.53±0.70 (19)	0.39±0.50 (18)	23.50±5.21 (6)	21.33±2.16 (6)
spm 4/3	0.53±0.70 (19)	0.40±0.60 (20)	23.50±5.21 (6)	23.33±6.98 (6)
spm 4/4	0.00±0.00 (24)	0.07±0.27 (14)	15.50±1.38 (6)	9.50±2.88 (6)[b]
spm 4/5	0.00±0.00 (24)	0.05±0.22 (21)	15.50±1.38 (6)	16.00±4.55 (4)
spm 4/6	0.12±0.40 (82)	0.45±1.53 (51)	15.27±9.94 (59)	0.96±1.57 (72)[c]
spm 4/7	0.18±0.48 (50)	0.41±0.64 (51)[a]	15.00±11.70 (35)	21.31±12.91 (32)[b]
spm 4/8	0.18±0.48 (50)	0.41±0.62 (32)	15.00±11.70 (35)	8.94±5.95 (34)

Table 7.4 Pooled data for the mean number (± standard deviation) of spermatozoa penetrating per egg in HEPT. The number of eggs used to test each antibody is shown in brackets. Levels of significance were tested using the Mann-Whitney *U* Test and indicated a, $p < 0.05$; b, $p < 0.01$; c, $p < 0.001$.

Antibody spm 4/7 had a stimulatory effect on the percentage of eggs penetrated and the mean number of spermatozoa/egg when the test was performed without A23187 and the increase in the number of spermatozoa/egg was also seen when A23187 was present. No increase in the penetration rate was seen in the presence of ionophore as the control rate was already 100%. Interestingly one antibody, spm 1/1, showed a stimulatory effect on both penetration and the number of spermatozoa/egg in the absence of A23187 but an inhibitory effect in the presence of A23187. Two of the antibodies, spm 1/2 and spm 4/6, caused highly significant decreases in both the penetration rate and mean number of spermatozoa/egg when the test was performed in the presence of A23187. The ability of these two antibodies to block the sperm-oocyte interaction as assessed by HEPT with A23187, was investigated at decreasing sperm concentrations of 10×10^6/ml, 5×10^6/ml and 1×10^6/ml. The results of these experiments, expressed as the percentage of eggs penetrated and the mean number of spermatozoa penetrating each egg respectively, are shown in Table 7.5 and Figure 7.3 for spermatozoa treated with spm 1/2 and in Table 7.6 and Figure 7.4 for spm 4/6 treated spermatozoa. Both antibodies caused significant decreases in the number of spermatozoa penetrating each egg and concomitant dose-dependent decreases in the penetration rate. The other seven antibodies, namely spm 2/1, spm 2/2, spm 4/1, spm 4/2, spm 4/3, spm 4/5 and spm 4/8, exhibited no effect on the HEPT.

A summary of the effects of the monoclonal antibodies on the biological assays is provided in Table 7.8.

7.4 Discussion

The importance of the acrosome for several steps in the fertilization process is highlighted by the occurrence of infertile men whose spermatozoa have no acrosome, a

Sperm Concentration	Control	Treated
10 x 10^6	100% (33)	36.4% (45)[a]
5 x 10^6	100% (27)	11.4% (44)[a]
1 x 10^6	100% (25)	0.0% (41)[a]

Table 7.5 Pooled data for the percentage of eggs penetrated in HEPT at various sperm concentrations for control and spm 1/2 treated spermatozoa performed in the presence of A23187. The number of eggs analysed is shown in brackets. Significance levels were determined by chi-square analysis — a, $p < 0.001$.

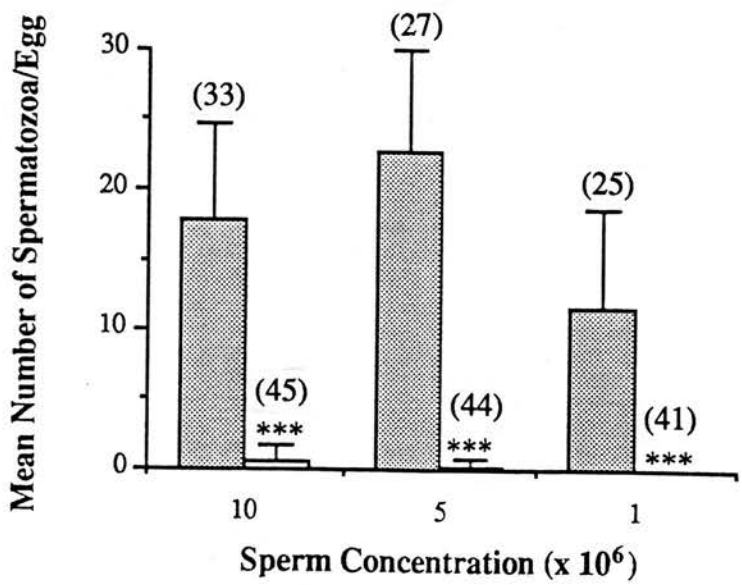

Figure 7.3 Pooled data for the mean number of spermatozoa penetrating each egg in HEPT performed with ionophore at various sperm concentrations in the presence or absence of monoclonal antibody spm 1/2. Significance levels were determined using the Mann-Whitney *U* test and are indicated ***, $p < 0.001$. Control spermatozoa are shown, ▨ and spm 1/2 treated spermatozoa, □ . The number of eggs analysed is shown in brackets.

Sperm Concentration	Control	Treated
10 x 10^6	100% (24)	21.2% (33)[a]
5 x 10^6	100% (25)	0.0% (28)[a]
1 x 10^6	100% (24)	0.0% (26)[a]

Table 7.6 Pooled data for the percentage of eggs penetrated in HEPT at various sperm concentrations for control and spm 4/6 treated spermatozoa both carried out in the presence of A23187. The number of eggs analysed is shown in brackets. Significance levels were determined by chi-square analysis — a, $p < 0.001$.

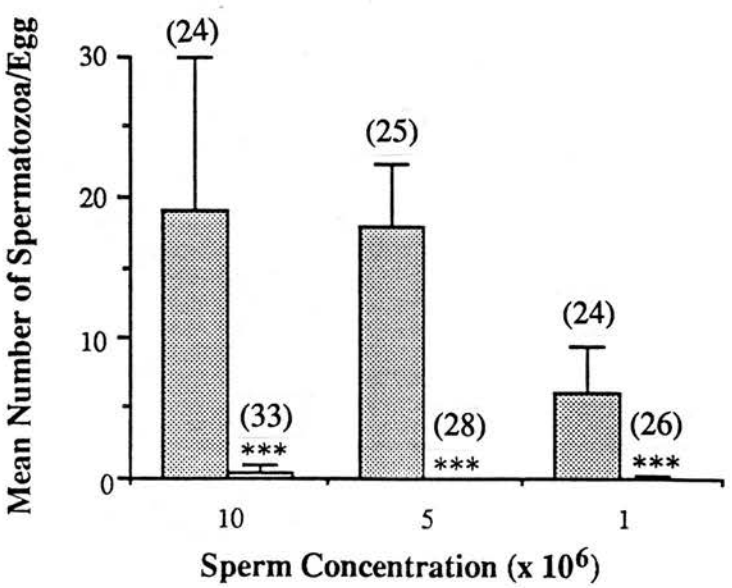

Figure 7.4 Pooled data for the mean number of spermatozoa penetrating each egg in HEPT performed with ionophore at various sperm concentrations in the presence or absence of monoclonal antibody spm 4/6. Significance levels were determined using the Mann-Whitney *U* test and are indicated ***, $p < 0.001$. Control spermatozoa are shown, ▨ and spm 4/6 treated spermatozoa, □. The number of eggs analysed is shown in brackets.

	- A23187				+ A23187		
		HEPT				HEPT	
	AR	pen	sp/egg	sp/ZP	AR	pen	sp/egg
spm 1/1	↓	↑↑	↑↑	-	↓↓↓	-	↓↓
spm 1/2	↓	-	-	↓↓↓	↓	↓↓↓	↓↓↓
spm 2/1	-	-	-	-	↓↓	-	-
spm 2/2	↓	-	-	-	-	-	-
spm 4/1	-	-	-	-	-	-	-
spm 4/2	-	-	-	-	-	-	-
spm 4/3	↓↓	-	-	-	-	-	-
spm 4/4	-	-	-	-	-	-	↓↓
spm 4/5	-	-	-	-	↓↓↓	-	-
spm 4/6	-	-	-	↓↓↓	-	↓↓↓	↓↓↓
spm 4/7	-	↑	↑	-	-	-	↑↑
spm 4/8	-	-	-	-	-	-	-

Table 7.7 Summary of results of biological assays. An increase is denoted by ↑, a decrease by ↓ and no significant change from control values by -. One arrow indicates a significant result of $p < 0.05$; two arrows, $p < 0.01$; three arrows, $p < 0.001$.

condition known as globozoospermia (Jeyendran *et al*, 1985; Aitken *et al*, 1990). The lack of an acrosome is incompatible with the participation of spermatozoa in the whole process of fertilization because a normal acrosome reaction has been shown to be a prerequisite for sperm penetration of the egg investments (Moore & Bedford, 1983; Katz *et al*, 1989a; von Bernhardi *et al*, 1990). Spermatozoa from globozoospermic men are therefore incapable of binding to the zona pellucida or fusing with the oolemma (Weissenberg *et al*, 1983; Syms *et al*, 1984; Lalonde *et al*, 1988; Aitken *et al*, 1990). The original definition of capacitation included all maturational changes which occurred in spermatozoa after they left the male reproductive tract and rendered them capable of penetrating eggs (Austin, 1952; Chang, 1984; Boatman & Robbins, 1991). This has gradually evolved and now capacitation is more commonly defined as the physiological changes which occur in spermatozoa and are prerequisites for the acrosome reaction (Yanagimachi, 1988b; Zinaman *et al*, 1989). However, with the possible exception of hyperactivated motility, no criteria for analysing capacitation and the acrosome reaction independently have been identified (Sidhu & Guraya, 1989) and the ability of spermatozoa to undergo the acrosome reaction is indicative of the fact that capacitation is complete.

Treatment of the spermatozoa with six of the monoclonal antibodies caused significant decreases in the percentage of spermatozoa which acrosome reacted. These six antibodies may therefore interfere with some process occurring either during the maturational changes that comprise capacitation or during the acrosome reaction itself. The fact that four of the antibodies affected the acrosome reaction under either spontaneous or inducing conditions but not both, highlights the differences between these two reactions. The use of A23187 leads to a progressive increase in cytoplasmic calcium and intracellular pH and a consequential increase in the proportion of cells undergoing the acrosome reaction (Aitken *et al*, 1984; Irvine & Aitken, 1986b).

Although the end result is the same, the mechanism of spontaneous and induced acrosome reactions may be different and differences have been identified in the acrosome reaction when induced naturally using follicular fluid or artificially using A23187 (Tesarik *et al*, 1990; Mahony *et al*, 1991b). Furthermore Leyton *et al* (1989) produced a monoclonal antibody, M42, raised against mouse spermatozoa which blocks the zona- and ZP3-induced acrosome reactions but not the ionophore induced reaction.

It is now thought that appropriate timing and location of the acrosome reaction is a requirement for successful fertilization (Wolf, 1989; Schill *et al*, 1988; Tesarik *et al*, 1990). Human spermatozoa can initiate binding to the zona pellucida after acrosome reacting (Morales *et al*, 1989) but only those which undergo this reaction at the zona pellucida seem to be capable of penetration (Tesarik *et al*, 1988; Tesarik, 1989). The human zona pellucida thus plays a significant role in the induction of the acrosome reaction (Cross *et al*, 1988; Aitken, 1989b). In the mouse, crosslinking of receptors on the sperm surface by one of the zona proteins, ZP3, has been shown to induce the acrosome reaction (Leyton & Saling, 1989). However the A23187- and zona pellucida-induced acrosome reaction are different in that the latter is induced on the zona pellucida and not in spermatozoa swimming free in medium (Liu & Baker, 1990) and thus a more physiological test of the ability of spermatozoa to acrosome react is by their ability to bind to and penetrate the zona pellucida.

The number of spermatozoa bound to the zona pellucida *in vitro* has been shown to correlate with the fertilization rate in IVF programmes (Kruger *et al*, 1986, 1988; Liu *et al*, 1988; Liu & Baker, 1988) and as such the number of spermatozoa bound to the zona is an important indicator of the fertilizing ability of spermatozoa (Liu *et al*, 1991). Two of the monoclonal antibodies, spm 1/2 and spm 4/6, caused significant decreases in the number of spermatozoa bound per mm^2 of zona surface

resulting in a level of binding that amounted to only 40% (spm 1/2) or 35% (spm 4/6) of the number bound to control zonae. This is similar to the degree of inhibition exhibited by another whole-sperm specific monoclonal antibody reported by Mahony *et al* (1991a) which inhibited zona binding by 70%. The mechanism by which the sperm-zona interaction was inhibited may have involved antigens that become expressed during sperm maturation, prior to this interaction (Trimmer *et al*, 1985; Saling, 1986). This may be the case for antibody spm 1/2 which also caused an inhibition of the acrosome reaction.

Although spermatozoa from proven fertile donors (men who have fathered a child within the last two years) were used for all the biological assays, there was still a degree of variation in the number of spermatozoa bound to the zonae on different test days. This was probably related to the different sperm samples used for the tests, as other workers have also identified the presence of significant inter-ejaculate variability in the functional competence of spermatozoa (Rogers *et al*, 1983; Irvine & Aitken, 1986a; Katz *et al*, 1989a). Oocyte quality may also influence the number of spermatozoa bound to the zona pellucida (Chen & Sathanathan, 1986; Mahadevan *et al*, 1987) but as pooled stored oocytes were used for each test and randomly assigned to control or antibody-treated sperm droplets, this variable should not have contributed to any significant differences found between the two sperm populations.

The influence of the monoclonal antibodies on sperm-oocyte fusion was assessed using the HEPT. The use of the calcium ionophore A23187 in this assay overcomes one of the major defects in this system in that it lacks the stimulus for the acrosome reaction which is normally delivered to the spermatozoon by the zona pellucida (Aitken *et al*, 1987b). Induction of the acrosome reaction using A23187 maximizes the number of spermatozoa fusing with the vitelline membrane often resulting in high penetration rates of 100% (Liu & Baker, 1990) and provides optimal

conditions under which antibodies exerting an inhibitory effect can be identified. Three of the antibodies exhibited significant inhibitions of HEPT only when the test was performed in the presence of A23187. Antibodies spm 1/2 and spm 4/6 caused decreases in both the penetration rate and the mean number of spermatozoa penetrating each egg whereas antibody spm 4/4 only caused a decrease in the number of spermatozoa/egg without a corresponding decrease in the penetration rate. This is consistent with the findings of Aitken & Elton (1984) who demonstrated that when penetration levels were high, the mean number of spermatozoa per egg rather than the penetration rate was a more sensitive measure of the fertilizing potential of sperm populations.

When the HEPT is performed in the absence of A23187, it relys on spontaneous acrosome reactions occurring in the sperm population to produce a fusogenic equatorial segment capable of initiating the sperm-oolemma interaction. Spontaneous acrosome reactions do not occur frequently (Mallet *et al*, 1985; Morales *et al*, 1988) and thus the penetration rate and mean number of spermatozoa penetrating each egg are low. This provides suitable conditions under which antibodies exerting a stimulatory effect can be identified. One such antibody is spm 4/7 which caused a significant increase in the penetration rate and mean number of spermatozoa/egg when the test was performed in the absence of A23187. This antibody also stimulated sperm-oocyte fusion in the presence of A23187 although a corresponding increase in the penetration rate was not seen as this was at its maximal 100% level. Spm 4/7 had no effect on the number of spermatozoa bound to the zona pellucida. However the sperm-zona binding assays were performed using spermatozoa from proven fertile men and therefore the number of spermatozoa attached to the zona would be nearly maximal. Although the zona bioassay provided a suitable test system for the presence of inhibitory antibodies, stimulatory antibodies would not be identified under such

conditions. This stimulatory effect of some antbodies on the HEPT has been reported previously. Bronson *et al* (1981) and Aitken *et al* (1987a) identified some sperm specific antibodies found in the sera of infertile people which stimulated sperm penetration of zona-free hamster oocytes. Subsequent publications from the former group have suggested that this increase in penetration is primarily exerted through the ability of the antibodies to increase the number of adherent spermatozoa via specific IgG Fc receptors (Fcγ receptors) on the vitelline membrane of zona-free hamster eggs or by immunoglobulin bridging if the antibody recognizes shared or cross-reacting epitopes on the plasma membranes of the gametes (Bronson *et al*, 1990a&b). It is interesting to note that spm 4/7 belongs to the IgM class of antibodies (Chapter 5) and would therefore not be expected to bind to IgG Fc receptors. Furthermore Fusi *et al* (1991) showed that control murine IgM did not interact with the Fcγ receptor on the hamster oolemma. An alternative mechanism of action for stimulatory antibodies was suggested by Aitken *et al* (1987a). This involved the stimulatory antibodies crosslinking receptor sites on the sperm surface thus activating the cell and initiating the biochemical events normally involved in the induction of membrane fusion. The ability of anti-receptor antibodies to mimic the action of the ligand upon binding subsequently leading to the activation of the cell, is well established (Schechter *et al*, 1979; Zick *et al*, 1984; Detmers *et al*, 1987; Ledbetter *et al*, 1987; O'Brien *et al*, 1987; Ikari *et al*, 1988). The recent discovery that aggregation of sperm receptors for the zona pellucida by one of the zona proteins, ZP3, is involved in the induction of the mouse sperm acrosome reaction (Leyton & Saling, 1989; Macek *et al*, 1991) coupled with the knowledge that as an IgM antibody, spm 4/7 is capable of receptor crosslinking, makes this an exciting possibility.

Although millions of spermatozoa are present in a normal ejaculate, only tens to hundreds can be found at any one time within the fallopian tube (Hafez, 1978;

Bronson *et al*, 1984; Chretien, 1989; Barratt & Cooke, 1991) and thus assessing the ability of the antibodies to block sperm-oocyte fusion in the presence of 10 x 10^6 spermatozoa/ml (equivalent to 1 x 10^6 spermatozoa in the test droplet) is rather an unnatural situation. It has been shown that the amount of antigen represented as the number of spermatozoa present in an assay, is highly critical in producing a positive or negative result (Dor *et al*, 1981) and there is a tendency for decreased penetration rates to result when decreased sperm densities are used (Overstreet *et al*, 1980; Tyler *et al*, 1981; Aitken, 1989a). However the densities used in the HEPT droplets are still in excess of those found at the natural site of fertilization in the ampulla of the fallopian tube (Hafez, 1978). Therefore the effect of the two antibodies spm 1/2 and spm 4/6, which showed the most marked inhibition of penetration and decrease in the number of penetrating spermatozoa per egg in the HEPT performed in the presence of A23187, were further analysed for their ability to exert this effect at decreasing sperm concentrations of 10 x 10^6/ml, 5 x 10^6/ml and 1 x 10^6/ml. Results of HEPT tests are often expressed in terms of the percentage of eggs penetrated and as such are correlated to the fertilizing potential of spermatozoa *in vitro* but the results can also be expressed in terms of the mean number of spermatozoa penetrating each oocyte and this is an even better predictor of fertility (Irvine & Aitken, 1986a). When penetration rates are high, the mean number of spermatozoa/egg rather than the penetration rate yields more information on the fertilizing ability of sperm populations (Aitken & Elton, 1984, 1986) and is demonstrated by the data presented here in that the value obtained for the number of spermatozoa penetrating each egg is more sensitive than the penetration rate to variations in the concentration of spermatozoa. No difference in the penetration rate was seen for untreated control spermatozoa at all three concentrations as this was at its maximal 100% level but the mean number of spermatozoa penetrating each egg did vary. It was similar for sperm concentrations of 10 x 10^6/ml and 5 x 10^6/ml but was

reduced at 1 x 10^6 spermatozoa/ml (Figures 7.3 & 7.4). This is consistent with previous data published by Tyler *et al* (1981) who also found that the results of HEPT varied with sperm concentration and that there was a marked decline in the penetration rate if the density of spermatozoa in the test droplet fell below 1 x 10^6/ml. This is because as the number of spermatozoa present in the assay droplet decreases, the frequency of sperm-oocyte collisions also decreases (Aitken, 1989a) and consequently the mean number of spermatozoa penetrating each egg and penetration rate also fall. At all three concentrations, antibody-treated spermatozoa showed both a significant inhibition of penetration and a marked decrease in the number of spermatozoa penetrating each egg. This may be due to there being less antigen present for the antibody to bind to at decreasing sperm concentrations and therefore a higher percentage of spermatozoa would be bound by antibody. Thus more spermatozoa are prevented from binding to and fusing with the vitelline membrane by either a direct or indirect block of the sperm component which binds to the oolemma. Alternatively at decreased sperm concentrations more of the spermatozoa may be agglutinated and less are therefore free to interact with the egg plasma membrane. It has previously been shown that it is the concentration of motile spermatozoa which is most important in determining HEPT results and below a concentration of 0.4 x 10^6 motile spermatozoa/ml, the penetration rate is greatly reduced (Aitken *et al*, 1982a; Barros *et al*, 1988). Thus if more spermatozoa are agglutinated, less are free and able to bind to and penetrate the oocyte. Both spm 1/2 and spm 4/6 caused some degree of sperm agglutination but the fact that other monoclonal antibodies tested also caused a similar degree of agglutination without a corresponding decrease in penetration or number of spermatozoa/egg (eg spm 2/1 and spm 4/2), indicates that the inhibitory effects of these antibodies cannot be due to their agglutinating activity alone.

Some monoclonal antibodies do not cause agglutination and this is thought to be due to a relatively low density of the antigen on the surface of the spermatozoa (Riedel *et al*, 1990). As all twelve of the monoclonal antibodies recognized components of the sperm surface which covered the whole surface and were not restricted to any particular domain (Chapter 6), it is not surprising that they all caused some degree of sperm agglutination. It has previously been shown that sperm agglutinating antibodies can result in a decrease in fertilization by interfering with a number of reproductive functions (Alexander, 1984; Menge *et al*, 1984) and thus any inhibition of sperm-zona pellucida or sperm-oolemma binding could be an indirect result of the agglutinating property of the antibody rather than a direct effect on the sperm surface molecule involved in that process (Moore & Hartmann, 1984; Mahony *et al*, 1991a). Furthermore Dor *et al* (1981) showed that the presence of sperm agglutinating antibodies in serum correlated with the interference of sperm fertilizing ability as assessed by the HEPT although they did not determine whether this effect was primarily due to the agglutinating properties of the antibody or the masking (directly or indirectly) of important sperm components. Thus as multivalent antibodies can exhibit non-specific actions due to their agglutination properties (Metz, 1972), care must be taken in the interpretation of inhibitory results of tests which assess the effect of these antibodies. The production of univalent Fab fragments by papain digestion and their subsequent use in the assays would resolve this problem allowing determination of whether agglutination and/or steric hindrance were factors which played a role in the inhibition (Menge & Black, 1979; Tzartos, 1979; Moore, 1981; Huang *et al*, 1981b; Mahony *et al*, 1991a). Although Fab preparations of the antibodies reported here were not made, this would be the next logical step to further characterize the antibodies showing inhibition of sperm-zona and sperm-oolemma binding, particularly spm 1/2 and spm 4/6.

The results of many sperm function tests often correlate because similar functions are measured by these tests (Liu *et al*, 1988). Sperm capacitation and a subsequent acrosome reaction are thought to be required for successful results in both the zona-binding assay and the HEPT (Overstreet *et al*, 1980; Yanagimachi, 1981) and thus antibodies interfering with these processes should be detected by both systems. However the zona-binding assay and HEPT do not measure exactly the same events occurring prior to fertilization and Bronson *et al* (1982) reported that antibodies previously shown to promote penetration of zona-free hamster eggs, blocked sperm-zona interaction indicating that antibodies can have differing effects on these two systems. Four monoclonals caused decreases in the percentage of spermatozoa acrosome reacted without exhibiting biological activity in any of the other tests. This may reflect the stringent conditions under which the sperm-zona binding assays and HEPT were performed as the number of spermatozoa used for each assay was in vast excess with respect to the natural situation. Also several workers have now suggested that spermatozoa displaying spontaneous acrosomal loss are inherently inferior defective spermatozoa whereas fertile spermatozoa are included in the unreacted population which respond to A23187 (Fukunda *et al*, 1989; Stock & Fraser, 1989; White *et al*, 1990; Fenichel *et al*, 1991; Cummins *et al*, 1991). Furthermore proven fertile donors have been shown to display a low spontaneous (5-10%) and a high inducible (15-70%) acrosomal loss (Tesarik, 1989; Stock & Fraser, 1989; Cummins *et al*, 1991; Fenichel *et al*, 1991; Ford *et al*, 1991) although a high degree of donor to donor variation in the number of spermatozoa exhibiting spontaneous or A23187-induced acrosome loss has been identified (Tesarik, 1985; Kallajoki *et al*, 1986b; Stock & Fraser, 1989). However spontaneously acrosome-reacted spermatozoa are still functionally competent and can fertilize zona-free eggs and produce healthy young when transferred to pseudopregnant mothers (Naito *et al*,

1992). Topfer-Petersen *et al* (1985) reported that infertile men with polyzoospermia (an increased number of spermatozoa in their ejaculate, usually >350 x 10^6/ml) have an abnormally high rate of spontaneous acrosome reactions and suggested that this contributed to their infertility. Thus the significance of the acrosome reaction measured *in vitro* in predicting the fertilizing ability of spermatozoa is still in dispute and has not clearly been demonstrated (Braun *et al*, 1991). Therefore antibodies affecting the number of spontaneously occuring acrosome reactions would not neccessarily be expected to show biological activity in the other tests of sperm function.

Intriguingly one antibody, spm 1/1, inhibited both spontaneous and A23187-induced acrosome reactions and the HEPT when performed in the presence of ionophore, but appeared to have a slight stimulatory effect on the HEPT in the absence of A23187. This stimulation of the HEPT is thought to be spurious as the non-specific stimulation of sperm-egg fusion by antibody coated spermatozoa has been previously reported (Aitken *et al*, 1987a; Bronson *et al*, 1990b). Thus this antibody is thought to be essentially an inhibitory antibody.

Although human sperm fusion to zona-free hamster oocytes occurs in a similar fashion to fusion with human oocytes in that it is initiated by the plasma membrane overlying the equatorial region (Koehler *et al*, 1982), it is still a heterologous system. Thus antibodies inhibiting sperm-oocyte fusion in the HEPT may not necessarily inhibit this interaction in the homologous situation. Although many antibodies which have been reported to inhibit the heterologous system have subsequently been shown to inhibit the homologous situation, occasionally this has not been so. Primakoff & Hyatt (1986) reported on a monoclonal antibody which was raised against guinea pig spermatozoa which inhibited the fusion of guinea pig spermatozoa to zona-free hamster oocytes in the HEPT. However this antibody did not block sperm-oocyte fusion when it was tested in the homologous situation using zona-free guinea pig eggs. This

indicates that some aspect of the fusion mechanism is different in the two systems and care should be taken not to extrapolate HEPT results to include the homologous system. Testing the homologous situation for the monoclonal antibodies reported here would require the use of mature living human ova with all the ethical and legal problems that this would entail. Such experiments have not been carried out but would be a necessary progression of the work once agglutination and steric hindrance factors had been eliminated.

7.5 Summary

Several *in vitro* tests of sperm function were used to assess the effects of the monoclonal antibodies on various processes involved in fertilization and a summary of the results is presented in Table 7.8. Antibodies spm 1/2, spm 4/6 and to a lesser extent spm 4/4 had inhibitory effects on the HEPT and the first two also inhibited sperm-zona pellucida binding. Monoclonal antibody spm 1/2 also decreased the number of spermatozoa in a population undergoing the acrosome reaction. Four further antibodies inhibited the acrosome reaction — antibodies spm 2/1 and spm 4/5 inhibited the A23187-induced acrosome reaction whilst antibodies spm 2/2 and spm 4/3 inhibited only the spontaneous acrosome reaction — but did not display any biological activity in the other tests of sperm function. One monoclonal antibody, spm 4/7, had a stimulatory effect on the HEPT.

8 Molecular Characterization of Sperm Antigen Recognized by Monoclonal Antibody spm 1/2

8.1 Introduction

The study of fertilization played a part in the discovery of chromosomes and DNA as the genetic material. In the 1850's, Rudolf Virchow recognized that the fertilized egg was the 'mother cell' of all the cells that make up an organism (Stent & Callendar, 1978). This led to the anomaly that although both spermatozoon and egg made equal hereditary contributions to the conceptus, their sizes were very disparate. It was noted that the egg contained an enormous amount of cytoplasm yet the spermatozoon was almost devoid of it and this led to the inference that the nucleus which was approximately the same size in both cells, was the component that contained the factors responsible for cellular heredity. Subsequently by the 1880's it had been discovered that the nuclei of the spermatozoon and egg contained an equal number of chromosomes which conferred hereditary material. Soon after this, Weissmann proposed a theory of heredity and development including the concept of a linear arrangement of hereditary units (genes) along the chromosomal threads (Weissmann, 1891-1892). The rediscovery of Mendel's classic genetics paper (Mendel, 1866) by Correns in 1900, led to intensive study of heredity and the birth of genetics as a discipline. It was not until 1944 that Avery, MacLeod and McCarty (1944) discovered that genes are embodied in deoxyribonucleic acid (DNA) and not until the 1950's was this generally accepted (Stent & Callender, 1978). The era of molecular genetics in which the DNA nucleotide was studied as the fundamental unit, had dawned. The elucidation of the double helix structure of DNA by Watson and Crick (1953) was quickly followed by the formulation of the hypothesis that the DNA nucleotide base

sequences specify the amino acid sequence in the corresponding peptide and that some sort of genetic code must exist (1953 Cold Spring Harbor Symposium; Stent & Callendar, 1978). In 1961, Crick *et al* provided evidence for a non-overlapping triplet code and it was finally broken by Nirenberg & Matthaei (1961) and Lengyel *et al* (1961).

Since then progress has been rapid and a range of powerful techniques have been developed. Using these techniques, genes for all types of proteins can be purified, sequenced, changed at will, reintroduced into individual cells of all kinds and expressed there as proteins (Darnell *et al*, 1990). Recombinant DNA technology has become an invaluable tool for research workers in a wide variety of disciplines. This is particularly true in fields such as reproductive biology where starting materials for identification and purification are limited. Indeed the techniques available are so efficient and relatively simple to perform that it is often easier (and quicker) to clone the gene for a protein, sequence it and predict the amino acid sequence of the corresponding protein than to purify the protein and sequence it directly.

The discovery by Campbell in 1971 that the central third of the bacteriophage lambda (λ) genome is not essential for lytic growth led to its development as a cloning vector and a large number of λ vectors have since been developed (Williams & Blattner, 1980; Sambrook *et al*, 1989). Vectors that have a single target site for insertion of foreign DNA are known as 'insertion' vectors and those that contain two sites flanking a segment of non-essential DNA that can be replaced by foreign DNA are known as 'replacement' vectors (Sambrook *et al*, 1989). One such insertion vector is λgt11 (Huynh *et al*, 1985) which carries a portion of the *E. coli* β-galactosidase gene which includes the upstream elements essential for its expression. Within the carboxy-terminal coding region of this gene is a single *Eco*R1 site into which foreign DNA can be inserted. In appropriate host cells, expression of the chimeric gene can be induced using isopropyl-1-thio-β-D-galactoside (IPTG) and a fusion protein

synthesized (Young & Davis, 1983a). Expression of the intact β-galactosidase gene results in dark blue plaques when the phage are plated in the presence of the chromogenic substrate 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal). When this gene is disrupted by the insertion of foreign DNA, the recombinant phage form colourless plaques in the presence of X-gal and thus can be easily identified (Miller, 1972; Sambrook *et al*, 1989). The fusion protein consists of the amino-terminal portion of β-galactosidase fused to the sequence encoded by the downstream open reading frame. Thus cDNA libraries constructed in the expression vector λgt11 may be screened immunologically for expression of specific antigens in *E. coli* (Young & Davis, 1983b). This technique was utilized to isolate the gene encoding the antigen recognized by monoclonal antibody spm 1/2.

8.2 Materials and Methods

8.2.1 Screening of Library

A commercially available human testicular cDNA library cloned into the expression vector, lambda gt11 (λgt11; Clontech, Cambridge, UK) was screened using the anti-human sperm plasma membrane monoclonal antibody spm 1/2 described previously in this thesis. A diagrammatic overview of the screening protocol is provided in Figure 8.1.

A single white colony of streaked out *E. coli* Y1090 from an LB plate (12.5g Luria broth base and 7.5g agar, both Gibco, Paisley, Scotland, UK; in 500mls water) containing 50μg/ml ampicillin (Penbritin; Beecham Research Laboratories, Bradford, UK), 30μg/ml 5-bromo-4-chloro-3-indolyl-ß-D-galactoside (X-gal; Gibco) and 30μg/ml isopropyl-1-thio-ß-D-galactoside (IPTG; Gibco), was grown up overnight in LB broth (12.5g Luria broth base in 500mls water) containing 0.2% maltose at 37°C with good aeration. For each 82mm plate, a 0.2ml aliquot of the Y1090 overnight

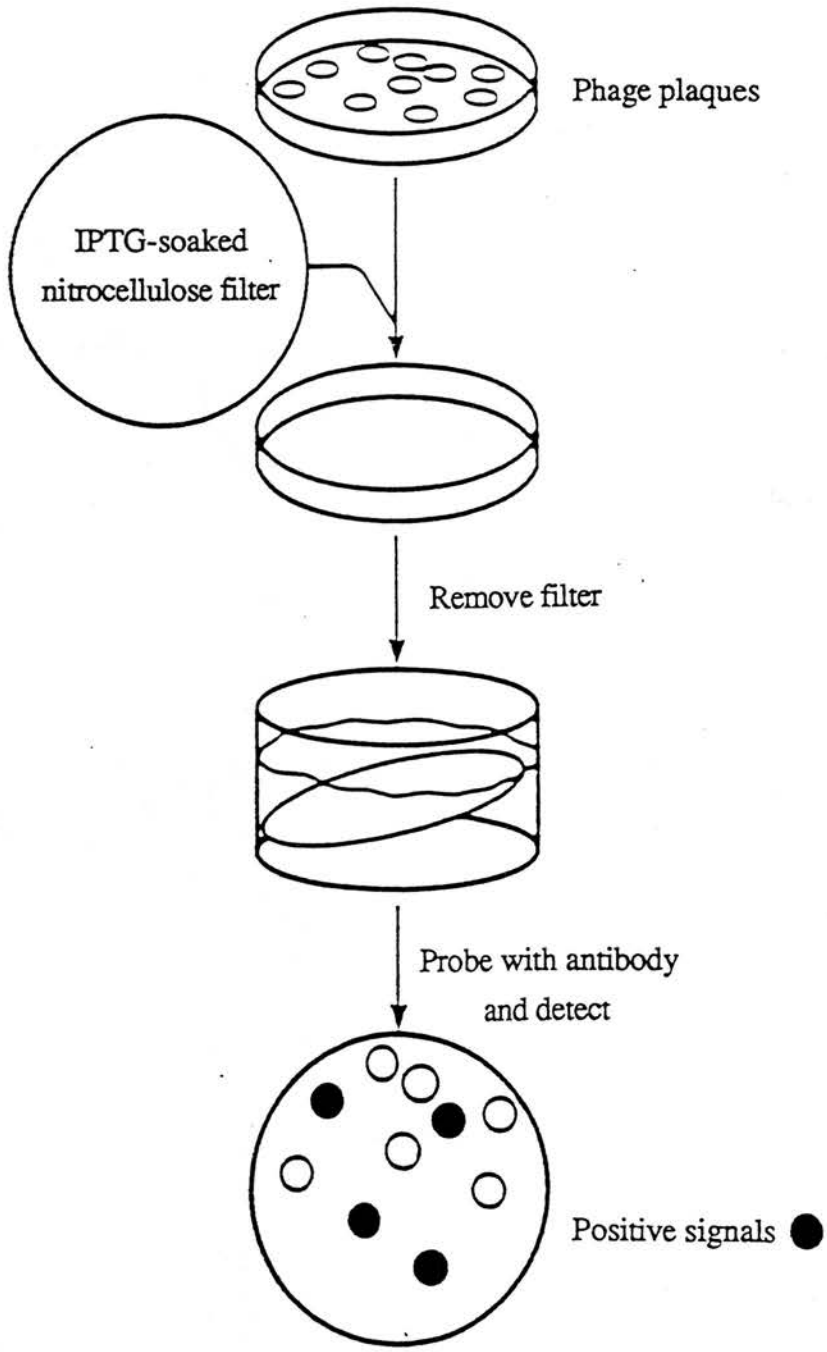

Figure 8.1 Diagrammatic representation of λgt11 cDNA library screening method. See text for details.

culture was mixed with 0.1ml of lambda diluent (10mM tris-HCl, pH7.5, 10mM $MgCl_2$, 0.1mM EDTA) containing 3 x 10^4 plaque forming units (pfu)/ml. The phage were allowed to adsorb to the cells at 37°C for 15 minutes. The cells were then plated onto an LB plate using 2.5mls LB top agar (12.5g Luria broth base and 3.6g agar in 500mls water) containing 560µg/ml X-gal. The plates were incubated at 42°C for 3.5 hours and then overlaid with a dry nitrocellulose filter (Hybond C-super; Amersham, Aylesbury, Bucks, UK) which had previously been saturated in 10mM IPTG. This induced expression of the β-galactosidase gene. After incubating the plates for a further 3.5 hours at 37°C, the first filter was removed and replaced with a second filter. The plates were again incubated for 3.5 hours at 37°C after which the second filter was removed. Phage plaques producing recombinant protein recognized by antibody spm 1/2 were identified as follows. The filters were blocked in 5% (w/v) dried milk (Marvel; Premier Brands, Knighton, Adbaston, Stafford, UK) in Tris buffered saline (TBS; 20mM Tris, 150mM NaCl) containing 0.05% (v/v) tween-20 for 1 hour. The filters were washed three times for 10 minutes in TBS-tween and incubated with the first antibody (diluted in TBS-tween) overnight at 4°C. Once again the filters were washed three times in TBS-tween and then incubated for 2-3 hours at room temperature with the second antibody, sheep anti-mouse immunoglobulin conjugated to horseradish peroxidase (Amersham) diluted 1/1000 in 5% (w/v) dried milk in TBS-tween. After the filters were washed three times in TBS-tween, positive plaques were identified using an enhanced chemiluminescence (ECL) detection system (Amersham).

8.2.2 Preparation of Lambda Lysates

In order to purify phage DNA, a phage lysate was prepared using a liquid culture method. A single well-isolated phage plaque was picked from an agar plate and placed in 100µl of phage buffer (20mM Tris-HCl, pH7.4, 100mM NaCl, 10mM $MgSO_4$). This was left overnight at 4°C for the phage to elute. A fresh culture of *E. Coli* strain

Y1090 was started by innoculating a single colony into 5mls of LB medium supplemented with 50µl of 1M $MgSO_4$ and 50µl of 20% (w/v) maltose and shaken (2 400 rpm) overnight at 37°C. The next day, 500µl of the overnight culture was added to the phage elution and incubated at 37°C for 20 minutes to allow the phage to adsorb to the bacteria. This was then used to innoculate 100mls of prewarmed LB medium (37°C) which was supplemented with 1ml of 1M $MgSO_4$. This was shaken (2 400 rpm) at 37°C until lysis occurred. If cell lysis had not occurred after 7 hours of incubation, 500µl of chloroform was added and the solution shaken for a further 15 minutes. The cellular debris was spun down at 8 000 x g for 10 minutes. The supernatant was transferred to a sterile container and stored at 4°C.

8.2.3 Isolation of Phage DNA

Phage DNA was isolated from the lysate medium using LambdaSorb phage adsorbent (Promega, Southampton, UK) according to the manufacturer's instructions. Briefly, 100µl of LambdaSorb was used for each 10mls of lysate. This solution was mixed for 30 minutes and then centrifuged at 10 000 x g for 15 minutes. The pellet was resuspended in 1ml of phage buffer (20mM Tris-HCl, pH7.4, 100mM NaCl, 10mM $MgSO_4$) per 10mls of initial phage lysate and transferred to a microcentrifuge tube. This was spun for 5 minutes at 12 000 x g in a microcentrifuge and the supernatant discarded. The pellet was once again resuspended in 1ml of phage buffer and spun at 12 000 x g for 5 minutes. The pellet was resuspended in 0.5mls release buffer (10mM Tris-HCl, pH 7.4, 10mM EDTA) per 10mls initial lysate. This solution was heated at 67°C for 5 minutes and spun again at 12 000 x g for 5 minutes. The supernatant was transferred to another tube and 5µl of 5M NaCl added per 0.5mls of supernatant. This was mixed and the DNA extracted with one volume of 1:1 phenol/chloroform (1 part TEN buffer-saturated phenol, pH7-8; 1 part chloroform). The solution was vortexed for 1 minute and then centrifuged at 12 000 x g for 5

minutes. The upper aqueous phase was transferred to a fresh tube and 1 volume of chloroform was added. This was also vortexed for 1 minute and centrifuged as above. The upper aqueous phase was again transferred to another tube and 2 volumes of 100% ethanol were added. This was placed at -20°C for at least 30 minutes. After centrifuging at 12 000 x g for 5 minutes, the supernatant was carefully removed and the pellet resuspended in 1ml of 70% ethanol. This was spun again, the supernatant carefully aspirated and the DNA pellet dried under vacuum. The pellet was resuspended in an appropriate volume (usually 50µl) of TE buffer (10mM Tris-HCl, pH7.8, 1mM EDTA). Before use, the isolated lambda DNA was treated with RNase A as detailed in Section 8.2.4.

8.2.4 RNase A treatment

Contaminating RNA in the isolated phage DNA, was removed by treatment with RNase A. DNase-free RNase A was added to the DNA solution to a final concentration of 100µg/ml and incubated at 37°C for 30 minutes. Sodium chloride was added to a final concentration of 50mM and the DNA extracted using 1 volume of 1:1 phenol chloroform (1 part TEN buffer-saturated phenol, pH7-8; 1 part chloroform). The solution was vortexed for 1 minute and then microcentrifuged at 12 000 x g for 5 minutes. The upper aqueous phase was transferred to a fresh tube and 1 volume of chloroform added. After vortexing for 1 minute, this was spun at 12 000 x g for 5 minutes. The upper aqueous phase was again transferred to a new tube and 2 volumes of 100% ethanol added. This was placed at -20°C for at least 30 minutes and then centrifuged at 12 000 x g for 10 minutes. The supernatant was carefully removed and the pellet resuspended in 1ml of 70% ethanol. The tube was centrifuged at 12 000 x g for 5 minutes and the DNA pellet dried under vacuum. The pellet was resuspended in an appropriate volume of TE buffer (typically 50µl).

8.2.5 *Eco*R1 Digestion

The restriction enzyme *Eco*R1 was purchased from Promega and used in conjunction with the restriction enzyme buffer supplied with it. Digests were prepared in 1.5 x restriction buffer and incubated overnight at 37°C.

8.2.6 Agarose Gel Electrophoresis

All agarose gels were run using a Minnie submarine agarose gel unit (model HE33; Hoefer Scientific Instruments, Newcastle, Staffs, UK). 0.8% agarose gels (SeaKem GTG Agarose; FMC Bioproducts, High Wycombe, Bucks, UK) in 0.5 x TBE containing 0.3µg/ml ethidium bromide (EtBr; Sigma, Poole, Dorset, UK) were run at a constant voltage of 80V for 1-2 hours using 0.5 x TBE as the running buffer. Low melting point agarose gels (typically 2%; Nusieve GTG agarose; FMC Bioproducts) in 0.5 x TAE containing 0.3µg/ml EtBr were run at a constant voltage of 45V for 2 hours at 4°C using 0.5 x TAE as the running buffer.

8.2.7 Primer Synthesis

Primers were made using an oligonucleotide synthesizer (Applied Biosystems, Warrington, Cheshire, UK). The primers were removed from the column using ice cold ammonia and deprotected by incubating overnight in a 55°C water bath. The deprotected primers were stored at -20°C and aliquots prepared by ethanol precipitation as required.

8.2.8 Polymerase Chain Reaction

The DNA insert of the positive phage was amplified using the polymerase chain reaction (PCR; Saiki *et al*, 1985, 1988; Mullis *et al*, 1986; Mullis & Faloona, 1987). PCR was performed using a GeneAmp PCR Reagent kit with AmpliTaq DNA Polymerase (Perkin Elmer Cetus, Norwalk, CT, USA) using two primers synthesized

as described in Section 8.2.7 and a programmable heating block (Hybaid, Teddington, Middlesex, UK). The forward primer was a 22-mer located 16 to 37 base pairs (bp) upstream of the EcoR1 site of λgt11 and had the sequence 5'-GGTGGCGACGACTCCTGGAGCC-3'. The reverse primer was also a 22-mer, located 23 to 44 bp downstream of the EcoR1 site of λgt11 and had the sequence 5'-GACACCAGACCAACTGGTAATG-3'. The final concentrations of the components in the 100µl reaction mixture were AmpliTaq DNA Polymerase, 2.5 Units; dNTPs, each 200µM; 1 x reaction buffer (10mM Tris-HCl pH8.3, 50mM KCl, 1.5mM $MgCl_2$, 0.001% (w/v) gelatin); forward primer, 0.5µM; reverse primer, 0.5µM. DNA from the phage lysate preparation was used as the DNA template. Prior to use 10µl lysate + 60µl water were mixed and heated to 70°C for 5 minutes then kept on ice until they were added to the other PCR components. The PCR reaction had an initial melt temperature of 94°C for 5 minutes and was followed by 30 cycles of annealing (1 minute at 50°C), extension (1 minute at 72°C) and melting (1 minute at 94°C). The final extension was carried out at 72°C for 7 minutes. The reaction products were checked by electrophoresis in a submerged 0.8% agarose gel in 0.5 x TBE buffer run at a constant voltage of 80V for 1 hour. The PCR fragment was then subcloned into a plasmid vector as described in Section 8.2.9.

8.2.9 Subcloning into pBluescript SK-

To enable sequencing, the PCR fragment was cloned into a phagemid vector, pBluescript SK- (Stratagene, Cambridge, UK) using a method involving proteinase K as described by Crowe *et al* (1991). The PCR product was phenol/chloroform extracted and then adjusted to 5mM EDTA, 10mM Tris (pH8), 0.5% SDS. Proteinase K (Promega) was added to a final concentration of 50µg/ml and the mixture incubated at 37°C for 30 minutes. The enzyme was denatured by heating to 68°C for 10 minutes. Phenol/chloroform extraction was followed by chloroform extraction and the DNA

precipitated using ethanol. The DNA was digested with the restriction enzyme *Eco*R1 as described in Section 8.2.5 and the products run on a 2% low melting point agarose gel in 0.5 x TAE buffer (Nusieve; FMC Bioproducts). The band composed of the *Eco*R1-digested PCR fragment was cut out of the gel and ligated to dephosphorylated *Eco*R1-cut pBluescript SK- overnight. The following day, XL1-B competent cells (Section 8.2.10) were electrotransformed with this ligation mixture using a Gene Pulser (BioRad, Hemel Hempstead, Hertfordshire, UK) as follows. Aliquots of competent cells (40µl) were thawed at room temperature and then immediately placed on ice. Between 1µl and 2µl of ligation mix was added to each cell suspension, mixed well and incubated on ice for 0.5-1 minute. The DNA-cell mixture was transferred to a cold 0.2cm sterile electroporation cuvette and pulsed for 15 seconds. The Gene Pulser apparatus was set at 25µF and 2.5kV and the Pulse Controller at 200 ohms. The cells were immediately resuspended in prewarmed (37°C) SOC medium (2% Bacto tryptone, 0.5% Bacto yeast extract, 10mM NaCl, 2.5mM KCl, 10mM $MgCl_2$, 10mM $MgSO_4$, 20mM glucose; 1ml) and the cells incubated at 37°C for 1 hour with shaking in 17 x 100mm polypropylene tubes. The cells were then plated on LB plates containing 50µg/ml ampicillin, 40µg/ml X-gal and 0.5mM IPTG (LAX plates) and grown overnight at 37°C. Cells transformed by wild type pBluescript SK- grow as blue colonies on these plates whereas those transformed with recombinant phagemid result in white colonies. Cells which contain no phagemid are sensitive to ampicillin selection and therefore die. Several white (putative positive) colonies were grown up and phagemid DNA purified using Magic Mini-Prep columns (Promega). To identify positive clones, the DNA was digested with *Eco*R1 (Section 8.2.5) to release the subcloned fragment and electrophoresed in a 0.8% agarose gel (Section 8.2.6).

8.2.10 Preparation of Competent Cells

E. coli XL1-Blue competent cells were prepared as follows. One litre of L broth was innoculated with 1/100 volume of a fresh overnight culture of XL1-B cells. The cells were grown at 37°C with vigorous shaking till the absorbance at 600nm was between 0.5 and 0.8. At this point the cells were harvested by chilling the flask on ice for 15-30 minutes followed by centrifugation in a cold rotor at 4 000 x g for 15 minutes. The supernatant was removed and the pellet resuspended in 1 litre of ice cold water. The suspension was then centrifuged as before and the pellet resuspended in 0.5 litres of ice cold water. After a third centrifugation, the pellet was resuspended in approximately 20mls of ice cold 10% glycerol. A final centrifugation step was performed and the cells resuspended in 2-3mls of ice cold 10% glycerol so that the final cell concentration was approximately 1-3 x 10^6 cells/ml. This suspension was frozen in aliquots (40µl) on dry ice and stored at -70°C until required.

8.2.11 Alkaline Denaturation of DNA

Prior to sequencing, phagemid DNA was denatured using alkaline treatment. The DNA was denatured in 0.2M NaOH, 0.2mM EDTA for 30 minutes at 37°C when it was neutralized by adding 0.1 volumes of 3M sodium acetate (pH4.5-5.5). The DNA was precipitated with 2-4 volumes of ethanol for 15 minutes at -70°C and then pelleted by centrifugation at 14 000 x g for 10 minutes. The pelleted DNA was washed with 70% ethanol and after a second centrifugation, the pellet was dried under vacuum. The DNA was then redisolved in water to be used for sequencing analysis (Section 8.2.12).

8.2.12 Sequencing

The nucleotide sequence of the subcloned PCR-amplified insert was determined according to the dideoxynucleotide chain termination method of Sanger *et al* (1977) using a commercially available sequencing kit (Sequenase; United States Biochemical

Corporation, Cambridge, UK) and ^{35}S-dATP (Amersham). The annealing reaction comprised 7µl DNA (or DNA plus water), 2µl Sequenase 5 x reaction buffer and 1µl primer (approximately 300ng). The tubes were warmed to 65°C for two minutes then allowed to cool slowly to <30°C (approximately 1 hour). The labelling reaction was carried out by adding 1µl dithiothreitol (DTT, 0.1M), 2µl diluted labelling mix (1:4), 0.5µl ^{35}S-dATP and 2µl diluted Sequenase enzyme (1:8) to the annealed template-primer mix and this was incubated at room temperature for 5 minutes. The reactions were terminated by adding 3.5µl of the sequencing reaction to each of the prewarmed (37°C) termination mixes containing a dideoxynucleotide (ddA, ddC, ddG and ddT; 2.5µl) and incubated for 5 minutes at 37°C. The reactions were finally stopped by adding 4µl of formamide stop dye to each termination mix and the tubes stored on ice until the gel was prepared. The sequencing reaction products were run on a Hydrolink Long Ranger sequencing gel (AT Biochem, Newcastle, Staffs, UK) prepared according to the manufacturer's instructions. Gels were run using an LKB power supply at 35W constant power for either 2.5 hours (for sequence close to primer) or 5.5 hours (for sequence distant form the primer). The gel was transferred to Whatman 3MM filter paper and carefully covered with cling film. The gel was dried under vacuum at 80°C for 30 minutes (Slab Gel Dryer, Hoefer Scientific Instruments, Newcastle, Staffs, UK) after which the cling film was removed and the gel exposed to X-ray film (Kodak X-OMAT AR; Kodak, Liverpool, UK) overnight.

8.2.13 Homology Analysis

Homology searches of the Genbank and Owl databases were kindly performed by Dr Philippa Saunders and Dr Phil Taylor.

8.3 Results

One million plaques were screened in duplicate and three putative positive clones were identified. After further rounds of screening only one proved to be a true positive and it was plaque purified. A lambda lysate was produced and the phage DNA purified using LambdaSorb. This DNA was digested with the restriction enzyme *Eco*R1, to release the insert and then electrophoresed on a 0.8% agarose gel (Figure 8.2) indicating that the insert was approximately 1.9kb in size and contained no internal *Eco*R1 sites. In order to obtain sufficient material for subcloning and subsequent sequencing, the DNA insert was amplified by the polymerase chain reaction (PCR) using a forward primer situated 16-37bp upstream and a reverse primer situated 23-44bp downstream from the *Eco*R1 site of λgt11. The sequence of these primers and their relative positions on λgt11 are depicted in Figure 8.3. The PCR products were electrophoresed on a 0.8% agarose gel (Figure 8.4) which indicated that the PCR product was approximately 1.9kb in size. This PCR product was subcloned into the phagemid vector pBluescript SK- (Figure 8.5) and this construct used for sequence analysis. As the sequence was elucidated, further primers were synthesized in order to obtain the nucleotide sequence of the whole insert (Figure 8.6). The insert comprized 1997 nucleotides and contained an open reading frame (ORF) from nucleotide 1 to nucleotide 660 coding for the carboxy-terminal 219 amino acids of the antigen recognized by spm 1/2 (Figure 8.7). The amino acid sequence Asn-X-[Ser or Thr] (where X is any other amino acid other than Pro) has been proposed as a possible consensus sequence for N-linked glycosylation (Kasahara *et al*, 1989; Wright *et al*, 1990; Schwoebel *et al*, 1991). The predicted amino acid sequence includes three of these possible sites for N-linked glycosylation at amino acid numbers 51, 81 and 145. The sequence Ser-[Asp or Glu]-X-X-Pro has likewise been suggested as a possible target site for O-linked glycosylation (Wright *et al*, 1990) and one possible site is present at amino acid number

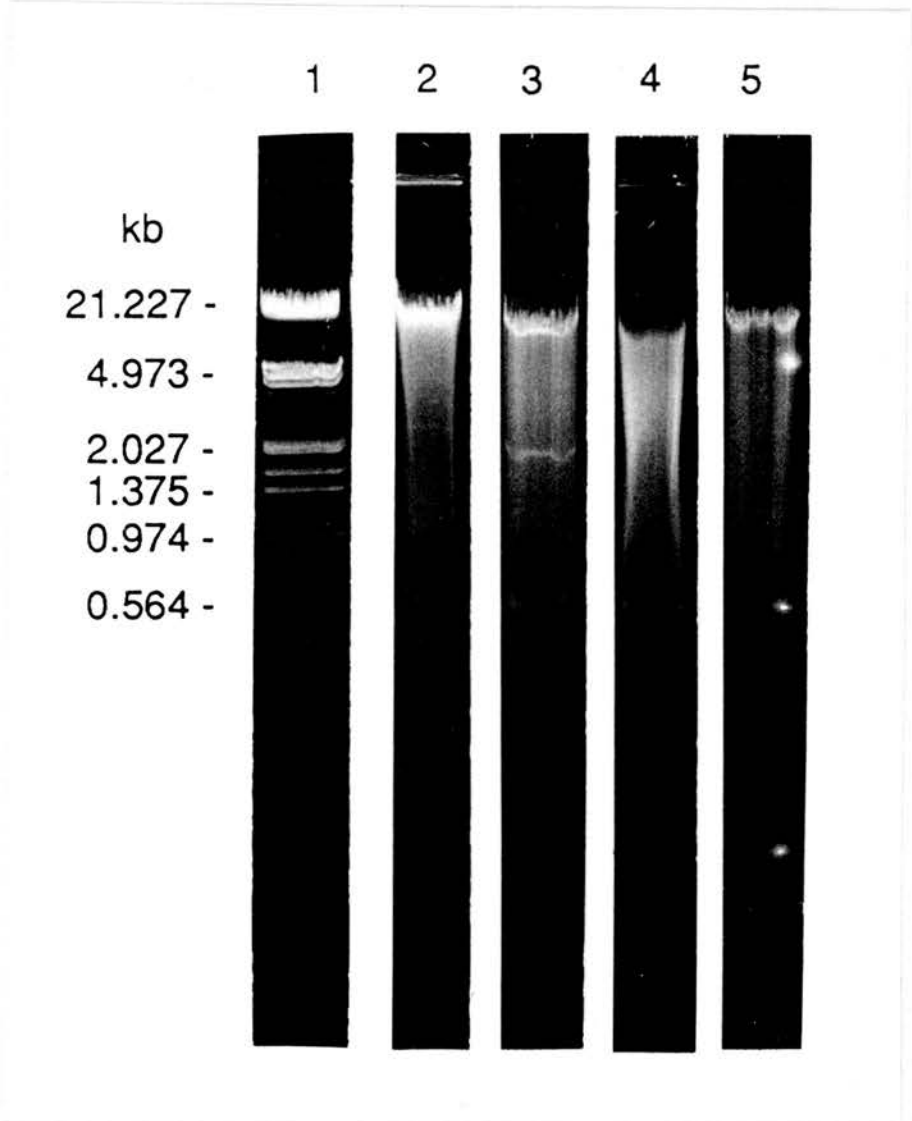

Figure 8.2 Electrophoresis in 0.8% agarose gel showing size of cloned insert. Lane 1, size markers, an *Eco*R1 and *Hind*III digest of lambda DNA producing bands of sizes 21 227b, 5 148b, 4 973b, 4 268b, 3 530b, 2 027b, 1 904b, 1 584b, 1 375b, 975b, 831b, 564b and 125b; Lane 2, undigested positive clone; Lane 3, positive clone digested with *Eco*R1 showing release of an insert of approximately 1.9kb; Lane 4, undigested cloning vector, λgt11; Lane 5, λgt11 digested with *Eco*R1. The gel was run at 80V for 1 hour in 0.5 x TBE.

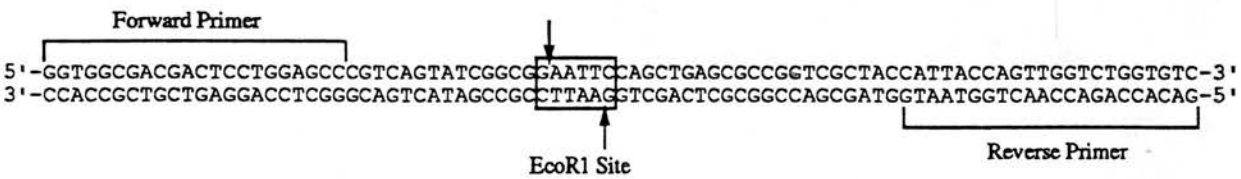

Figure 8.3 DNA sequence of λgt11 around the *Eco*R1 restriction site showing the sequence and position of the primers used during PCR amplification of the insert DNA.

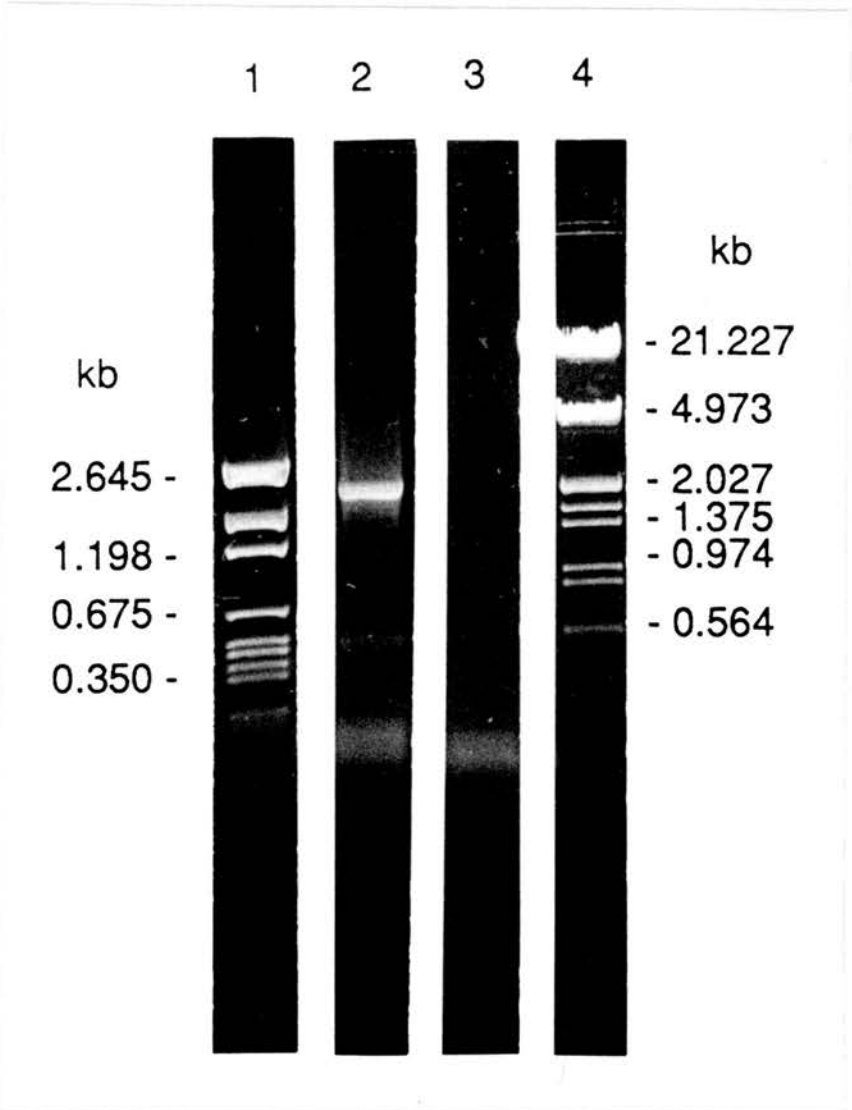

Figure 8.4 Electrophoresis of PCR products in 0.8% agarose gel. Lane 1, pGEM DNA size markers (Promega) producing bands of sizes 2 645b, 1 605b, 1 198b, 675b, 517b, 460b, 369b, 350b, 222b, 179b, 126b, 75b, 65b, 51b and 36b; Lane 2, PCR products from amplification of λgt11 positive clone; Lane 3, same as lane 2 but no template was added; Lane 4, size markers, an *Eco*R1 and *Hind*III digest of lambda DNA producing bands of sizes 21 227b, 5 148b, 4 973b, 4 268b, 3 530b, 2 027b, 1 904, 1 584b, 1 375b, 975b, 831b, 564b and 125b. The gel was run at 80V for 1 hour in 0.5 x TBE.

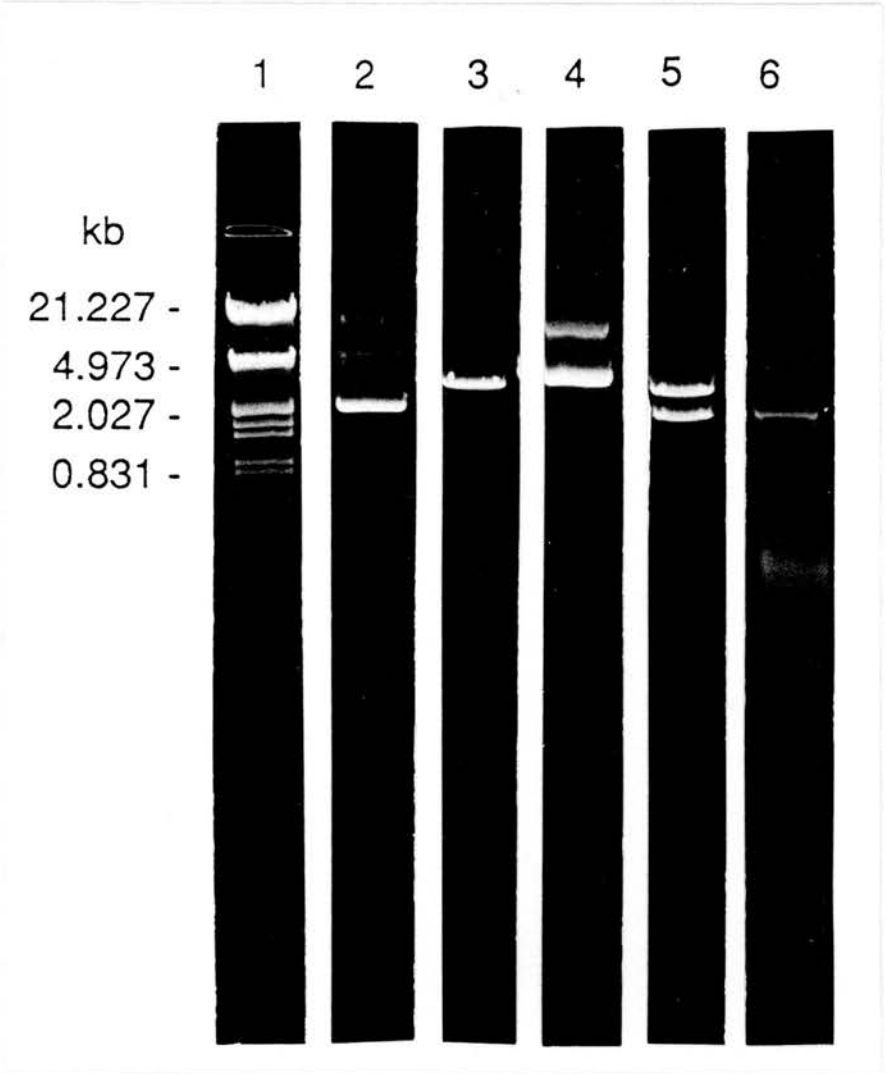

Figure 8.5 Electrophoresis of subcloned PCR fragment in pBluescript SK- in an 0.8% agarose gel. Lane 1, DNA size markers, an *Eco*R1 and *Hind*111 digest of lambda DNA producing bands of sizes 21 227b, 5 148b, 4 973b, 4 268b, 3 530b, 2 027b, 1 904b, 1 584b, 1 375b, 975b, 831b, 564b and 125b; Lane 2, uncut cloning vector pBluescript SK-; Lane 3, cloning vector pBluescript SK- digested with *Eco*R1; Lane 4, uncut positive subclone; Lane 5, positive subclone digested with *Eco*R1 producing vector band at 3kb and release of insert of 1.9kb; Lane 6, PCR fragment. The gel was run at 80V for 1 hour in 0.5 x TBE.

```
   1  GAATTCGGGA AGTGTGTCCG TGATTGTGAA AAGTACAAGC CTGGCGTGTT
  51  GCTGGGGTTT AACATGAAAG AATTAAATAA AGTCAAGCAT GAGATGGATT
 101  TTGATGCTGA GTGGCTATTT GAAAATCATG ATGAGAAGGA TTCCAGAGTC
 151  AATGCTAGTA AGACTGCTGA CAAAAAGCTC AACCTTGGGA AGGAAGATGA
 201  CAGTTCCTCA GACAGTTTTG AAAATTTGGA AGAAGAATCC AATGAAAGTG
 251  GTAGCCCTTT TGACCCTGTT TTTGAAGTTG AACCTAAAAT CTCTAACGAT
 301  AACCCAGAGG AACATGTACT GAAGGTAATT CCTGAGGATG CTTCAGAATC
 351  TGAGGAGAAG CTAGACCAAA AAGAGGATGG TTCAAAATAC GAAACTATTC
 401  ATTTGACTGA GGAACCAACC AAACTAATGC ACAATGCATC TGATAGTGAG
 451  GTTGACCAAG ACGATGTTGT TGAGTGGAAA GACGGTGCTT CTCCATCTGA
 501  GAGTGGGCCT GGATCCCAAC AAGTGTCAGA CTTTGAGGAC AATACCTGCG
 551  AAATGAAACC AGGAACCTGT CTGACGAGTC TTCCCAAAGC GAAGATGCAA
 601  GGAGCAGTAA GCCAGCTGCC AAAAAAAAGG CTACCATGCA AGGTGACAGA
 651  GAGCAGTTGA AATGGAAGAA TAGTTCCTAT GGAAAAGTTG AAGGGTTTTG
 701  GTCTAAGGAC CAGTCACAGT GGAAGAATGC ATCTGAGAAT GATGAGCGCT
 751  TATCTAACCC CCAGATTGAG TGGCAGAATA GCACAATTGA CAGTGAGGAT
 801  GGGGAACAGT TTGACAACAT GACTGATGGA GTAGCTGAGC CCATGCATGG
 851  CAGCTTAGCC GGAGTTAAAC TGAGCAGCCA ACAGCGCTAA GTGCAGGTTC
 901  CCTGTTGGTG ACATGCTGCA GCCTGGAACT CTGATCTCCA GTGTGACTGC
 951  AAAGCTGTCT TCTCACTGGT ACTGCCTTGT GAGTACTGGT TGGACTGTGG
1001  GGCATGTGGC GCTGCAGTTC CAGTGGTTAT TTCTAAGTCT ATGACAGGAC
1051  AGGCTGTTCT TGCTTCAGAA CCTTCTCTGA CAGACACGGT AACTAAATGT
1101  GAAAAACCAA TAAGCTGGTG ACTCATGAAT ACACACGAGG AAAAGCAGAG
1151  GTTTATTTTA TCTGCCTTTT CAACATTTCT TTCCCTCTGT GAAATGATTG
1201  GTCAGATGTC TTTGAGAAGT GTTAAACTAA TTCACATGGT AGTGTAGGGC
1251  CAACATACAA GCTACCAGTC TAATGTGTAT AGTAGACTTT GGGAAAAGCG
1301  ATTTTTTTTC ATGTATTCAT TCTGATAGTT GAAATGTATA TTTGTACAGT
1351  CTTTTAGACC TATTCAAGTG ATGCTCATGA TCCTGCTACT GTGTGCCCAT
1401  CATAGATTTC TTTTTTTAGT GTTGCCCTTG CTGTGTAATA AACGCTCTAT
1451  CTAGTTTACC TAGCAAAAGC TCAAAACTGC GCTAGTATGG ACTTTTTGGA
1501  CAGACTTAGT TTTTGCACAT AACCTTGTAC AATCTTGCAA CAGAGGCCAG
1551  CCACGTAAGA TATATATCTG GACTCTCTTG TATTATAGGA TTTTTCTTGT
1601  TCTGAATATC CTTGACATTA CAGCTGTCAA AAACAAAAAC TGGTATTTCA
1651  GATCTGTTTT CTGAAATCTT TTAAGCTAAA ATCACATGCA AGAATTGACT
1701  TTGCAGCTAC TAATTTTGAC ACCTTTCAGA TCTGTATAAA AGTGTGTTGT
1751  GTTGAAGCAG CAAACCAATG AGTGCTGCAT TTTGGATATT TAGTTTTATC
1801  TTTAGTTCAA CACCATCATG GTGGATTCAT TTATACCATC TAATATATGA
1851  CACACTGTTG TAGTATGTAT AATTTTGTGA TCTTTATTTT CCCTTTGTAT
1901  TCATTTTAAG CATCTAAATA AATTGCTGTA TTGTGCTTAA AAAAAAAAAA
1951  AAAAAAAAAA AAAAAAAAAA AAAAAAAGAA AAAAAAAACC CGAATTC
```

Figure 8.6 Nucleotide sequence of cloned cDNA showing position and sequence of primers used for sequencing. The nucleotides are numbered on the left and arrows indicate the direction of the primer.

```
    Glu Phe Gly Lys Cys Val Arg Asp Cys Glu Lys Tyr Lys Pro Gly Val Leu Leu Gly Phe Asn Met    22
  1 GAA TTC GGG AAG TGT GTC CGT GAT TGT GAA AAG TAC AAG CCT GGC GTG TTG CTG GGG TTT AAC ATG

    Lys Glu Leu Asn Lys Val Lys His Glu Met Asp Phe Asp Ala Glu Trp Leu Phe Glu Asn His Asp    44
 68 AAA GAA TTA AAT AAA GTC AAG CAT GAG ATG GAT TTT GAT GCT GAG TGG CTA TTT GAA AAT CAT GAT

    Glu Lys Asp Ser Arg Val Asn Ala Ser Lys Thr Ala Asp Lys Lys Leu Asn Leu Gly Lys Glu Asp    66
134 GAG AAG GAT TCC AGA GTC AAT GCT AGT AAG ACT GCT GAC AAA AAG CTC AAC CTT GGG AAG GAA GAT

    Asp Ser Ser Ser Asp Ser Phe Glu Asn Leu Glu Glu Glu Ser Asn Glu Ser Gly Ser Pro Phe Asp    88
200 GAC AGT TCC TCA GAC AGT TTT GAA AAT TTG GAA GAA GAA TCC AAT GAA AGT GGT AGC CCT TTT GAC

    Pro Val Phe Glu Val Glu Pro Lys Ile Ser Asn Asp Asn Pro Glu Glu His Val Leu Lys Val Ile   110
266 CCT GTT TTT GAA GTT GAA CCT AAA ATC TCT AAC GAT AAC CCA GAG GAA CAT GTA CTG AAG GTA ATT

    Pro Glu Asp Ala Ser Glu Ser Glu Glu Lys Leu Asp Gln Lys Glu Asp Gly Ser Lys Tyr Glu Thr   132
332 CCT GAG GAT GCT TCA GAA TCT GAG GAG AAG CTA GAC CAA AAA GAG GAT GGT TCA AAA TAC GAA ACT

    Ile His Leu Thr Glu Glu Pro Thr Lys Leu Met His Asn Ala Ser Asp Ser Glu Val Asp Gln Asp   154
398 ATT CAT TTG ACT GAG GAA CCA ACC AAA CTA ATG CAC AAT GCA TCT GAT AGT GAG GTT GAC CAA GAC

    Asp Val Val Glu Trp Lys Asp Gly Ala Ser Pro Ser Glu Ser Gly Pro Gly Ser Gln Gln Val Ser   176
464 GAT GTT GTT GAG TGG AAA GAC GGT GCT TCT CCA TCT GAG AGT GGG CCT GGA TCC CAA CAA GTG TCA

    Asp Phe Glu Asp Asn Thr Cys Glu Met Lys Pro Gly Thr Cys Leu Thr Ser Leu Pro Lys Ala Lys   198
530 GAC TTT GAG GAC AAT ACC TGC GAA ATG AAA CCA GGA ACC TGT CTG ACG AGT CTT CCC AAA GCG AAG

    Met Gln Gly Ala Val Ser Gln Leu Pro Lys Lys Arg Leu Pro Cys Lys Val Thr Glu Ser Ser Stop  219
596 ATG CAA GGA GCA GTA AGC CAG CTG CCA AAA AAA AGG CTA CCA TGC AAG GTG ACA GAG AGC AGT TGA

662 AATGGAAGAATAGTTCCTATGGAAAAGTTGAAGGGTTTTGGTCTAAGGACCAGTCACAGTGGAAGAATGCATCTGAGAATGATGAGC

749 GCTTATCTAACCCCCAGATTGAGTGGCAGAATAGCACAATTGACAGTGAGGATGGGGAACAGTTTGACAACATGACTGATGGAGTAG

836 CTGAGCCCATGCATGGCAGCTTAGCCGGAGTTAAACTGAGCAGCCAACAGCGCTAAGTGCAGGTTCCCTGTTGGTGACATGCTGCAG

923 CCTGGAACTCTGATCTCCAGTGTGACTGCAAAGCTGTCTTCTCACTGGTACTGCCTTGTGAGTACTGGTTGGACTGTGGGGCATGTG

1010 GCGCTGCAGTTCCAGTGGTTATTTCTAAGTCTATGACAGGACAGGCTGTTCTTGCTTCAGAACCTTCTCTGACAGACACGGTAACTA

1097 AATGTGAAAAACCAATAAGCTGGTGACTCATGAATACACACGAGGAAAAGCAGAGGTTTATTTTATCTGCCTTTTCAACATTTCTTT

1184 CCCTCTGTGAAATGATTGGTCAGATGTCTTTGAGAAGTGTTAAACTAATTCACATGGTAGTGTAGGGCCAACATACAAGCTACCAGT

1271 CTAATGTGTATAGTAGACTTTGGGAAAAGCGATTTTTTTTCATGTATTCATTCTGATAGTTGAAATGTATATTTGTACAGTCTTTTA

1358 GACCTATTCAAGTGATGCTCATGATCCTGCTACTGTGTGCCCATCATAGATTTCTTTTTTTAGTGTTGCCCTTGCTGTGTAATAAAC
                                                                                  ******

1445 GCTCTATCTAGTTTACCTAGCAAAAGCTCAAAACTGCGCTAGTATGGACTTTTTGGACAGACTTAGTTTTTGCACATAACCTTGTAC

1532 AATCTTGCAACAGAGGCCAGCCACGTAAGATATATATCTGGACTCTCTTGTATTATAGGATTTTTCTTGTTCTGAATATCCTTGACA

1619 TTACAGCTGTCAAAAACAAAAACTGGTATTTCAGATCTGTTTTCTGAAATCTTTTAAGCTAAAATCACATGCAAGAATTGACTTTGC

1706 AGCTACTAATTTTGACACCTTTCAGATCTGTATAAAAGTGTGTTGTGTTGAAGCAGCAAACCAATGAGTGCTGCATTTTGGATATTT

1793 AGTTTTATCTTTAGTTCAACACCATCATGGTGGATTCATTTATACCATCTAATATATGACACACTGTTGTAGTATGTATAATTTTGT
                                          ^^^^^

1880 GATCTTTATTTTCCCTTTGTATTCATTTTAAGCATCTAAATAAATTGCTGTATTGTGCTTAAAAAAAAAAAAAAAAAAAAAAAAAA
                                          ******

1967 AAAAAAAAAAAAGAAAAAAAAAACCCGAATTC
```

Figure 8.7 Nucleotide sequence of cloned cDNA and deduced C-terminal amino acid sequence of the antigen recognized by spm 1/2. The deduced amino acid sequence is shown above the nucleotide sequence. The nucleotides are numbered on the left and amino acids are numbered on the right. The open reading frame contains three potential N-linked glycosylation sites which are shown in solid boxes, and one potential O-linked glycosylation site shown in a broken box. The 3' untranslated region contains two polyadenlation siganls (AATAAA) which are underscored with the symbol ****** and a mRNA degradation signal indicated by the symbol ^^^^^.

166. A triple serine residue sequence which occurs at positions 68-70 has also been suggested as a potential phosphorylation site (Yan *et al*, 1990). The insert has a long 3' untranslated region of 1357 nucleotides which contains a putative mRNA degradation signal (ATTTA; Caput *et al*, 1986; Shaw & Kamen, 1986) at nucleotides 1830-1834. Two polyadenylation signals (AATAAA; Berget, 1984; Joshi, 1987) are also present at positions 1438-1443 and 1918-1923. A poly-A tail begins at nucleotide 1940, 17bp downstream from the second polyadenylation signal. A hydrophobicity plot (Kyte & Doolittle, 1982) of the predicted amino acid sequence was generated, averaging six amino acids for each point (Figure 8.8). This indicated that the carboxyl terminus of spm 1/2 antigen is mainly hydrophilic. The DNA and amino acid sequences were checked for homology by carrying out searches of the Genbank and Owl databases respectively. Neither the nucleic acid nor amino acid sequences showed any significant homology with the sequences contained in the banks.

8.4 Discussion

One positive clone was identified after screening the human testicular cDNA library and the insert was amplified using the polymerase chain reaction. Direct sequencing of PCR products is possible (Wrischnik *et al*, 1987; Innis *et al*, 1988; Winship, 1989) although often some difficulty is encountered. It was therefore decided to subclone the PCR-amplified insert before sequence analysis was undertaken. Subcloning of PCR products is relatively inefficient even when restriction sites have been incorporated into the PCR primers (Lew, 1991). This may be due to inefficient cleavage of restriction sites at or near termini and one method used to overcome this problem utilizes long oligonucleotide primers that contain sequences complementary to the vector (Shuldiner *et al*, 1990). An alternative theory as to why PCR products are often difficult to subclone was proposed by Crowe *et al* (1991) who suggested that the *Taq* polymerase

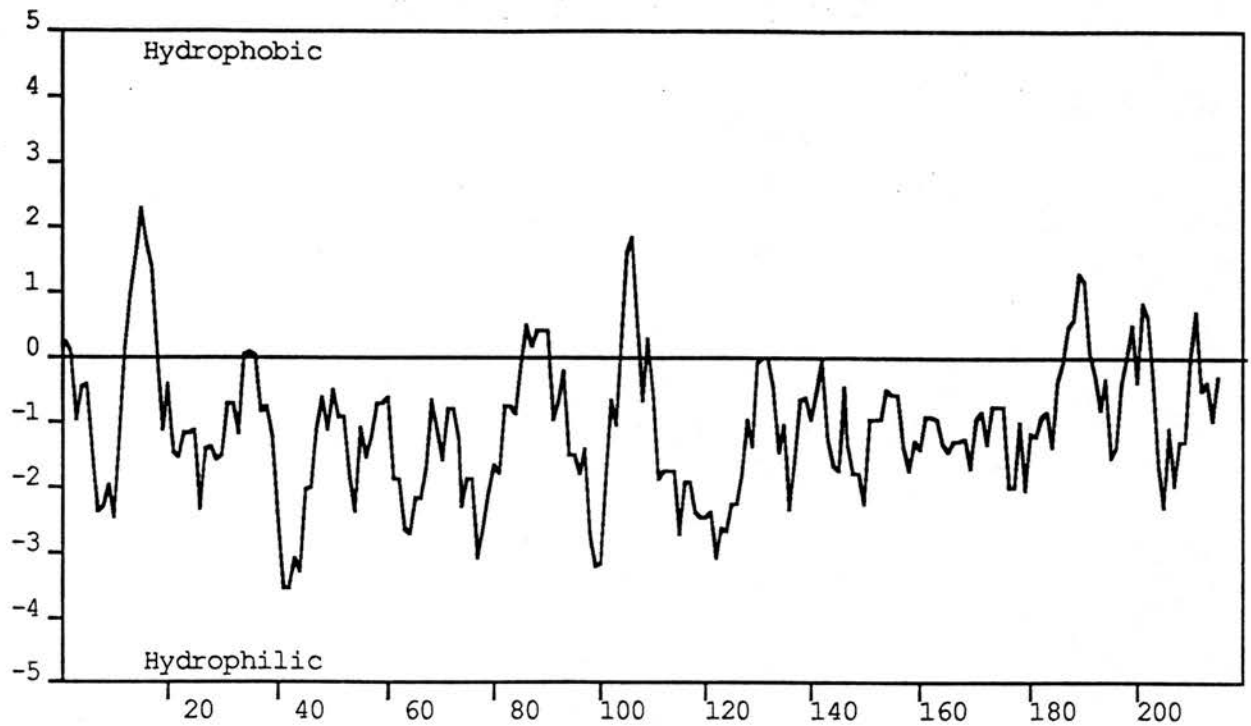

Figure 8.8 Hydrophobicity plot generated from the deduced amino acid sequence of the C-terminus of the antigen recognized by spm 1/2.

used during the PCR remains bound to the DNA and inhibits restriction endonuclease activity. Thus increased cloning efficiency was achieved after incorporating a proteinase K digestion step into the cloning protocol prior to endonuclease digestion and this method was therefore followed to subclone the PCR-amplified insert reported here.

DNA fragments up to 7.2kb can be cloned into the *Eco*R1 site of λgt11 (Huynh *et al*, 1985; Sambrook *et al*, 1989) as to be packaged efficiently, the length of the phage genome must be between 78% and 105% of that of wild-type bacteriophage lambda (Kaiser & Murray, 1985; Sambrook *et al*, 1989). The insert of 1.9kb isolated here falls well within this range. The sequence contains an open reading frame from nucleotide 1 to nucleotide 660 which encodes the C-terminal 219 amino acids of spm 1/2 antigen. The long 3' untranslated region contains a putative mRNA degradation signal and two polyadenylation signals. Only one polyadenylation signal appears to function in the testis as only one poly-A tail is found, 17bp downstream from the second signal. Welch *et al* (1990) also discovered two polyadenylation signals in the cDNA encoding a sperm-specific antigen although, as reported here, only one of these appeared to function.

Analysis of the deduced amino acid sequence revealed three possible N-linked and one possible O-linked glycosylation sites. It is quite probable that carbohydrates are added to the protein at these positions as many sperm proteins are glycosylated and carbohydrates have been shown to be important in mammalian fertilization (Ahuja, 1982; Lambert, 1984; Boldt, 1989; Shur, 1989; Wassarman, 1989; Kimber, 1990; Miller & Ax, 1990). The predicted amino acid sequence has a molecular weight of 24.5kD and this is less than the estimated molecular weight of 36kD obtained from gel electrophoresis and blotting studies (Chapter 5). When a full length sequence is obtained, the molecular weight calculated from the predicted sequence is often less than that estimated on gels due to glycosylation of the protein (Adham *et al*, 1989; Baba *et*

al, 1989; Wright *et al*, 1990). However the discrepency found here is mainly because only the carboxy-terminal region of the protein has been cloned. In order to obtain clones which contain the amino terminus and 5' untranslated region sequences, the library must be rescreened using the cDNA sequence as a probe.

8.5 Summary

The gene encoding the antigen recognized by antibody spm 1/2 was isolated from a human testicular cDNA library cloned into the expression vector λgt11. The insert DNA was amplified using the polymerase chain reaction and then subcloned into the phagemid pBluescript SK- for sequence analysis. The whole insert was sequenced and found to be 1997 nucleotides in length. It contains an open reading frame from nucleotide 1 to nucleotide 660 which encodes the carboxy-terminal 219 amino acids of the antigen recognized by spm 1/2. The predicted amino acid sequence contains three possible sites for N-linked glycosylation and one possible site for O-linked glycosylation. The insert has a long 3' untranslated region of 1337 nucleotides which contains a mRNA degradation signal, two polyadenylation signals and a poly-A tail which starts 17 nucleotides downstream from the second polyadenylation signal. The hydrophobicity plot generated from the predicted amino acid sequence indicated that the C-terminus of spm 1/2 antigen was mainly hydrophilic. Screening of the Genebank and Owl databases for homologous sequences, indicated that the protein recognized by spm 1/2 has not previously been described.

9 General Discussion

This thesis describes the production and characterization of twelve monoclonal antibodies raised against the human sperm plasma membrane. It is thought that most sperm antigens are not directly involved in the fertilization process (Mahony & Alexander, 1991) as only a small proportion of antibodies which react with the sperm surface affect fertility as assessed using a variety of *in vitro* tests of sperm function (Anderson *et al*, 1987). Similarly only three of the twelve antibodies described here were shown to be strongly biologically active. Two of these antibodies, spm 1/2 and spm 4/6, caused a significant inhibition of fertilization and are thus of interest in the development of a contraceptive vaccine. The other antibody, spm 4/7, stimulated fertilization and may have a clinical application in the treatment of subfertile men.

Any animal used for a fusion must contain cells expressing antibodies directed against the desired molecule otherwise it is impossible to successfully produce monoclonal antibodies against this molecule (Milstein, 1982). Thus the animal requires to be primed with the desired antigen prior to the fusion. Although the immunogen does not have to be pure, numerous hybrids have to be screened in order to identify those which are positive if it is not. This is often a time-consuming and labour-intensive procedure. The probability of obtaining biologically active monoclonal antibodies may be increased by partially purifying the immunogen so that it contains a relatively higher amount of active antigen. Thus a purifed sperm plasma membrane preparation was used as the immunogen in the production of the antibodies reported here (Chapter 4).

Since this work was initiated, alternative methods to increase the proportion of positive clones have been developed. One such method utilizes biotin-streptavidin-mediated electrofusion (Lo *et al*, 1984; Wojchowski & Sytkowski, 1986; Hewish & Werkmeister, 1989). This protocol involves biotinylating the desired antigen (against

which the monoclonal antibodies are to be directed) and mixing this with B-lymphocytes from a preimmunized animal. B-lymphocytes which produce antibody against the desired antigen will then bind to the labelled antigen. This B-lymphocyte-antigen-biotin complex is then brought into close proximity with biotinylated myeloma cells using a streptavidin bridge. The cells are then fused using electrical pulses and cultured as per the classical monoclonal antibody production technique (Chapter 4). The vast majority of the resulting hybrids secrete antibodies against the desired antigen. There are two main reasons for this. Firstly the streptavidin bridge ensures that the two cells which require to be fused to produce the specific monoclonal antibodies, are closely linked when the electric current is passed. Only a relatively small number of other cells are close enough to each other, by chance, when the pulse is applied, to fuse and therefore the background rate of negative fusions is low. Secondly, Chang (1989) reported that electrofusions are more effective when they involve cells of a similar size and non-activated spleen cells are generally smaller than myeloma cells whereas activated B-lymphocytes are approximately the same size as myeloma cells. Thus most of the hybrids produced using this method produce antibodies directed against the desired antigen. Modifications of the basic hybridoma technique such as this one, make the process more efficient and less demanding. A larger panel of antisperm antibodies can then be produced from which biologically active ones can be identified.

Although whole spermatozoa can produce antibodies that induce infertility, there are many sperm antigens which have been shown to be shared with other somatic tissues and cells in the body (Freund *et al*, 1955; Kerek, 1974; Jacob, 1977; Chaffee & Schachner, 1978; Menge & Fleming, 1978; Mathur *et al*, 1981; Jorgensen & Moller, 1983). Therefore sperm-based contraceptive vaccines must utilize sperm-specific antigens as cross-reactivity with somatic cells could interfere with other cellular processes leading to serious side effects. While spm 4/6 cross-reacted with various leucocyte subsets tested, spm 1/2 did not (Chapter 6). Although these preliminary

results suggest that spm 1/2 is tissue specific, further investigation using a more extensive panel of somatic tissues is required to establish this unequivocally. On Western blots, spm 4/6 stained a large smear and is thus thought to recognize a commonly occurring epitope such a carbohydrate (Chapter 5). Carbohydrates have been shown to be important in fertilization (Shur, 1989; Wassarman, 1989; Miller & Ax, 1990) and recently Phillips (1991) suggested that sperm-zona pellucida binding may involve a simple relatively non-specific binding mechanism utilizing sugars. It is unlikely that this is the only mechanism for sperm-zona pellucida binding but such an interaction may be involved in the maintenance of sperm binding to the zona pellucida after the acrosome reaction has occurred.

Multivalent antibodies can exert non-specific actions due to their agglutinating ability (Metz, 1972; Moore & Hartmann, 1984). Antibodies can prevent the interaction between spermatozoa and eggs by agglutinating most of the spermatozoa and leaving few free in solution to interact with the oocyte. Such antibodies have an inhibitory effect on the sperm-zona binding assay and HEPT but represent 'false positives' because the antibody does not recognize a biologically active protein. Antibodies can mask receptors in the sperm surface by steric hindrance as immunoglobulin molecules are relatively large and this also gives a false positive result in these *in vitro* tests of sperm function (Moore & Hartmann, 1984). Any role of agglutination and steric hindrance in the inhibition of the sperm-zona pellucida and sperm-oolemma interactions by monoclonal antibodies spm 1/2 and spm 4/6 was not directly assessed (Chapter 7). However none of the antibodies were tested at very high concentrations and other antibodies caused a similar degree of agglutination but had no affect on fertilization (Chapter 7). Spm 1/2 also inhibited the acrosome reaction indicating that the antigen recognized by this antibody may be involved in events during capacitation and/or the acrosome reaction. This suggests that neither steric hindrance nor agglutination played a role in the spm 1/2 block of fertilization. On the other hand, monoclonal antibody

spm 4/6 did not have any effect on the acrosome reaction although it inhibited both sperm-zona pellucida binding and sperm-oolemma fusion. Although spm 4/6 belongs to the IgG_{2a} subclass and is therefore smaller than the pentameric IgM antibodies, it recognizes a commonly occurring epitope which is thought to be a carbohydrate (Chapter 5) and thus the spm 4/6 block to fertilization may be partly attributable to steric hindrance. These problems can be overcome by digesting the antibody molecules with papain to produce Fab fragments. These Fab fragments only contain one antigen binding domain and are therefore unable to crosslink. They are also much smaller than whole immunoglobulins and thus lose their ability to mask other molecules. Preparation of Fab fragments would be the next logical step to determine the potential of these antibodies and their corresponding antigens for contraceptive vaccine development.

Passive immunization with antibodies raised against sperm-specific antigens is a feasible approach to the development of contraceptive vaccines as it has been shown that monoclonal antibodies passively administered to female mice have an antifertility effect (Saling & Waibel, 1985; Saling *et al*, 1986; Saling, 1990). Similar studies in males have also reduced fertility although the effect was less dramatic in males than females (Lee *et al*, 1987). This is probably due to the action of the blood-testis barrier which sequesters much of the male reproductive tract from the immune system. Most monoclonal antibodies raised against human sperm antigens have been produced in mice and these murine antibodies have limited potential for transfer to humans because of their xenogeneic status. Although human monoclonal antibodies have been made, many problems have been encountered and frequently the resulting human antibodies are not of the appropriate isotype or do not possess the desired specificity (James & Bell, 1987; Winter & Milstein, 1991). Transforming murine antibodies into chimeric antibodies by combining the variable domain of the murine antibody with constant

domains from human antibodies is not sufficient to overcome this problem (Morrison *et al*, 1984; Boulianne *et al*, 1984; Bruggemann *et al*, 1989). In order to reveal the full potential of such antibodies, they must be fully humanized. This involves reshaping both the variable and constant domains of the antibody to make them 'human-like'. This was first successfully achieved by Reichmann *et al* (1988) and a few humanized antibodies have since been produced (Queen *et al*, 1989; Brown *et al*, 1991; Co *et al*, 1991). Generation of humanized antibodies has proved difficult (Co *et al*, 1991; Winter & Milstein, 1991) although this technique is still in its infancy and has yet to realize its potential.

Until recently, the production of monoclonal antibodies has required preimmunization of animals (usually rodents). However towards the end of last year, a new technique for monoclonal antibody production which bypassed the need for both immunization and classic hybridoma technology, was published (Marks *et al*, 1991). This involved amplification of antibody genes from human B-lymphocytes using PCR and cloning the DNA fragments into phage DNA. This was done in such a way that when the phage infected a bacterium, Fab fragments were made and displayed on the phage surface. A 'phage display library' was thus created where every phage carried a different antibody on its surface. Marks and colleagues were than able to select and culture phage that bound a particular antigen and so produce human monoclonal antibodies of defined specificity without the need for immunization. The development of techniques such as these hold great promise for the future and is essential if the production of a contraceptive vaccine by passive immunization is to become a reality.

The alternative to passive immunization is to administer a contraceptive vaccine using active immunization with purified protein. Preliminary investigations with sperm-specific antigens have been performed and indicate that a long lasting but reversible contraceptive effect can be obtained in both males and females although this effect is titre dependent and varies from one animal to the next (Naz, 1987b; Primakoff

et al, 1988b; Liu *et al*, 1989c; Shaha *et al*, 1990). In order to purify bulk quantities of a sperm antigen, vast amounts of spermatozoa would be required and this is impractical. The impact of molecular techniques on this problem has been profound. Once the gene encoding the desired sperm antigen has been cloned, unlimited supplies of recombinant protein can be produced thus alleviating the need for large quantities of starting material. Recombinant proteins have been used in several small scale active immunization trials. Although they are less effective than the native protein in stimulating an immune response, an antifertility effect was still seen (Liu *et al*, 1990; Wright *et al*, 1990). This indicates that it is plausible to induce infertility using recombinant proteins.

A partial clone of the gene encoding the antigen recognized by monoclonal antibody spm 1/2 has been identified and the sequence elucidated (Chapter 8). In order to carry out further studies to evaluate the contraceptive potential of the spm 1/2 antigen, this sequence may be used to isolate a full-length clone from a human testicular cDNA library. Recombinant spm 1/2 protein could then be produced and preliminary trials conducted to identify if it can induce infertility.

Synthetic peptides can also be used as the basis of vaccines and they may help reduce side effects by increasing specificity (Arnon, 1986). Suitable peptide sequences can be identified by epitope mapping studies. This method involves systematically synthesizing all the overlapping short peptides (of say 7 or 8 amino acids in length) in the entire protein sequence and measuring their reactivity with antibodies raised against the native protein (Geysen *et al*, 1984). A monoclonal antibody can also be used to identify the short peptide which has greatest binding and this is called a 'mimetope' (Geysen *et al*, 1985). The protein recognized by spm 1/2 can be epitope and mimetope mapped and the short peptides thus identified can then be chemically synthesized and used as the basis for a contraceptive vaccine (Vanage *et al*, 1992).

From the original twelve antibodies raised against the human sperm surface one antibody, spm 4/7, had a stimulatory effect on fertilization (Chapter 7). This antibody had no effect on the acrosome reaction nor on the interaction between spermatozoa and the zona pellucida but significantly stimulated sperm-oolemma fusion. It has previously been suggested that such antibodies exert their stimulatory effect by increasing the number of spermatozoa adherent to the oolemma via specific Fcγ receptors which are present on the vitelline membrane of hamster oocytes (Bronson *et al*, 1981, 1990a&b). However spm 4/7 is an IgM antibody (Chapter 5) and therefore does not bind to Fcγ receptors (Fusi *et al*, 1991). Alternatively spm 4/7 may recognize an epitope which is common to both spermatozoon and egg and function by antibody bridging (Bronson *et al*, 1990b). This may fortuitously increase sperm-egg fusion by simply increasing the number of sperm adherent to the oolemma. It is not known whether spm 4/7 recognizes any molecules on the hamster oolemma but this would be a relatively simple experiment to perform in order to provide evidence for or against this mechanism of action. A third possible mechanism by which spm 4/7 may exert its stimulatory effect is by crosslinking receptors and activating the sperm cell thereby initiating the normal biochemical events which occur upon sperm contact with the oolemma (Aitken *et al*, 1987a). This mechanism requires that spm 4/7 is a multivalent antibody and could be tested by preparing Fab fragments. If this antibody works by receptor clustering, these Fab fragments alone should not be able to activate spermatozoa but the addition of a second antibody to crosslink the first antibody should and this would provide substanciating evidence for this mechanism. This is an exciting possibility and would provide a tool with which the biochemical events occurring in the spermatozoon upon contact with the oolemma could be probed and investigated.

The use of monoclonal antibody technology has permitted the identification of molecules important in the fertilization process and in conjunction with molecular

studies, it has allowed a more detailed insight into the intricacies of the sperm-egg interaction. This has important implications for both the treatment of infertile couples and the development of novel forms of contraception.

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