

**PUTATIVE IMMUNOSUPPRESSIVE MOLECULES ASSOCIATED
WITH IN VITRO FERTILIZED EMBRYOS
MAY BE ESSENTIAL GROWTH FACTORS**

By

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**IMMUNOSUPPRESSIVE MOLECULES AS
ESSENTIAL GROWTH FACTORS**

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TITLE: Putative Immunosuppressive Molecules Associated with
In Vitro Fertilized Embryos may be Essential Growth
Factors

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ABSTRACT

There is a high rate of pregnancy failure in humans. The greatest loss occurs at the time of implantation or immediately after the embryo has implanted. Up to 50-60% of this loss can be attributed to embryonic chromosomal abnormalities. The absence or anomalous amounts of physiologic factors which are necessary for implantation and early embryonic development may be the cause of pregnancy failure. Since the embryo is foreign, it is specifically necessary to explore the role of rejection and failure of mechanisms that suppress rejection at implantation.

The murine system has been used to investigate the identity of immunosuppressive molecules produced during the process of preimplantation embryo development. Supernatants from mouse *in vitro* fertilized (IVF) embryo cultures can suppress *in vitro* lymphocyte proliferation stimulated by the mitogen concanavalin A. Medium conditioned by incubation with mouse epididymal spermatozoa alone were even more inhibitory to mitogen stimulated lymphocyte proliferation. Thin layer chromatography detected the polyamines spermine in sperm, and spermidine as well as spermine in IVF embryo culture supernatants. Evidence was obtained that these were possibly the *in vitro* molecules that were immunosuppressive and were likely produced by the embryo. The diamine

putrescine was also detected but was not immunosuppressive.

The conclusion from the studies suggest that polyamines may have a role *in vivo* in suppressing uterine immune response thereby assisting the embryo in the process of implantation. Failure of embryos to produce sufficient amounts of polyamines perhaps due to chromosome abnormalities, may explain failure of embryo implantation. As well, the failure of IVF embryos to produce adequate quantities of polyamines which are known to be essential for cell proliferation, could lead to embryo division arrest. In this broad sense polyamines may be viewed as "growth factors" i.e. defined molecules essential for cell division.

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LIST OF ABBREVIATIONS USED

μ Ci	-	microcurie
AB	-	Blood group AB
Ab	-	antibody
AG	-	aminoguanidine
Con A	-	concanavalin A
CL	-	corpus luteum
cpm	-	counts per minute
CTL	-	cytotoxic T lymphocytes
CSF	-	colony stimulating factor
DFMO α	-	DL- α -difluoromethylornithine
EGF	-	epidermal growth factor
EPF	-	early pregnancy factor
ET	-	embryo transfer
FBS	-	fetal bovine serum
GIFT	-	gamete intra-fallopian transfer
GM-CSF-	-	granulocyte-macrophage colony stimulating factor
H-2	-	the major histocompatibility complex of the mouse
HA	-	hyaluronic acid

hCG	-	human chorionic gonadotropin
HLA	-	human major histocompatibility complex
HPLC	-	high performance liquid chromatography
hr	-	hour
Ig	-	immunoglobulin
IFN	-	interferon
IL	-	interleukin
IP	-	Intraperitoneal
IU	-	International units
IVF	-	<i>in vitro</i> fertilization
LH	-	luteinizing hormone
LAK	-	lymphokine activated killer
LPD	-	luteal phase defect
μ l	-	microlitre
mg	-	milligram
MGBG	-	methylglyoxal- bis-(guanylhydrazone)
MHC	-	major histocompatibility complex
ml	-	millilitre
mRNA	-	messenger ribonucleic acid
NK	-	natural killer

ODC	-	L-ornithine decarboxylase
PAF	-	platelet activating factor
PAO	-	polyamine oxidase
PBL	-	peripheral blood leukocytes
PBS	-	phosphate buffered saline
Ped	-	preimplantation embryo development gene
PG	-	prostaglandin
PMSG-		pregnant mare serum gonadotropin
R_f	-	distance traveled by solute / distance traveled by mobile phase
rRNA	-	ribosomal ribonucleic acid
SF	-	Suppressive factor
SAM-D	-	S-adenosyl-L-methionine decarboxylase
TLC	-	Thin layer chromatography
TF	-	Transferrin
TFRC-		Transferrin receptor
TGFB	-	Transforming growth factor- β
TNF α	-	Tumor necrosis factor- α
TP-1	-	Trophoblast protein-1
TPB	-	Trypan blue

tRNA - transfer ribonucleic acid

v/v - volume per volume

1. LITERATURE REVIEW

1.1 INTRODUCTION

For successful establishment of mammalian pregnancy, an oocyte must be fertilized, develop into a blastocyst, and in most species the blastocysts must attach to and embed into the wall of the uterus, and then produce factors that maintain secretion of maternal hormones to support the pregnancy. The embryo is derived genetically from both parents, and thus bears paternal antigens foreign to the mother. To prevent rejection of the fetus, adjustments in the maternal immune system, and locally at the uterine implantation site may be required (Innes et al., 1989). Communication between the mother and the implanting embryo is critical, or else loss of the pregnancy due to either endocrine or immunologic factors can occur.

Female sterility can emanate from vaginal, cervical, uterine, tubal and

endocrine factors (Rahwan, 1985). Equally important, failure of sperm transport, and of fertilization can be the cause of unexplained infertility (Ramsewak et al., 1990). Failure of embryonic development prior to implantation, is usually viewed as "infertility". In normal *in vivo* reproduction approximately 80% of human embryos are unsuccessful at or before implantation.

Embryonic mortality (occurring from syngamy to the time of implantation) could result from structural and chromosomal abnormalities in the fertilizing gametes, or from expression of lethal genes early during development (Hanly, 1961; Kalter, 1980; Iannaccone et al., 1987). Although the data are still meagre, studies on human *in vitro* fertilized embryos that have failed to divide have shown a high frequency (50-60%) of chromosomal aberrations (Angell et al., 1983, 1986; Rudak et al., 1984, 1985; Papadopoulos et al., 1989). In mice, a low percentage of preovulatory oocytes are abnormal. *In vivo*, as many as 50% of human pregnancy losses can be attributed to chromosomal abnormalities of the embryo, or to anatomical defects (Hertig et al., 1959), which is higher than in most species. In the mouse the highest percentage of abnormalities is approximately 32% (Badenas et al., 1989). In sheep up to 25% abnormal chromosome complements in embryos were reported (Murray et al., 1986a, 1986b). About 6-10% of fertilizable oocytes in rabbits were found to be abnormal (Luckett and Mukherjee, 1986).

Following fertilization, inappropriate hormonal support and harmful changes in the oviductal or in the uterine environment may be detrimental to embryonic development. Most embryonic mortality in humans occurs during cleavage, and is associated with abnormalities in the uterine environment (Rogers et al., 1986). Similarly, embryonic mortality in dairy cows is associated with abnormal uterine environment (Wiebold, 1988). It has also been demonstrated in the mouse that alterations in genital tract function will alter embryonic implantation rates (Singh et al., 1983). Other factors that may be responsible for prenatal loss are the embryonal stage of development, and asynchrony between the embryonic and the maternal environment in sheep (Wilmot et al., 1985a, 1985b; Wilmot et al., 1986; Wilmot et al., 1988).

There is a high rate of pregnancy failure in humans as already mentioned. The greatest loss occurs at the time of implantation or immediately after the embryo has implanted (Wilcox et al., 1988). The highest pregnancy success rates in humans appears to be in the range of 20%, highlighting our lack of understanding of many of the mechanisms involved in successful establishment of pregnancy (Roberts and Lowe, 1975).

The main focus of this work addresses the effect and identity of factors in *in vitro* fertilization (IVF) embryonic culture medium that may be relevant to

implantation. First, the basics of natural and assisted reproduction are reviewed. Subsequently, the biology and immunology of implantation in mammals is described in some detail. Note is made of the role of growth factors in these reproductive processes, especially the polyamines. Finally, the use of the mouse as a model for pregnancy failure, and ageing mice as a model for the mechanisms of reproductive failure are described.

1.2 BIOLOGY OF IMPLANTATION

1.2.A Preimplantation development

Following successful fertilization, the activated oocyte develops into a blastocyst, which will attach to a variety of surfaces, but not normally to the endometrium. In most species, there is only a narrow "time window" during which embryonic attachment to the endometrium and implantation can occur. The timing of embryonic development with respect to events in the oviducts and the endometrium must be precise. It appears that essential factors which participate in the process of early development may be available only for a limited period of time. After this period, one or more essential components may become non-functional or absent.

The structural system for the transport of nutrients from the cumulus cells surrounding the oocyte to the oocyte is provided by a network of connecting cytoplasmic processes and gap-junctions (Andersen and Albertini, 1976; Brower and Schultz, 1982). Cumulus cells contain abundant amounts of hyaluronic acid (Eppig, 1979), and appear to protect the oocyte against degeneration (Sato et al., 1987). At a later stage, during the late 8-cell stage when the embryo undergoes compaction, the cells of the embryo become polarized, resulting in apical localization of microvilli on each blastomere, and gap-junctions which allow rapid molecular transfer between the cells are formed (Buehr et al., 1987; Lee et al., 1987).

It has been suggested that initiation of blastocyst implantation involves several factors (Figure 1). In mice and rats, activation of the endometrium for implantation is triggered by secretion of estradiol (Myers, 1970). The events that follow include synthesis of embryonal and uterine estrogen and progesterone receptors, ovulation, followed by secretion of progesterone by the corpus luteum, and transformation of the proliferative phase endometrium into a secretory tissue, capable of supporting implantation (Levy et al., 1980; Puri and Roy, 1980).

A deficiency of progesterone steroidogenesis by the corpus luteum (CL), either in amount or duration, leads in humans to luteal phase defect (LPD). LPD has been related to post-implantation pregnancy failure (Witten and Martin, 1985;

Seif et al., 1989). Treatment of women suffering from LPD with an antiestrogen, Tamoxifen, has been successful, resulting in pregnancies possibly due to an increase in the effective ratio of progesterone to estrogen (Fukushima et al., 1982). A similar progesterone deficiency in the preimplantation period has been produced in the mouse by injection of antiprogestosterone antibodies, and this treatment produces division abnormalities (Heap et al., 1989).

The receptive "implantation window" is activated by an estradiol pulse. In the majority of mammalian species, the normal cyclic regression of the CL after embryonic attachment must be prevented in order to ensure continued production of progesterone. Progesterone supports continued endometrial function. This maintenance of ovarian function is a phenomenon called "maternal recognition of pregnancy" (Short, 1969). The conceptus provides the signal to maintain continuous progesterone secretion by the CL, and in this sense, "recognition" is a passive act by the mother (Browning and Wolf, 1981).

Stage-specific proteins synthesized and secreted by peri-implantation embryos have been shown to correlate with changes in specific proteins synthesized and released by the uterus in the mouse. These stage-specific proteins may be important in either signalling for embryonic implantation or in embryonic growth (Nieder et al., 1987).

It has been suggested that prior to embryonic attachment, embryo-

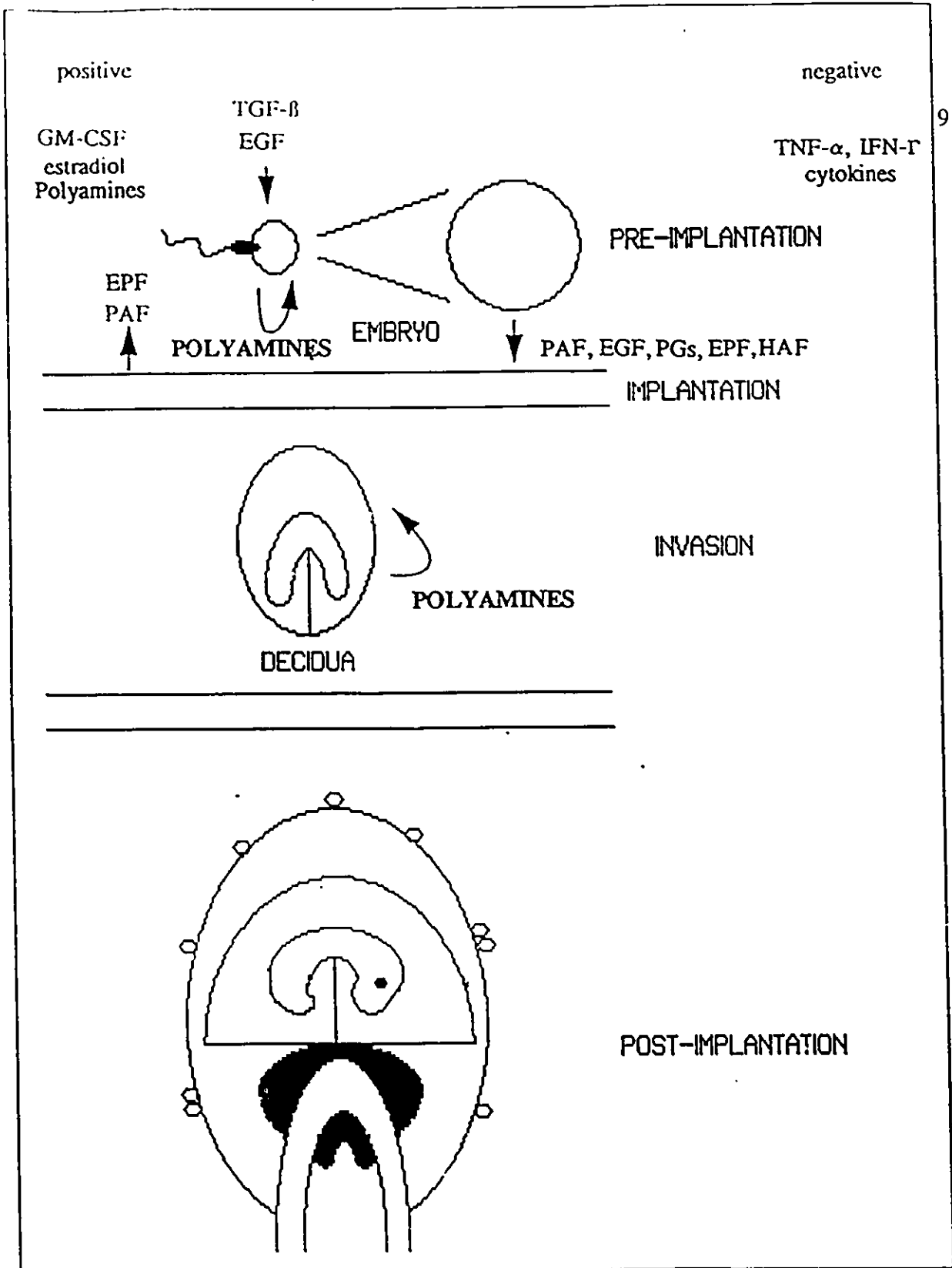
derived platelet-activating factor (PAF), and PAF-like materials are released, and may function as an important signal between the embryonic and the maternal tissues in rats, mice and humans (Kennedy, 1983; O'Neill 1985; O'Neill et al., 1985, 1989). Support for this suggestion in humans has come from the observation that human IVF embryos may produce PAF (O'Neill et al., 1987). This PAF production has been reported to correlate with the potential of IVF embryos to implant (O'Neill et al., 1987). However, recently Milligan and Finn (1990) failed to inhibit embryonic implantation in mice using specific PAF inhibitors. This suggests the embryonic-derived molecules described by O'Neill (1987) may be different from PAF.

PAF is present in the uterus in the mouse, and its release into the lumen of the oviduct (uterine tube) may be important in mammalian fertilization (Kuzan et al., 1990). PAF may also have a direct role in regulating embryonic development in the mouse (Ryan et al., 1989). Further, its administration triggers the appearance of early pregnancy factor (EPF) in the serum of estrous mice (Orozco et al., 1986). EPF is a glycoprotein associated with pregnancy and found in mammalian serum and urine during very early stages of pregnancy, even before implantation in a number of species including the pig, rabbit, rodents and humans (Morton et al., 1976, 1977, 1983; Sueoka et al., 1988b, 1989). In the

FIGURE 1:

Factors involved in blastocyst implantation

DEVELOPMENT OF CONCEPTUS



preimplantation period, EPF is produced by an interaction of factors derived from the ovary and the oviduct in the rabbit (Sueoka et al., 1988b). Secretion of PAF-like material from the fertilized oocyte of the rabbit, while it is still in the oviduct, may trigger EPF secretion, and thus explain the observed EPF activity from oviduct perfusions within 3 hours of fertilization (Sueoka et al., 1988a). Uterine PAF and EPF may be important for support of the pregnancy just prior to implantation (Clarke et al., 1987), but there are also potential roles for these mediators after implantation.

During the peri-implantation period, the uterine glands maintain synthesis and secretion of proteinaceous uterine fluid, and may play a role in early embryonic development and implantation (Given and Enders, 1980, 1981). For successful implantation, the uterine glands must be at a suitable stage of differentiation. The endometrial stroma undergoes reorganization in preparation for implantation (Dockery et al., 1990).

Using genetic, biochemical and immunological probes it has been demonstrated that galactosyltransferase, a cell surface enzyme involved in cell binding, functions as a recognition molecule during fertilization (Bayna et al., 1986), early embryonic development, cell migration and cell-matrix interactions (Sato et al., 1984; Bayna et al., 1986; Chavez, 1986). Other molecules, localized to the surface of the blastocyst, such as heparin/heparan sulphate and proteoglycans may

serve as ligands to their receptors on the uterine epithelium (Farach et al., 1987). The complex structure of the glycoprotein-bound large carbohydrate chains (glycans) found on surface of early embryonic cells is well suited to be recognized during intercellular communication (Ozawa et al., 1985).

1.2.B Attachment phase and early invasion of trophoblast into maternal endometrium

It has been proposed that embryonic cell surface recognition is mediated by protein-carbohydrate interactions. Changes in cell surface carbohydrates occur during early embryonic development and may be responsible for the stage-specific capacity of the outermost cells to attach to the uterine wall and become invasive (Pinsker and Mintz, 1973; Marticorena et al., 1983). Changes in embryonic surface glycoproteins can be the result of oviductal glycoproteins that become associated with embryos in their passage through the oviduct (Kapur and Johnson, 1985).

The process of blastocyst implantation, in humans and in rodents, entails adhesion to and invasion of the endometrium by the blastocyst followed by decidualization of the uterine stroma. Ligand-receptor binding reactions such as binding to laminin (Armant et al., 1986) occur while systemic endocrine, and local

paracrine and autocrine mechanisms at or near the implantation site are working in concert. By attaching to the receptors of target cells, a ligand can induce changes in conformation and in the cytoskeletal morphology of cells (Lawrence et al., 1979). Modifications in cytoskeletal function of the endometrial epithelium, caused by the adhesion of its membrane to the trophoblast membrane can thus result in accessibility of the human blastocyst to the endometrial basal lamina and permit penetration.

The trophoblastic epithelium must affix firmly to the uterine epithelial surface glycoproteins (Sretarugsa et al., 1987), penetrate this initial barrier and gain access to the endometrial stroma where the necessary components for placentation are available (Schlafke and Enders, 1975). Early abortions following human *in vitro* fertilization and embryo transfer (IVF-ET) showed histologic evidence of trophoblastic insufficiency, leading to failure of gestation (Nebel et al., 1985).

Alignment of the blastocyst over the implantation site denotes the oppositional stage of nidation in the rat. Trophoblast pseudopodia are very active metabolically and may serve to align the blastocyst over the stroma regions (Garris, 1984). Concomitantly, the luminal epithelial cells in the rat undergo a process of differentiation accompanied by progressive development of morphological and functional polarity (Glasser et al., 1988). Initial embryo-uterine interactions require

heterotypic cell adhesion between the apical membranes of the two polarized epithelial cell populations; that is, the interacting cells are genetically different, and from differing tissue types. Loss of surface negativity (Hewitt et al., 1979), and stage-specific alterations in protein and saccharide composition of the apical surface of the endometrial epithelium occurs in rabbits prior to blastocyst implantation (Anderson et al., 1986). These alterations may have a role in mediating signal transduction between the implanting embryo and the endometrium.

Transglutaminase (EC 2.3.2.13) is an enzyme that is able to bind proteins covalently by forming cross-bridges and by cross-linking through diamines and polyamines (Folk, 1980; Williams-Ashman and Canellakis, 1980). Transglutaminase is present in the rabbit uterus and its activity is increased 5-fold during early pregnancy (Alving and Laki, 1967), suggesting that the enzyme may play a role in adhesion of the blastocyst to the uterine luminal epithelial cell surface. At present, a ligand-receptor binding system between the trophoblast and the uterine epithelial plasma membranes is obscure. Polyamines, such as spermine and spermidine localized on the surface of the blastocyst may play important roles in blastocyst adhesion to the uterine epithelium by serving as ligands to their receptors on the endometrial epithelial surface. Transglutaminase has also been suggested to play a role in hormone binding in the uterus in cows (Grasso et al., 1987), in sperm maturation in semen in humans (Porta et al., 1986), and in suppression of

epididymal sperm antigenicity *in vitro* in rabbits (Mukherjee et al., 1983).

Some of the factors involved in the ligand-receptor binding mechanism may have multiple functions. For example, hyaluronic acid (HA), a glycosaminoglycan present in the endometrium, is known to be involved in cell-cell adhesion (Hamasima, 1982). Another suggested function for murine endometrial HA is to promote the differentiation of extra-embryonic tissues of peri-implantation embryos (Hamasima, 1982). HA and ovarian (follicular fluid) glycosaminoglycans have also been shown to prevent spontaneous degeneration of mouse oocytes *in vitro* (Sato et al., 1987).

A variety of extracellular matrix proteins may support attachment. Collagen is associated with the 2-cell stage embryo of the mouse, and may be involved in blastocyst adhesion to the endometrium during implantation (Sherman et al., 1980). Fibronectin, an extracellular matrix and cell surface-associated glycoprotein detected in late blastocysts, is implicated in mediation of cell adhesion to extracellular matrix in the mouse embryo (Wartiovaara et al., 1979). Endogenous fibronectin-blastomere interaction may be crucial for preimplantation mouse embryonic development. A ligand-receptor mechanism involving laminin is also involved in the attachment and outgrowth of murine trophoblast on extracellular matrix components *in vitro* (Tsuiki et al., 1989).

All basal laminae contain laminin, collagen IV and various heparan

sulphate proteoglycans. It is known that penetration of the basal lamina by invading tumor cells involves laminin receptor production by the tumor cells (Liotta, 1986). It was hypothesized that in the mouse tumor cells anchor to the basal lamina and secrete or induce other cells to secrete hydrolytic enzymes, which in turn break down the local matrix to allow tumor cell penetration. Trophoblastic cells seem to pause at the basal lamina and send a signal to the decidual cells to secrete proteolytic enzymes, so that the basal lamina is invaded from the stromal side by the decidual cells (Schlafke and Enders, 1975). Prostaglandins (PGs), known to be involved in implantation and decidualization, may also be involved at this point (Kennedy, 1983). Once the trophoblast attaches to the subepithelial matrix, it penetrates by digesting its way using proteolytic enzymes (Schlafke and Enders, 1975) and continues growing to form the placenta, and maternal stroma is transformed into decidual cells of the placenta.

In the peri-implantation period, the blastocyst of humans (Weisburger et al., 1978; Heap et al., 1979; Sauer, 1979; Kennedy, 1983), of the rabbit (Harper et al., 1983), and of the mouse (Neider et al., 1987; Weitlauf and Suda-Hartman, 1988; Weitlauf, 1989a, 1989b) synthesizes and releases several soluble factors, including steroids, PGs, histamine and proteins that may influence metabolic activity in the uterus. Blastocyst-derived PGs in the rabbit appear to be important for the increase in vascular permeability that occurs in the stroma underlying the implanting

blastocysts (Jones et al., 1986; Harper et al., 1987). As a result of blastocyst interaction with the uterine epithelium by release of these PGs, uterine PAF may also be released thus increasing the vascular permeability at the implantation site. Steroids originating from the blastocyst increase the number of nuclear receptors for estrogen and progesterone in the endometrial implantation site (Logeat et al., 1980; Ghosh and Sengupta, 1988).

At least one embryo product maintains ovarian hormone secretion. Bovine trophoblast protein-1 (TP-1) has been implicated as the bovine conceptus signal mediating maternal recognition of pregnancy by acting as a paracrine hormone directed locally to the uterine endometrium where it apparently influences both protein synthesis and prostaglandin production (Bartal et al., 1985; Bazer et al., 1986; Helmer et al., 1987; Vallet et al., 1987; Kazemi et al., 1988). In cattle, TP-1 complex has been shown to exert an antiluteolytic effect to extend corpus luteum lifespan (Helmer et al., 1989). Recently, TP-1 has been identified as an interferon-alpha II ($\text{INF-}\alpha_{\text{II}}$) (Roberts et al., 1989). Since TP-1/ $\text{INF-}\alpha_{\text{II}}$ is immunosuppressive it may be involved in immunoprotection of the conceptus (Bazer et al., 1986; Murray et al., 1987). It has been suggested that interferon production by embryonal trophoblast cells and by placental structures suppresses the induction of uterine PGs (Fowler et al., 1980; Chard et al., 1986; Fincher et al., 1986; Helmer et al., 1987). In other species such as the human, human chorionic

gonadotrophin hCG), synthesized by the syncytiotrophoblast, rather than IFN- α_1 , appears to be responsible for the maintenance of the CL (Atkinson et al., 1975).

1.3 ASSISTED REPRODUCTION

Assisted reproduction provides an opportunity to look at the mechanisms involved in preimplantation embryonic development and embryonic implantation. IVF-ET have been achieved with many mammals, including humans. Modern technology has allowed couples who were previously considered infertile to conceive by permitting the first stages of conception to take place in the laboratory. Using these modalities, the frequency of chromosomal abnormalities, growth retardation, factors involved in the implantation process and underlying physiologic mechanisms that fail frequently in natural mating, can be recorded. Moreover, failure of fertilization can be excluded as an explanation for infertility in IVF-ET patients.

In vitro cultures of dividing embryos can be efficient for monitoring factors affecting embryonic survival and development. Under these conditions the embryo is neither exposed to maternal tubal secretions and cells, nor does it interact with the uterine milieu including products of the maternal immune system.

Some of the above secretions may include growth factors from the mother and/or immunosuppressive molecules that, in part, act to suppress maternal cytotoxic cells. Some factors may be produced by the *in vitro* fertilized embryo itself.

Approximately 85% of human oocytes fertilize *in vitro* (Lopata, 1983; Edwards and Steptoe, 1983). Following IVF the human cells are incubated using conditions that simulate those of the human oviduct. At 48 hours following oocyte retrieval, the embryos normally reach 4-8 cells, and they may be transferred to the uterus (ET). However, embryos that had successfully fertilized and divided *in vitro* do not appear to implant in the endometrium as well as *in vivo* fertilized embryos (Papaioannou and Ebert, 1986; Fischer, 1987). A considerably higher than normal failure rate has been experienced by women suffering from blocked tube infertility who participate in IVF-ET programs (Rudak et al., 1984, 1985).

Chromosomal aberrations originating from the spermatozoa may contribute to some of cases of infertility. In large scale studies of human sperm chromosomes visualized following IVF, structural aberrations were found at a frequency of 6.2-13.0% (Martin and Radmaker, 1987; Brandiff et al., 1985). In the mouse, 0.7-8.0% aberrations were reported for sperm following IVF (Hansmann and Jenderny, 1983; Martin-DeLeon and Boice, 1985).

In humans, four embryos are normally transferred to the uterus following an IVF procedure, provided there are sufficient viable embryos to be transferred.

Assuming a 50% chance of chromosomal abnormality (Hertig, 1959), the probability of transferring at least one normal embryo is theoretically $(1 - (0.5)^4) \times 100 = 93.8\%$. *In vivo*, if 20% of human embryos lead to live born fetuses (Roberts and Lowe, 1975), and 60% are chromosomally defective, then 50% (20/40) of normal embryos should succeed in implanting. Hence, IVF should give a $93.8 \times 0.5 = 46.9\%$ success rate. However, the percentage of take-home-babies per IVF trial is approximately 15% when at least 3 embryos are transferred (Lopata, 1983; Edwards and Steptoe, 1983). Thus, most transferred IVF embryos fail to implant.

The causes for subnormal embryonic implantation rates could be similar for embryos fertilized *in vivo* and those fertilized *in vitro*. In addition, a decline in fruitful implantation could be attributed to the laboratory manipulations leading to fertilization, to faulty *in vitro* embryonic development, to the embryonic transfer procedure, or to events following the transfer of the embryo to the uterus. These may include an inadequate uterine milieu, a deficiency caused by the embryo, or both. Asynchrony between embryonic development and uterine characteristics may lead to implantation failure (Vanderhyden et al., 1986; Fischer 1987).

In vitro culture techniques for preimplantation mouse embryos have been utilized to study the effects and mechanisms of action of drugs, and to identify embryotoxicity of samples based on their effect on developmental and cytological parameters (Spielmann et al., 1982; Abraham et al., 1986). It has been

demonstrated that the effect of chemical exposure on mouse preimplantation embryos may manifest long after the time of exposure (Iannaccone et al., 1987). Hence, early injury to the embryo may explain in part the high abortion rate (30-40%) of IVF embryos which do not manage to implant; in normal *in vivo* pregnancy in humans, the abortion rate is believed to be lower (i.e 5-15%), and even if chemical pregnancies are included, does not exceed 20-25% (Chemical pregnancies are defined as one or two β -hCG readings above 10 mIU/ml, or two readings showing a positive and increasing β -hCG value taken at least 10 days post oocyte retrieval) (Jones et al., 1983; Liu et al., 1988).

1.4 THE IMMUNOLOGY OF IMPLANTATION

In theory, all embryonic cells in contact with maternal tissues should be subject to rejection for the following reasons:

- 1) A body is foreign if it displays antigens other than "self".
- 2) Under conditions which allow recognition and suitable response by the mother, foreign bodies are rejected by the maternal immune system.
- 3) The embryo is a foreign body because it expresses paternal alloantigens.
- 4) In the preimplantation phase the embryo is in contact with oviductal fluid which contains maternal lymphocytes and macrophages. After attachment, the embryo is in direct contact with the maternal endometrium which contains lymphocytes and macrophages.

However, blastocysts that succeed in implanting are not rejected, or else eutherian reproduction would be impossible. How then can we explain the success of at least a percentage of embryos?

The defence response in humans includes both specific immunity and

a nonspecific resistance component. The specific processes consist of humoral (antibody) and cellular (cell-mediated) components. The humoral processes involve the interactions between antigens and antibodies. The cellular processes entail interactions between antigens and thymus-derived lymphocytes, which act both directly and through elaboration of substances such as cytokines and lymphokines. The nonspecific mechanisms of the immune system, such as phagocytosis by macrophages and mast cell degranulation, are often involved in antibody and lymphocyte reactions against antigenic substances. Macrophages and natural killer cells may also identify and kill virus-altered cells.

Generally, T-lymphocytes are responsible for regulation of the immune response. Using surface markers the regulatory cells can be divided into two major subsets: CD4⁺ (helper-T cells) and CD8⁺ (cytotoxic- and suppressor-T cells). Macrophages are necessary for processing and presenting antigens to T-cells. Activated T-cells mediate cellular immunity by direct toxic effects, reacting directly with cell-membrane-associated-antigens, or by releasing various lymphokines. The suppressor T-cells can down-regulate antibody production by inhibiting helper T-cells, and can down regulate cellular immune responses by inhibiting cytotoxic cells and the production of lymphokines. This suppressive effect may involve direct cell contact and/or production of suppressive factors.

The major histocompatibility complex (MHC) regulates immune responses and is determined by a group of genes that code for cell surface antigens, known as the human leukocyte antigens (HLA) in humans. Class I MHC antigens regulate immune responses by restricting antigen recognition by CD8⁺ (suppressor and cytotoxic T-lymphocytes (CTLs)). Class II MHC antigens regulate immune responses by restricting antigen recognition by CD4⁺ cells. It has also been suggested from studies in mice, that class I genes may regulate cell-cell interactions during embryonic development (Warner et al., 1987).

1.4.A Immunological aspects in the pre-attachment period

Maternal humoral immunity directed primarily against products of the paternal MHC component of the fetus has been demonstrated in some pregnancies in mice and in humans (Innes et al., 1989). However, blastocysts transplanted from one inbred strain of mice to the pseudopregnant uterus of another hyperimmune strain implanted and developed (Kirby et al., 1966). Tubal eggs and blastocysts that were transplanted to the kidney or to the peritoneal cavity failed to develop. Thus, in preimmunized recipients a local protective function was found only in the uterus, that allowed blastocysts to implant (McLaren and Tarkowski, 1963; Kirby et al., 1966).

Human blastocysts and cultured human trophoblast cells secrete immunosuppressive factors that induce suppressor T-lymphocytes, and activity increased during the early phase of embryonic development (Dey et al., 1979). Similar data was obtained by Mayumi et al. (1985) using mouse blastocysts. No such immunosuppressive factors were detected in pseudopregnant, and in non-pregnant animals, where no embryo was present. This factor could be directly suppressive by inhibiting the intra-cytoplasmic signal transduction that triggers the T-cell proliferation after the interaction between interleukin-2 (IL-2) and its receptor. Alternately, the factor could suppress indirectly by activating suppressor T-cells.

1.4.B Immunological aspects during the post-attachment stage

The fetal component of the maternal-fetal interface during pregnancy is comprised of three types of trophoblast cells: placental syncytiotrophoblast, placental cytotrophoblast cells and chorionic membrane cytotrophoblast cells. Syncytiotrophoblast cells are exposed to maternal blood and chorionic membrane, while trophoblast cells reside in intimate association with maternal tissues.

Ober and Weitkamp (1990) from their studies of Hutterites concluded that '...normal pregnancy requires maternal recognition of, and response to,

paternally derived fetal antigens. Thus, pregnancy failure could result from (a) inadequate or inappropriate maternal immune response to fetal antigens, or (b) failure of the maternal immune system to recognize fetal antigens. The former may be related to maternal MHC haplotypes carrying particular immune response genes, while the latter may result from maternal-fetal histocompatibility....'. It is plausible that a combination of factors cause pregnancy failure.

Invasive extravillous cytotrophoblast express novel class I MHC antigens in humans (Head et al., 1987). Class II MHC genes restrict antigen recognition by CD4⁺ T-cells, and are not expressed on any trophoblast cell population (Head et al., 1987). The expression of MHC antigens *in vitro* and *in vivo* can be regulated by a variety of lymphokines and by IFN- γ (Halloran et al., 1986). Class I antigens but not class II antigens can be induced on both murine and human trophoblast cells by IFN- γ (Zukerman and Head, 1986). Trophoblast cells are highly resistant to the induction of MHC antigens. Mouse trophoblast cells are resistant to MHC-specific cell-mediated lysis by CTLs, even when Class I expression is augmented with IFN- γ (Zukerman and Head, 1987). Mouse trophoblast is also resistant to natural killer (NK) cells and to cytokine mediated killing (by tumor necrosis factor alpha (TNF- α)) (Koyama et al., 1986; Drake and Head, 1990). However, both murine and human trophoblast are killed by IL-2 activated NK-type cells (lymphokine activated killer (LAK) cells) (Drake and Head, 1989).

HLA-DR antigens are expressed on T cells when they are activated. IL-2 is thought to be produced by helper T-cells that have been stimulated by a combination of IL-1, and antigen bound to HLA-DR gene products (class II MHC) on the cell surface of antigen presenting cells. NK cells also produce a large quantity of IL-2 upon activation. A significant number of the mature T-cells in the decidua were found to be CD8⁺ (T8⁺), and express HLA-DR antigens, suggesting they were activated suppressor T-cells and CTLs. About half of the T-cell population in the normal decidua were found to be immature, tissue NK-like cells, and they are thought to cause high proliferative activity to IL-2 (Kanzaki et al., 1989).

It has been suggested that genes in the HLA-DR region may affect fertilization, implantation and gestational processes (Ober and Weitkamp, 1990). In decidua of first trimester pregnancy, leucocytes expressing HLA-DR antigens were mostly tissue macrophages, and only a small proportion of T cells were positive for HLA-DR (Bulmer and Johnson, 1985). A large number of HLA-DR positive macrophages were found in women who suffered from endometriosis (Haney et al., 1983). Women suffering from endometriosis proved to have high numbers of peritoneal lymphocytes, and of oviductal macrophages that exhibited more phagocytizing capacity than macrophages from fertile women (Muscato et al., 1982; Haney et al., 1983; Hill and Anderson, 1989). Infertility due to failure of

implantation in endometriosis, may be due to maternal production of factors that impede embryonic development (Hahn et al., 1986).

The presence of activated macrophages expressing class II antigens and of helper-T cells in the human oviduct suggested cytokines, such as gamma-interferon (IFN- γ) and IL-1 were involved. IL-1 and TNF- α released by human maternal macrophages can lead to embryonic damage (Haney et al., 1983; Hill et al., 1987). However, some uterine mechanisms for protection of the blastocyst appear to be active during delay of implantation in the mouse (Hakansson et al., 1978). Knowing the response of endometrial cells to hormonal stimulation at the molecular level would help in understanding the regulated and the diseased states of the endometrium.

The lack of certain pregnancy-associated immunologic responses, such as production of lymphotoxic alloantibodies and serum suppressor factors (elicited by fetal lymphocytes that cross the placenta), may be the result of genetic variability and underlie reproductive disorders (Ober and Weitkamp, 1990). HLA-B8 and HLA-DR3 haplotypes have been implicated in autoimmune-associated spontaneous abortions. However, in non-autoimmune patients, no association between particular antigens and spontaneous abortion has been found (Giordano, 1990).

The embryo may synthesize and secrete factors which locally suppress maternal rejection mechanisms, thus providing a safer environment at the maternal-

fetal interface (Murray et al., 1987; Panadian et al., 1988). Inhibition of the effector phase of the maternal cell-mediated immune response would prevent damage to the fetus. An additional mechanism providing protection from immunological attack was suggested by Kirby et al. (1964, 1966), who observed that trophoblast cells are surrounded by a layer of fibrinoid material. It was proposed this layer prevents trophoblast histocompatibility antigens from reaching and sensitizing the host. This layer may also provide resistance to lysis. Its existence has, however, been disproved.

Steroid hormones have been found to have immuno-suppressive activity. Sera from pregnant women and from women taking oral contraceptives inhibit lymphocyte activity and induce an increase in some glycoproteins (Bousquet and Fizet, 1984). Immunoregulation in humans may also be attributed to the binding of EPF to lymphocytes, thereby stimulating the release of soluble effector molecules (Rolfe et al., 1989).

Progesterone may be crucial for stimulation of stromal cells to produce stimulatory and suppressive factors to assist in blastocyst interaction with the endometrial epithelium. In rats, progesterone promotes a massive infiltration of the uterine cervix by eosinophilic polymorphonuclear leucocytes (Luque and Montes, 1989). In humans, administration of the progesterone antagonist mifeprisone (RU486), and of the progesterone synthesis inhibitor epostane, has been effective

in the termination of early pregnancy (Couzinet et al., 1986; Crooij et al., 1988; Bulieu, 1989; Cekan et al., 1989; Silvestre et al., 1990).

Recently it has been demonstrated that progesterone binding capacity of lymphocytes from pregnant women (first trimester) is higher than that of lymphocytes from non-pregnant women (Szekeres-Bartho et al., 1990). This difference in progesterone binding capacity is due to specific progesterone receptors on these lymphocytes. A component of RU486 activity may therefore contribute to pregnancy disruption by acting on lymphocyte progesterone receptors.

Local immune modulation by maternal immunosuppressive factors may be macrophage-derived, and may cause down-regulation of the activities of other immunocompetent cells. The local immune response involving macrophages present in the oviduct and in the endometrium is likely elicited by the implanting blastocyst in the mouse (Tachi et al., 1981; Haney et al., 1983; Tachi and Tachi, 1986; Tachi, 1990), and in guinea-pigs (Sype et al., 1989). Maternal decidual macrophages may play a role in phagocytosis, antigen-presentation in the rat and in the mouse (Tachi and Tachi, 1989) and in humans (Oksenberg et al., 1986), and may have suppressor functions mediated by prostaglandin E_2 (Lala et al., 1986). By removing antigen-antibody complexes, macrophages may facilitate uptake of maternally-derived immunoglobulin G (IgG) to provide passive immunity to the embryo.

Human decidual macrophages were HLA-DR positive, while fetal

macrophages in the chorionic villi (in first trimester) were rarely HLA-DR positive (Bulmer et al., 1988). A large number of HLA-DR positive macrophages were associated with ectopic and intrauterine pregnancy tissues (Earl et al., 1987).

Cytokines may regulate preimplantation and postimplantation cell proliferation and differentiation. Regulation of postimplantation events by cytokines is suggested by the presence of cytokine receptors on decidual cells and on the trophoblast. It has also been demonstrated that the ability of human sperm to penetrate zona-free hamster egg is negatively affected by lymphokines and monokines (Hill et al., 1989). The ability of the sperm to penetrate a zona-free hamster egg is a correlate of the ability to fertilize a human egg.

In abortion-prone murine CBA/J x DBA/2 matings, it was shown that TNF- α , IFN- γ and IL-2 caused an increase in fetal resorption rate, while cytokines of the colony stimulating factor (CSF) family, such as GM-CSF, released by trophoblast cells and by decidua cells, increased the chance of fetal survival (Clark et al., 1989a; Chaouat et al., 1990). It was suggested that GM-CSF can down-regulate TNF- α production and thus support placental growth.

Transferrin (TF) is a serum β -globulin that transports iron and zinc and binds to transferrin receptor (TFRC) on the trophoblast. It has been suggested that

by binding maternal TF to trophoblast antigens, these antigens are masked from maternal immuno-surveillance (Faulk and Galbraith, 1979). Other proteins expressed on trophoblast cells such as insulin and insulin-like growth factor-I receptors may also be involved in interactions at the maternal-trophoblast interface and regulate and promote cell growth (Murphy et al., 1987).

Some reproductive dysfunctions are explained by embryotoxic activity in aborting mother's serum. It has been shown that at least some women who suffer from recurrent abortions or unexplained infertility may have embryotoxic or mutagenic factors in their sera (Porter et al., 1988). Exposure of mouse preimplantation trophoblast cells to sera from women with reproductive dysfunction resulted in inhibition of attachment or disruption of cytotrophoblastic layer (Chavez and McIntyre, 1984).

1.5 GROWTH FACTORS AND THE REPRODUCTIVE PROCESS

1.5.A Growth factors during preimplantation embryonic differentiation

Biochemical processes in mammalian cells, including membrane transport and rates of RNA and protein synthesis and degradation, are influenced by growth promoting agents. Factors related to transforming growth factor-beta (TGF- β), epidermal growth factor (EGF), insulin and interleukins are associated with cell growth and differentiation in a variety of systems (Metcalf, 1989). It is highly likely that these growth factors are associated with embryonic development (Sporn et al., 1986; Simmen et al., 1988; Rizzino, 1988; Gupta and Dey, 1989). EGF receptors are detected on mouse trophoblast outgrowths (Wood and Kaye, 1989; Paria et al., 1989; Paria and Dey, 1990), and insulin receptors are detected on human morulae (Thraill et al., 1988). Besides embryonic-derived molecules, many specific proteins and factors, including EGF, are synthesized and secreted by human decidua of early pregnancy (Linzer and Nathans, 1985; Bell, 1986; Yamamoto et al., 1989).

Polyamines are defined molecules essential for cell proliferation and differentiation, and are present in all living cells (Janne et al., 1973; Rees et al.,

1979; Williams-Ashman and Canellakis, 1979; Heby, 1981; Heby and Emanuelsson, 1981; Oka et al., 1981; Ito and Igarashi, 1990). It is likely that low, constant levels of polyamines are essential for cell growth, while higher levels are transiently required during cell differentiation. (Williams-Ashman and Canellakis, 1979). In this sense, one can refer to polyamines as "growth factors".

A brief description of the chemical properties of polyamines is followed by an outline of their role in fertilization, in early embryonic development and in embryonic implantation.

FIGURE 2:

Polyamines: structure and molecular weights

$\text{NH}_2-(\text{CH}_2)_3-\text{NH}-(\text{CH}_2)_4-\text{NH}-(\text{CH}_2)_3-\text{NH}_2$	Spermine	MWt=202.34
$\text{NH}_2-(\text{CH}_2)_3-\text{NH}-(\text{CH}_2)_4-\text{NH}_2$	Spermidine	MWt=145.24
$\text{NH}_2-(\text{CH}_2)_4-\text{NH}_2$	Putrescine	MWt= 88.15

FIGURE 3:

Polyamine metabolites and related enzymes

ADH - aldehyde dehydrogenase

ODC - L-ornithine decarboxylase (EC 4.1.1.17)

DAO - diamine oxidase (EC 1.4.3.6)

2 - S-adenosyl-L-methionine decarboxylase (EC 4.1.1.50)

3 - spermidine synthase (EC 2.5.1.16)

4 - spermine synthase (EC 2.5.1.22)

5 - spermidine/spermine N¹-acetyltransferase (no EC number)

6 - polyamine oxidase (FAD-dependent) (no EC number)

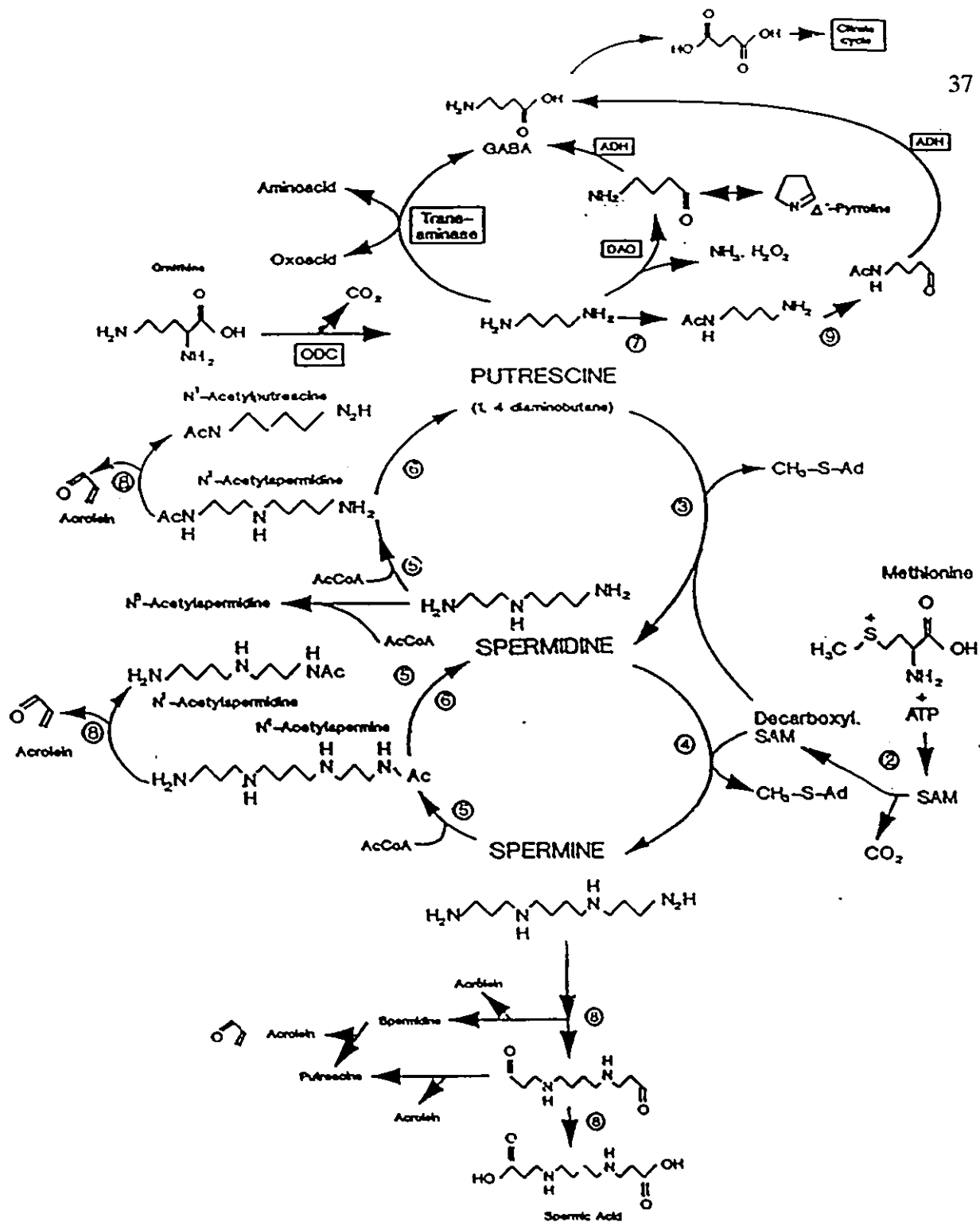
7 - acetyl-CoA-1,4-diamine N-acetyltransferase

8 - serum spermine oxidase (EC 1.5.3.3)

9 - monoamine oxidase (EC 1.4.3.4)

POLYAMINE METABOLITES AND RELATED ENZYMES

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1.5.A.i The chemical properties of polyamines

Polyamines are low molecular weight, polycationic, non-protein nitrogenous bases (Figure 2). There are two polyamines: spermine (N,N'-bis(3-aminopropyl)-tetramethylene-1,4-diamine) and spermidine (N-(3-aminopropyl)-tetramethylene-1,4-diamine). Putrescine (1,4-tetramethylenediamine), a diamine, is included in this research as a polyamine, because it is the immediate precursor in the biosynthesis of the polyamines (Figure 3), and closely implicated in reactions mediated by spermine and spermidine (Tabor et al., 1956; Pegg, 1970; Seiler et al., 1981; Pegg and McCann, 1982).

The metabolism, interconversions and distribution of the polyamines have been explored extensively (Rosenthal and Tabor, 1956; Tabor and Tabor, 1964, 1976, 1984; Pegg et al., 1982; Scalabrino and Ferioli, 1984; Pegg, 1986). Polyamines are present in acetylated, oxidized, and protein-bound forms in virtually all living cells (Aigner-Held and Daves, 1980; Tabor and Tabor, 1985). As cellular cations, polyamines mediate cellular and extracellular processes. Spermine, for example, being a tetra-amine, carries four positive charges at pH values near neutrality and therefore has a high affinity for anions such as nucleic acids and phospholipids (Pegg, 1986).

Although the details of the molecular mechanisms of polyamine

associations with nucleic acids are still unclear, it is known that polyamines stabilize DNA and enhance DNA synthesis (Allison et al., 1965; Feuerstein et al., 1990; Smith and Walker, 1990). It is conceivable that low polyamine concentrations lead to charge neutralization and high polyamine concentrations result in charge reversal, thus affecting DNA condensation and conformation. Polyamines interact with tRNA, mRNA and rRNA in most somatic cells (Pegg, 1970; Russell, 1970; Chiu and Sung, 1972; Tabor and Tabor, 1972; Konecki et al., 1975), and their interactions with other cell components can promote changes in membranes and enzymes (Rottenberg and Marbach, 1990; Votyakova et al., 1990).

1.5.A.ii Polyamines, fertilization and early embryonic differentiation

During laboratory handling in human IVF-ET procedures, preimplantation embryos are usually transferred to fresh culture medium once every 24 hours. This can be reviewed from two perspectives. The cultured medium, containing factors released by the dividing embryos is discarded in the process, leading to the loss of these factors. On the other hand, the embryo is bathed in waste components that surround it for 24 hours without being cleared or detoxified. In addition, the insemination, growth and embryonic transfer media may be

deficient in significant ions, growth factors and enzymes critical for normal embryonic development. A change in medium to mimic tubal fluid partially improves implantation rates (Quinn et al., 1984; Quinn et al., 1985). Higher success rates are also obtained when fertilization occurs *in vivo*, following gamete intra-fallopian transfer (GIFT) (Asch et al., 1988). These observations suggest that essential factors are lost in IVF, and that their preservation is important for improved implantation rates following IVF.

Polyamines may have several roles in relation to fertilization and embryo development. The use of polyamine-synthesis inhibitors has shown that particular polyamines are required in specific situations. In the sea-urchin, there is an increase in oocyte polyamine levels immediately following fertilization (Oberdorf et al., 1989). High concentrations of polyamines are found in embryonic and fetal tissues of rats (Maudsley and Kobayashi, 1977a; Andersson et al., 1978; Lundgren and Oka, 1978; Snyder and Russell, 1970). Increased accumulation of putrescine and spermidine as well as an early enhancement in the activity of polyamine-synthesizing enzymes usually precedes the growth of any tissue (Heby, 1981). During exponential *in vitro* growth of cells, cellular polyamine content normally increases and with continued time in culture gradually decreases. During normal human pregnancy there is a rise in polyamine levels in amniotic fluid, plasma, and urine (Russell et al., 1978; Russell, 1984). Consequently, the physiologic function of the

polyamines in mammalian germ cell differentiation and in early embryonic development was studied (Heby and Emanuelsson, 1981; O'Toole et al., 1989).

Heby and Emanuelsson (1981) noted that the major ultrastructural effect causing *in vitro* mouse embryonic arrest by spermine deprivation, was an interference with nucleolar formation. It was suggested that polyamine synthesis is essential for the expression of ribosomal genes associated with oogenesis and embryogenesis. Using a polyamine-dependent mutant of *Escherichia coli*, polyamine stimulation of ribosomal synthesis and activity has been demonstrated (Kashiwagi et al., 1989).

Methylglyoxal- bis-(guanylhydrazone) (MGBG) is an inhibitor of S-adenosyl-L-methionine decarboxylase (SAM-D), a key enzyme in polyamine biosynthesis. Addition of MGBG to *in vitro* cultures of mouse preimplantation embryos led to arrest of early embryonic development, and strongly reduced embryonal DNA synthetic rate (Zwierzchowski et al., 1986). L-ornithine decarboxylase (ODC) (EC 4.1.1.17) is the first and rate-limiting enzyme for polyamine synthesis (Figure 3) (Russell and Snyder, 1969; Russell, 1985). Because of its key role, ODC is essential for the growth and development of newly implanted embryonic tissue (Fozard et al., 1980a). The effects of inhibition of polyamine biosynthetic enzymes on embryonic growth at differing stages of gestation have been demonstrated by several research groups (Heby, 1981; Marton and

Morris, 1987). Studies of cells treated with ODC inhibitors, such as DL- α -difluoromethylornithine (DFMO- α), showed that the inhibited cells grew at a normal rate if spermine was added (Mamont et al., 1980; Porter and Bergeron, 1983).

All cellular functions of polyamines can theoretically be fulfilled by spermine (Heby and Persson, 1990). Addition of exogenous spermidine will also support cell growth (Pegg, 1986). Polyamines have been implicated in cavitation of mouse 2-cell preimplantation embryos cultured *in vitro* (Alexandre, 1979). The inhibition of blastocyst formation and DNA synthesis by MGBG was readily reversible by an exogenous supply of spermine and/or spermidine to the culture medium. In contrast, DFMO- α had no effect on embryos cultured for 1 or 2 days. On the third day, embryonic DNA synthesis in the presence of DFMO- α was significantly depressed. These observations suggest that during early development of the preimplantation mouse embryo, spermine and spermidine are involved in regulating embryonic growth and DNA synthesis. They may also indicate a role for putrescine at a later stage of mouse embryonic development.

1.5.B Growth factors regulation of peri-implantation and post-attachment embryonic functions

Polyamines are present in the human placenta (Porta et al., 1978), and

there is a close relationship between the levels of polyamines and RNA levels of the placenta. Ovarian and uterine polyamine metabolism are known to be influenced by prolactin, luteinizing hormone (LH), as well as by estrogens (Kobayashi et al., 1971). Antisera to LH and prolactin interfere with placental polyamine content and placental function in the mouse (Thakur et al., 1975), and there are fluctuations of unbound whole blood polyamine levels during the menstrual cycle in humans (Lundgren et al., 1976).

Metabolic transformation of polyamines may have potential regulatory functions. For example, excessive transformation of putrescine to gamma-aminobutyric-acid (GABA) noted in early neural development, may imply that it is the factor controlling cellular differentiation and modulation of protein synthesis in the central nervous system (Seiler, 1980). Addition of spermine to nuclei isolated from decidual tissue produced an increase in the rate of RNA synthesis (Hoshiai et al., 1981). Spermidine in these nuclei is involved in elongation of RNA chains, but not in initiation of new RNA chains (Hoshiai et al., 1981).

Early mammalian embryogenesis represents one of the most actively dividing and differentiating cell systems, and numerous studies have described a substantial increase in ODC activity during embryogenesis (Fozard et al., 1980b). In the rat ovary, administration of LH or hCG caused the induction of ODC (Kobayashi et al., 1971; Maudsley and Kobayashi, 1977b). In the mouse uterus,

ODC activity begins to increase shortly after nidation and reaches a peak on day 8 of gestation. The biochemical changes peak in correlation to embryonic growth (Hiramatsu et al., 1981; Hoshiai et al., 1981). Treatment of mouse embryos with DFMO- α during days 5 to 8 of gestation showed that polyamine concentrations were diminished, embryonic development failed to progress resulting in arrested embryos that were subsequently resorbed (Fozard et al., 1980b; Fozard and Koch-Weser, 1982).

Administration of DFMO- α via the drinking water of pregnant rats and rabbits, significantly reduced maternal food and water consumption and body weight gain, and all conceptuses were aborted or resorbed (O'Toole et al., 1989). By using DFMO- α , peak ODC activity in the mouse uterus was localized to the developing decidual tissue (Barkai and Kraicer, 1978), and the embryo (Fozard et al., 1980b). The embryotoxicity and severe growth retardation demonstrated in these studies imply that adequate polyamine levels are also essential for normal embryonic and fetal postimplantation development.

1.6 POLYAMINES - THE FACTORS LINKING GROWTH AND IMMUNOLOGY?

In comparison to other cell types, cells of the immune system appear to be especially susceptible to polyamines (Byrd et al., 1977; Theoharides, 1980). Numerous studies have reported the influence of polyamines on *in vitro* parameters of immunity. Induction of ODC may lead to proliferation and activation of lymphocytes, and may be controlled by polyamines (Kay and Cooke, 1971; Kay et al., 1972; Kay and Lindsay 1973a, 1973b). Micromolar quantities of spermine and spermidine inhibit both lymphocyte transformation (Murray et al., 1977), and responses of primary cultures of murine spleen cells (Byrd et al., 1977).

Bovine or fetal bovine serum (FBS), a common culture medium supplement, contains several amine oxidases. A number of studies have reported that spermine and spermidine suppress mitogen-stimulated lymphocyte proliferation in the presence of FBS (Byrd et al., 1977; Gaugas and Curzen, 1978; Morgan and Illei, 1980; Quan et al., 1990). Spermine incubation in the presence of amine oxidases has been shown to produce six major oxidation products (Labib and Tomasi Jr., 1981). Oxidized polyamines generate the relevant aminoaldehydes, which

are highly reactive (Blaschko, 1962; Kimes and Morris, 1971a; Holtta, 1977). Acrolein formation by these unstable products might be prevented *in vivo* by binding of the unstable aminoaldehydes to proteins to yield complexes. *In vitro* acrolein is extremely toxic (Alarcon, 1970). It has been suggested that it is an oxidized polyamine product which potently arrests cell proliferation (Gaugas and Dewey, 1979).

The reversibility of lymphocyte proliferation suppression by polyamines, argues against a cytotoxic effect of polyamines (Byrd et al., 1977; Patt et al., 1981; Patt et al., 1982). It is, of course, possible that both cytotoxic and immunosuppressive factors are formed by polyamines.

1.7 POLYAMINES AND IN VITRO FERTILIZATION

Daya and Clark (1986) reported that lymphocyte proliferation is suppressed in the presence of culture medium conditioned by human IVF embryos. This suppression correlated with successful embryonic implantation (Bose and Mahadevan, 1990). Suppressible molecules in embryonic culture supernatants could originate from dividing embryos, oocytes, sperm, or a combination of the above. In order to determine the origin of these IVF-associated suppressible molecules, human sperm culture supernatants were tested for their ability to inhibit lymphocyte proliferation. Indeed, suppressible molecules were present, and the molecular weight range of these sperm-derived molecules was similar to that of suppressible molecules found in IVF embryonic culture supernatants (Clark et al., 1989b).

Approximately 40% of human IVF oocytes (embryos) liberated low molecular weight immunosuppressive factors into the surrounding medium. These correlated with the ability of the fertilized oocytes to implant and produce pregnancy (Daya et al., 1986; Daya and Clark, 1986; Daya and Clark 1988). The suppressible activity acted in an entirely non-specific manner, i.e. it inhibited the growth of mouse lymphocytes and the murine P815 mastocytoma more effectively

than it inhibited human PBL cells (Daya et al., 1986), and was present in supernatants conditioned by sperm alone (Schechter et al., 1988; Clark et al., 1989b). Further studies have suggested that spermine and/or spermidine, or non-covalently bound polyamines in polyamine-complexes are likely responsible for this activity (Remacle-Bonnet et al., 1985; Lea et al., 1990; Porat and Clark, 1990a).

Several other embryo-associated immunosuppressive molecules have been described. Bose (1989) identified two high molecular weight, embryo-associated immunosuppressive factors in human IVF embryonic culture supernatants of preimplantation embryos. A different high molecular weight glycoprotein that suppresses lymphocyte activation, is released from preimplantation ovine and porcine embryos (Murray et al., 1987).

As will be later discussed polyamines and polyamine interactions with degrading enzymes may be responsible not only for embryonic growth and differentiation, but also for the protection of the fetus against maternal immune rejection (Morgan and Illei, 1980; Gahl et al., 1982a, 1982b; Morgan, 1983), for the regulation of placental growth and the attachment of the blastocyst to the endometrium.

1.8 AGEING MAMMALS AS A MODEL FOR MECHANISMS OF REPRODUCTIVE FAILURE

There is a decline in mammalian multiparous females in litter size at advanced age (Finch, 1978). This may result from steroid and peptide hormone dysfunctions, failure of ovulation, a decrease in the number of eggs ovulated, deterioration in oocyte quality, genetic malfunction in the ovum or embryo, changes in the uterine milieu, including infection, or lack of a suitable fetoplacental response to hormones and other growth and immunosuppressive factors (Harman and Talbert, 1970; Fabricant and Schneider, 1978; Holinka et al., 1979; Brook et al., 1984; Chandley, 1985). Consequently, aged animals can provide a model to study mechanisms of pregnancy failure. This work is concerned with embryo-associated age-related implantation dysfunctions.

Age-related reproductive failure and immunologically-related infertility may be closely linked. Immunologic factors and the factors leading to ageing may be identical. It is also possible that immunologic factors may be the cause of the increase of reproductive failure with age. Genetic variations associated with murine H-2 complex genes were shown to influence reproductive senescence of female

mice (Lerner et al., 1988).

Maternal environment is a major factor for the reduction in litter size noted in females at advanced age (Talbert and Krohn, 1966; Holinka et al., 1979; Miller et al., 1979; Menken et al., 1986). Chronic exposure to ovarian hormones cause advancement of the onset of age-related changes in the murine reproductive system (Finch et al., 1984). On the other hand, long term ovariectomy and chronic food restriction were shown to extend intrinsic uterine function of aging mice (Goodrick and Nelson, 1989). Sera from old female mice significantly reduced the rate of fertilization and blastocyst development *in vitro* (Tsunoda and Chang, 1979).

There are changes in polyamine content and levels of activity of the enzymes involved in the polyamine biosynthetic pathway in organs of ageing mammals (Tabor and Tabor, 1984). The levels of putrescine and spermidine decrease in different mammalian organs with ageing, and polyamine levels in serum and urine of healthy humans decline progressively with increasing age. The enzymes ODC and SAM-D, involved in the polyamine biosynthetic pathway, show similar trends. There is also evidence for an age-related decrease in the level of ODC and its inducibility in mammalian cells cultured *in vitro*. Another interesting phenomenon is that hormonal induction of ODC activity is strongly reduced in organs of aged animals, as it is in neoplastic organs (Scalabrino and Ferioli, 1984).

1.9 THE MOUSE AS A MODEL FOR HUMAN PREGNANCY

FAILURE

For ethical reasons, experimental use of human oocytes and embryos is unacceptable and only discarded supernatants of human cultures can be tested. It is unacceptable to add factors to the embryonic cultures to test for growth promoting activity or to inject factors into the patient's uterus in order to evaluate effects on immune function. If a common mechanism for communication between the preimplantation embryo and its mother is conserved throughout the class Mammalia, investigations using animal models, such as the mouse, can provide a focus for clinical approaches to infertility and the regulation of fertility. In comparison to the human system, a mouse model is less limited in the availability of oocytes and sperm, it allows for experimentation with IVF as well as *in vivo* fertilized embryos, and enables testing of the effects of suppressive factors on IVF oocytes and embryos *in vitro* and *in vivo*.

Successful fertilization of mouse eggs *in vitro* was first achieved by incubating freshly ovulated eggs with capacitated spermatozoa recovered from the uterus of mated animals (Whittingham, 1968). The fertilization of mouse eggs by

epididymal spermatozoa *in vitro* is now a routine procedure (Iwamatsu and Chang, 1970; Tsunoda and Chang, 1976). *In vitro* fertilization rates in mice are about 90% (Tsunoda and Chang, 1979).

The mouse lacks the high frequency of chromosomal problems found in human IVF, but mimics other aspects of human reproductive problems such as implantation failure and spontaneous abortion / resorption (Santalo et al., 1986). A mouse model, mimicking human IVF failure was described by Abe (1986). In this model Abe reported *in vitro* fertilized embryos transferred to a host had a lower implantation rate and a higher abortion rate than embryos fertilized *in vivo*.

Murine CBA females resemble human females in that their ovaries are depleted of oocytes by the end of their reproductive life (Jones and Krohn, 1962). Their litter size declines with ageing and embryonic malformations increase (Eichenlaub-Ritter et al., 1988). The murine immune system is similar to the human immune system. The murine MHC is called the H-2 complex (HLA in humans). The preimplantation-embryonic-development gene (*Ped*) has been associated with the H-2 complex in the mouse. *Ped* influences the rate of cleavage of preimplantation mouse embryos (Warner et al., 1987). Murine blastocysts have been shown to have histamine receptors type H-2 and histamine forming capacity (not to be confused with the H-2 complex) (Dey et al., 1979; Dey and Johnson, 1980). Lymphocytes also have histamine receptors and can be stimulated to produce

suppressor factors (Dey et al., 1979). In this way a murine blastocyst could stimulate suppressor cell activity.

1.10 STUDY OBJECTIVES

Culture supernatants of human IVF embryos demonstrated immunosuppressive activity which was correlated with the ability of the embryo to implant and produce a pregnancy (Clark et al., 1989b). In assuming that an embryo produces immunosuppressive factors which assist in its implantation process, it is also assumed these factors are released to the surrounding medium and thus condition the medium.

The specific objectives pursued in this thesis include:

- (1) To determine if immunosuppressive molecules similar to those found in human IVF embryonic cultures are present in human sperm cultures.
- (2) To examine murine IVF embryonic culture fluids for the presence of immunosuppressor molecules.
- (3) To utilize the murine IVF system as a model for investigating the nature and function of factors produced by growing embryos.

(4) To identify the origin of the suppressive activity in murine IVF embryonic culture supernatants by examining conditioned media from incubations of oocytes and sperm alone.

(5) To characterize the immunosuppressive molecules found in IVF cultures.

(6) To examine any association between polyamine deficiency in IVF embryonic culture supernatants and age-onset infertility in CBA/J mice.

In this thesis, it is shown that murine IVF embryonic culture supernatants contain low molecular weight inhibitory peaks which can also be found in murine 24 hour sperm culture supernatants and appear to be polyamines (Porat and Clark, 1990a, 1990b).

2. MATERIALS AND METHODS

2.1 Mice

Mice of strains C3H/HeJ, CBA/J, aged CBA/J, DBA/2J, Balb/CJ and Swiss Webster (obtained from the Jackson Laboratories, Bar Harbor, ME, Charles River and from Iffa Credo, France), C.B-17 and C.B-17 scid (SCID) (obtained from Dr. R.A. Phillips, Toronto) were used. The mice were housed 4-5 animals per cage, were allowed chow and water ad lib, and were kept under constant environmental conditions of light and temperature (12 hour light/dark cycle and $22 \pm 2^{\circ}\text{C}$). C.B-17 and SCID mice were kept in sterile microfilter capped cages and were given autoclaved chow and water.

2.2 Culture Media

All supernatants for gamete and IVF experiments were prepared using Whitten's defined medium (Whitten, 1971). Crystalline bovine serum albumin (BSA, Fraction V, Calbiochem) at a concentration of 4.0 mg/ml, 100 IU penicillin and streptomycin 50 $\mu\text{g/ml}$ were added. Before culture, the osmolality of the media and the pH were adjusted to

280-285 mOsm/kg and 7.4 respectively. The media were equilibrated in the incubator in 5% CO₂ before use.

The alpha-MEM medium (Grand Island Biol Co, NY) for lymphocyte proliferation assays was supplemented with fetal bovine serum (FBS) or human blood type AB serum. Lymphocyte culture medium was supplemented to a final 10% concentration (v/v) of non-heat-inactivated or heat-inactivated (30 min 56°C) serum.

2.3 Murine sperm culture supernatants

Spermatozoa were obtained from the *cauda epididymis* of mice. Male DBA/2J mice 3 to 5 months of age were sacrificed by cervical dislocation. The *epididymes* and *vasa deferentia* were removed, carefully cleaned and washed several times in culture medium pre-equilibrated in 5% CO₂ at 37°C. In a Falcon (# 3037) tissue culture dish containing 0.5 ml of medium, sperm was expressed from two *vasa deferentia* and two *epididymes* using watchmakers forceps. This resulted in a concentrated sperm suspension that was incubated at 37°C, in a humidified atmosphere of 5% CO₂ in air for 15 minutes. Seventy five microliters of the suspension were removed and diluted in 0.5 ml of fresh equilibrated medium and the sperm concentration was determined. A sperm suspension of 1-2x10⁶ cells/ml was allowed to complete capacitation for further 45 minutes before insemination, or was incubated for 24 hours for generation of supernatants. Before assay, supernatants were collected by centrifugation of the cultures 5-10 minutes at 12,000 rpm and removal

of the fluid phase which was then filter sterilized (Millex - GV, 0.22 μm , Millipore). Supernatants were used for testing or stored at 4°C.

2.4 Oocyte supernatants

CBA/J females age 8-10 weeks (young) and 20-30 weeks (old) were superovulated by injecting 5 IU pregnant mare serum gonadotropin I.P (PMSG, Sigma, Saint Louis, MO) followed by 5 IU human chorionic gonadotropin (hCG, Sigma) 48 hours later I.P. The mice were sacrificed 15-18 hours after injection of hCG by cervical dislocation. Oviducts were dissected out into pre-equilibrated Whitten's medium. Oviductal oocytes surrounded by cumulus cells were released into the medium by puncturing the swollen ampullae using two sterile 26 Gauge needles. All manipulations were carried out at 37°C on a heated stage. The oviducts were discarded, and the oocytes which had been released were transferred into medium equilibrated with 5% CO₂ to be incubated or inseminated.

2.5 Culture of oocytes and insemination for IVF

For fertilization *in vitro*, approximately equal numbers of cumulus masses were used as for oocyte incubation alone. Each cumulus mass contained 4-7 oocytes. Cumulus masses were placed into 1 ml medium (pre-equilibrated with CO₂), inseminated with 75 μl of the capacitated spermatozoa, and incubated at 37°C in a humidified atmosphere of 5% CO₂ in air. At 5 hours after insemination the oocytes were transferred, using a pulled glass

mouth-pipette, into fresh medium for further culture under conditions similar to those used above. Twenty four hour supernatants were collected and the cultures were examined for cleavage using a dissecting microscope. Culture medium surrounding mouse embryos during the second 24 hours after insemination (Group A) was centrifuged, filtered and tested for the presence of immunosuppressive activity. All supernatants not tested at the day of collection were stored at 4°C until tested. Two groups of oocytes, those obtained from young females and these obtained from old females, were incubated without being inseminated (Group B). These were used for testing for oocyte-derived immunosuppressive activity and as parthenogenetic controls. Sperm were also cultured separately for 24 hours to generate supernatants (Group C).

2.6 Human IVF embryo culture supernatants

IVF embryo culture supernatants were kindly provided by Dr. S. Daya from the McMaster IVF Clinic (Daya and Clark, 1986; Clark et al., 1989). Briefly, infertile patients were hormonally stimulated to superovulate, ovulation was induced and oocytes retrieved. Prior to in-vitro insemination, sperm was collected, washed, and subjected to "swim-up" to select the viable subpopulation. Following a 12-24 hour incubation period, fertilization was confirmed microscopically. Embryos were transferred into 3 ml of growth medium (Gibco) supplemented with 10% heat inactivated patient serum and were incubated further 30-32 hours before embryo transfer (ET) into the patient's uterus. The growth phase media were tested for inhibitory activity (Daya and Clark, 1986; Clark et al., 1989) and positive

samples were pooled. In order to increase potency, the pool of suppressive IVF growth phase supernatants was lyophilised to dryness by speed vacuum centrifugation, and reconstituted to 1/3 its original volume.

2.7 Assay for suppressive activity

A series of lymphocyte proliferation assays were performed to test for suppressive activity in supernatants derived from cultures of murine or human in-vitro-fertilized embryos, murine oocytes alone and murine or human sperm alone. Each sample was tested in four replicate cultures. Twenty five microliters of murine test supernatants were added to 75 μ l assay medium containing 4×10^5 mouse lymph node cells (LNC) obtained from C3H/HeJ virgin females. Cultures were performed in 96-well round bottomed culture trays (Linbro, Flow Laboratories). Lymphocyte proliferation was stimulated by Concanavalin A (Con A, Sigma) at a final concentration of 5-10 μ g/ml. Assay medium (pH, 7.4) was alpha-MEM (Gibco) supplemented so as to contain 10% FBS (Gibco), glutamine and antibiotic (48 μ g/ml Garamycin, Schering, Canada). Following 48 hours incubation at 37°C in 5% CO₂, 1 μ Ci/well tritiated (³HTdR) thymidine was added in 25 μ l assay medium and incubation was carried out for an additional 18 hours. The cells were then harvested onto glass filter mats and radioactivity was determined in a liquid scintillation counter. Twenty five microliters Whitten's medium, or buffer added to 75 μ l assay medium were used as the medium control cultures.

Degree of suppression was calculated according to the formula:

$$100 \times \frac{(\text{cpm control} - \text{cpm test supernatant})}{\text{cpm control}}$$

in which cpm represents mean incorporation of quadruplicate cultures with concanavalin A minus cpm mean incorporation of quadruplicate cultures incorporated by LNC incubated in medium alone.

In certain experiments suppression of proliferation was also assayed using P815 mouse mastocytoma cells or using 100 μ l cultures containing 1×10^5 - 2×10^5 human peripheral blood mononuclear cells (PBL) stimulated by Con A. Cells were cultured in a similar manner to the above described except that no concanavalin A was added to the P815 cells.

2.8 High performance liquid chromatography (HPLC) separation

A 100 to 300 μ l aliquot of embryo culture medium from separate embryos, from pools of embryos, or from oocyte or sperm cultures was loaded on to a Spherogel TSK gel G3000SW column (Toyo Soda, USA, Inc., Atlanta, GA), pre-equilibrated with Tris (0.01 mol/l) buffered sodium chloride (0.1 mol/l) pH 7.0 (HPLC buffer). One ml fractions were collected at a flow rate of 1 ml/min at room temperature. For some of the samples, 1 ml of supernatant pool, or of the polyamine sample was separated by preparative HPLC

using the Spherogel TSK 2000SW column (Beckman) using the same buffer and run at 5 ml/min. The column effluent was monitored at 280 nm using a Gilson 111B ultraviolet detector (Mandel Scientific Co. Ltd.), connected to a Linear 1200 chart recorder. Twenty five microliters of each eluate fraction were tested in culture (final concentration of 25% (1:4)), and 25 μ l HPLC buffer was used as a control. The columns were calibrated using the following markers: Alcohol dehydrogenase (150,000 daltons), transferrin (90,000 daltons), bovine serum albumin (63,000 daltons), ovalbumin (44,000 daltons), cytochrome C (12,200 daltons), vitamin B₁₂ (1355 daltons), and phenol red (354 daltons).

2.9 Test for polyamine (spermine and spermidine) related suppression

Spermine (spermine tetrahydrochloride), spermidine (spermidine trihydrochloride; N-[3-Aminopropyl]-1,4-butanediamine), putrescine dihydrochloride (tetramethylenediamine); aminoguanidine (bicarbonate salt) and hydroxylamine, amine oxidase inhibitors, were purchased from Sigma (St. Louis) and tested in the lymphocyte proliferation assay. In experiments where aminoguanidine was added to the culture medium, serum was incubated with aminoguanidine for at least an hour prior to addition to culture medium. In other experiments FBS-containing medium was incubated for 30 min at 37°C with 0.1 mM (final dilution) of hydroxylamine (Sigma) before the addition of lymphocytes and mitogen. The results were compared with controls without hydroxylamine.

2.10 Thin layer chromatography (TLC) of culture supernatants

HPLC fractions of the IVF embryo culture supernatants and of sperm culture supernatants were tested for the presence of polyamines by thin layer chromatography. Ten to 20 μ l of single HPLC fractions were mounted on TLC silica-gel plates (Uniplate, Analtech, DE, USA). The running solvent, consisting of 1-butanol, pyridine, acetic acid and H₂O (4:1:1:2, v/v/v/v), was freshly prepared and equilibrated prior to each run. When dry, the plates were sprayed with ninhydrin (200 mg) in isopropyl alcohol (100 ml) containing 2,4,6 collodine (100 μ l). Polyamines were detectable as brown spots and photographed.

2.11 Toxicity Assays

Mouse spleen cells were incubated at 37°C with unseparated culture supernatants, with HPLC purified inhibitory fractions, and with polyamines. Cell viability was estimated by (0.4%) Trypan blue (TPB) exclusion at various time points. Percentage cytotoxicity was calculated from:

$$\% \text{ kill} = \frac{\% \text{ TPB with test supernatant} - \% \text{ TPB with control}}{100 - \% \text{ TPB with control}}$$

2.12 Statistical tests

The significance of differences was determined by Student's t-test or by Fisher's Exact test as appropriate.

3. RESULTS

3.1 CONCANAVALIN A DOSE RESPONSE STUDIES

It has been shown previously that culture medium collected from human *in vitro* fertilized embryo cultures suppressed human peripheral blood lymphocyte proliferation stimulated by 10 $\mu\text{g/ml}$ Con A (Clark et al., 1989b).

A dose response curve was determined with respect to final Con A concentration in the murine lymphocyte proliferation assay as shown in Table 1. A concentration of 5 $\mu\text{g/ml}$ appeared sufficient to stimulate proliferation. 10 $\mu\text{g/ml}$ was used for some experiments to ensure that inhibition of proliferation by test supernatants was not due to carbohydrate factors in test supernatant binding to and blocking the mitogen.

3.2 SUPPRESSION OF CON A STIMULATED LYMPHOCYTES BY MURINE IVF OOCYTE CULTURE SUPERNATANTS

Figure 4 illustrates the degree of suppression produced by three groups of supernatants: those collected from the second 24-hour incubation of IVF (CBA/J oocytes fertilized by DBA/2J sperm) embryos (A), from 24-hour cultures of CBA/J oocytes only (B), and 24-hour culture medium of DBA/2J sperm (C). Whitten's medium alone was used as control. IVF embryo culture supernatants (A) fell into two sets. Immunosuppressive activity was readily detected when oocytes from young female donors had been fertilized. Stimulatory, or very slightly suppressive activity (in one case) appeared when supernatants were obtained from embryos where the oocytes had been retrieved from aged (20-30 weeks) CBA/J mice and fertilized by young DBA/2J sperm. In column B, the supernatants obtained from the incubation of oocytes alone, showed either immunostimulatory or slightly immunosuppressive activity. None of these oocytes showed evidence of division.

Column C shows that supernatants obtained from the incubation of DBA/2J epididymal sperm alone contained potent immunosuppressive activity (97.3 ± 1.9 % suppression). The potency of the sperm-conditioned supernatants and quantity available was sufficient to allow dose response testing. A dose response relationship was readily demonstrated for epididymal sperm culture supernatants (Figure 5).

3.3 HPLC ANALYSIS OF SUPPRESSIVE MOLECULES IN SPERM AND IVF EMBRYO CULTURE SUPERNATANTS

HPLC separation of suppressive IVF embryo culture medium obtained from young female donors (from Figure 4) showed several peaks (significant by Student's t-test) of immunosuppressive activity (Figure 6) at approximate molecular weights of 100 kD (peak "a"), 1 to 7 kD (peak "c") and 0.3 to 0.4 kD (peak "d").

HPLC fractionation of a sperm culture supernatant showed immunosuppressive activity associated with a similar set of molecules plus a 20 to 30 kD peak (peak "b"). These results suggested sperm released similar sets of molecules to those detected in 48 hour (24 to 48 hour collection period) IVF embryo culture supernatants. It is noted that the low molecular weight factors (peaks "c" and peak "d") were similar in size to those of the suppressive molecules found in human IVF embryo culture supernatants (Clark et al., 1989; Lea et al., 1990) that have been correlated with successful implantation. It was, therefore, of interest to determine the reproducibility of the low molecular weight peaks "c" and "d", the nature of these molecules, and their relationship to embryo implantation.

Figure 7 shows the HPLC suppression profile of three independent murine IVF embryo culture supernatants, and Figure 8 shows three murine epididymal sperm culture supernatants (HPLC analysis) experiments. In the IVF embryo culture supernatants peaks "c" and "d" were reproduced; with respect to peak "d",

TABLE 1

DOSE RESPONSE OF MURINE LNC TO CON A IN CULTURES
CONTAINING 10% FBS OR 10% AB SERUM

Con A concentration (final)	Serum Type	mean cpm \pm 1 SEM
20 μ g/ml	10% FBS	73 \pm 21
10 μ g/ml	10% FBS	2365 \pm 89
5 μ g/ml	10% FBS	69173 \pm 508
20 μ g/ml	10% AB	9581 \pm 485
10 μ g/ml	10% AB	25855 \pm 1769
5 μ g/ml	10% AB	26330 \pm 935

FIGURE 4:

Distribution of percent suppression of thymidine uptake obtained with different test supernatants. Column A - values from incubations of fertilized oocytes: oocytes fertilized from aged (20-30 weeks) CBA/J (o); oocytes fertilized from young CBA/J (•). Column B - values for supernatants from incubations of ovulated oocytes only ([]). Column C - values of sperm culture supernatants (▼). Control value SEM averaged $\pm 5\%$. All data obtained using Con A $10 \mu\text{g/ml}$.

FIGURE 5:

Dose response curve for suppressive sperm culture supernatants. Values represent mean \pm 1 SEM. All data were obtained using Con A 10 $\mu\text{g/ml}$. Suppression was measured as described in Materials and Methods. The response in the presence of 1.5 - 25 $\mu\text{l/well}$ (100 μl total volume/ well) is shown.

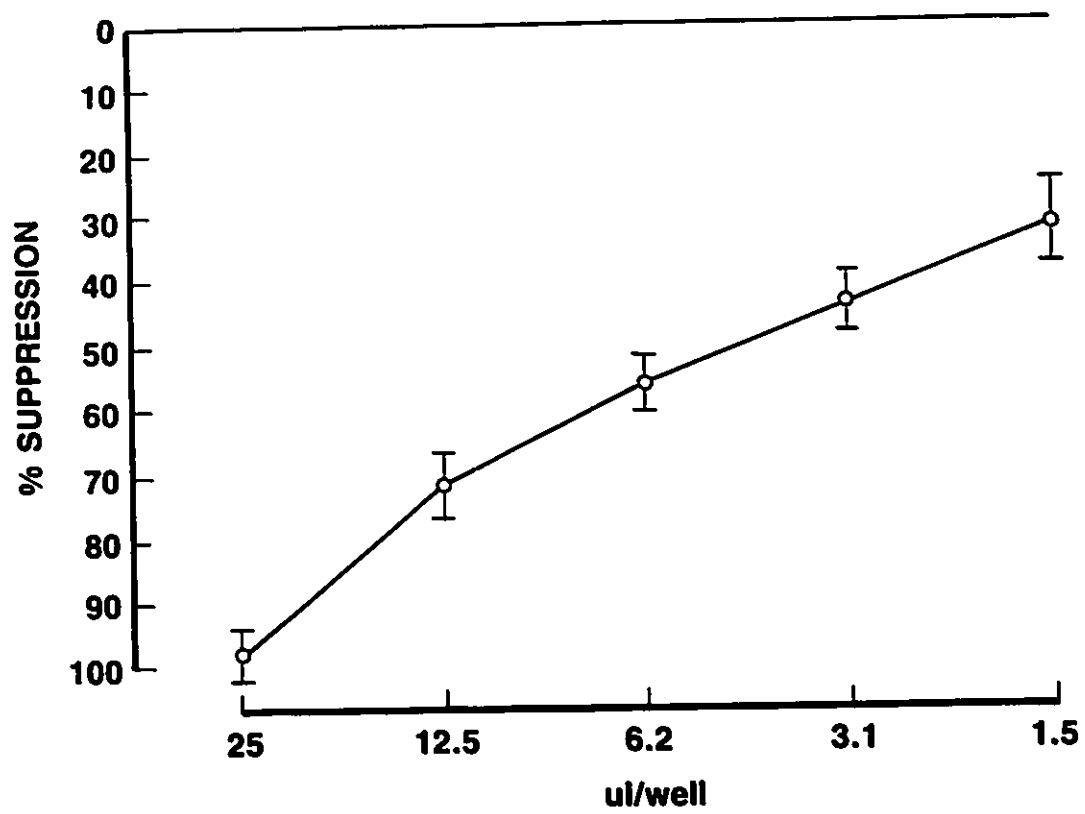


FIGURE 6:

HPLC analysis of a suppressive IVF embryo culture supernatant (\bullet), (45% suppression unseparated) and epididymal sperm culture supernatant (\blacktriangledown) (90% suppression unseparated). The supernatants were separated and each fraction tested as described in Materials and Methods. The lower part of the figure shows the optical density profile. The dotted line shows ± 1 SEM for buffer control proliferative response. All data obtained using Con A 10 $\mu\text{g/ml}$.

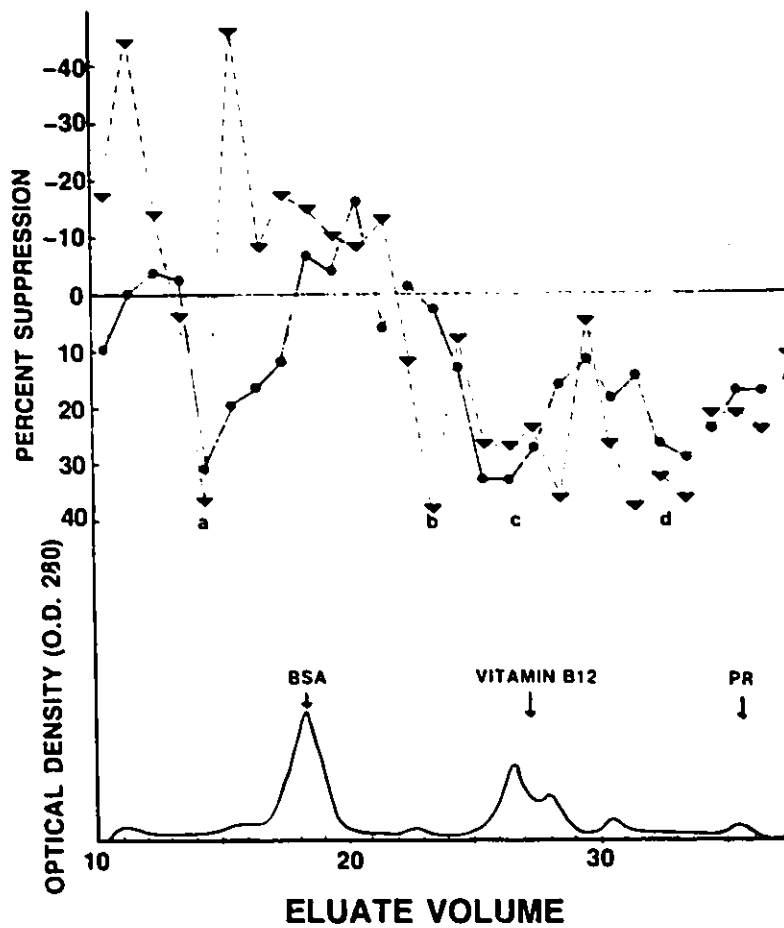


FIGURE 7:

HPLC analysis of 3 suppressive IVF embryo culture supernatants. The supernatants were separated and each fraction tested as described in Materials and Methods. The lower part of the figure shows the optical density profile. Peak "c" (as shown in figure 6) is at approximate molecular weight 1 to 7 kD (vitamin B₁₂ marker), and peak "d" (as shown in figure 6) at 0.3 to 0.4 kD (PR marker). The dotted line shows ± 1 SEM for buffer control. Data obtained using Con A 5 μ g/ml, data for (•) using Con A 10 μ g/ml.

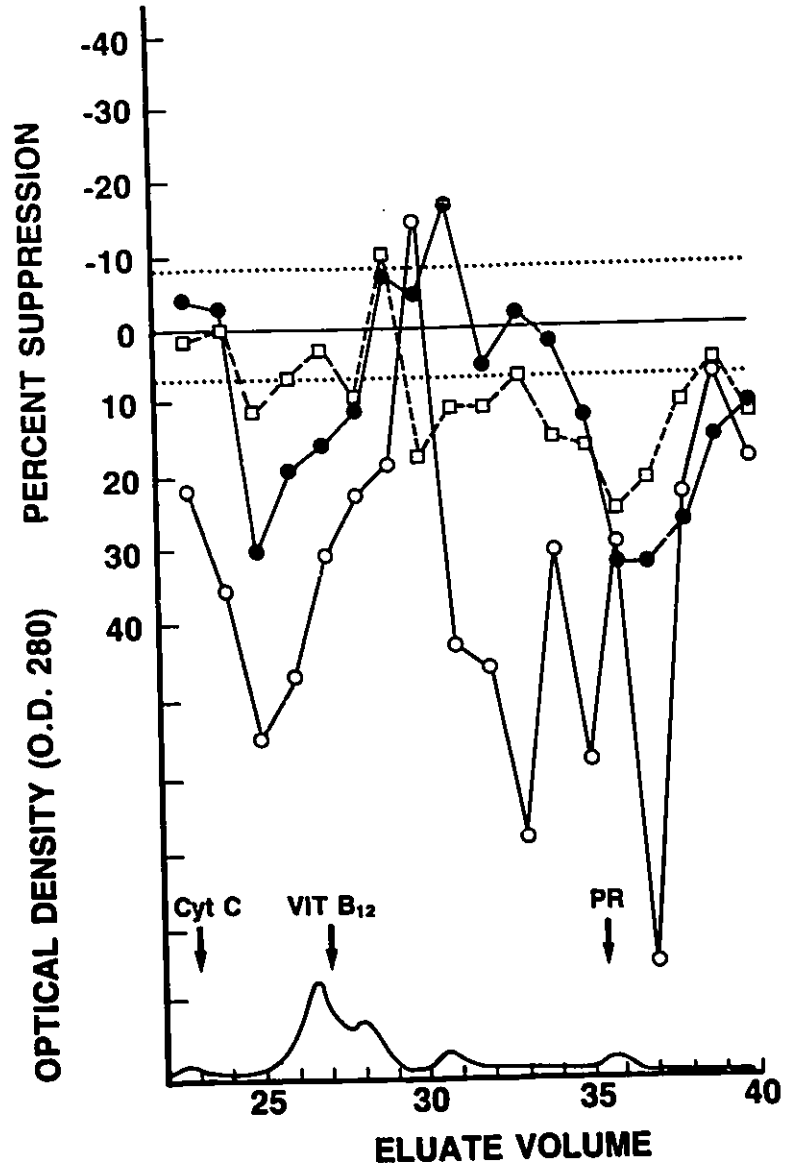
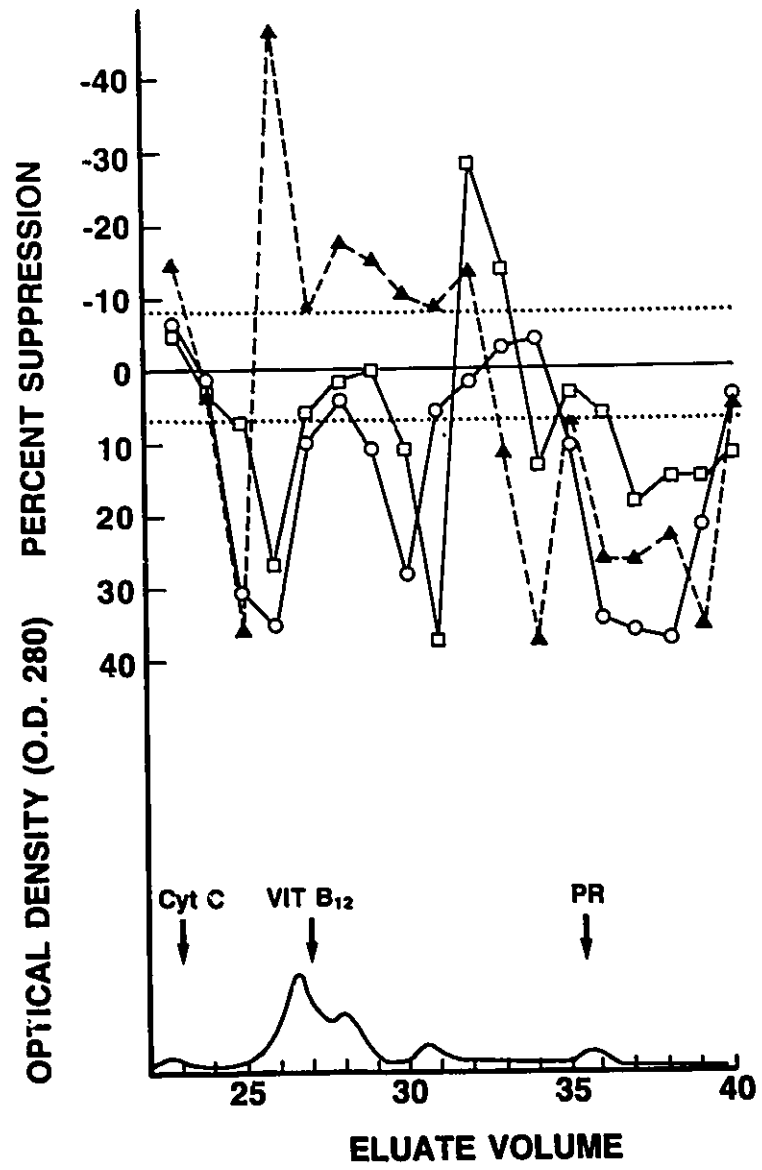


FIGURE 8:

HPLC analysis of suppressive activity of 3 suppressive sperm culture supernatants. The supernatants were separated and each fraction tested as described in Materials and Methods. The lower part of the figure shows the optical density profile. Peak "c" (as shown in figure 6) is at approximate molecular weight 1 to 7 kD (vitamin B₁₂ marker), and peak "d" (as shown in figure 6) at 0.3 to 0.4 kD (PR marker). The dotted line shows ± 1 SEM for buffer control. All data was obtained using Con A 5 μ g/ml.



this appeared to be a complex of several peaks where IVF supernatants were studied, and one IVF embryo culture supernatant showed an additional peak of activity at 31-34 ml elution volume. A similar set of peaks was also seen in 3/3 of sperm culture supernatants (Figure 8), as was the "c" peak. These data suggest the low molecular weight inhibitory molecules in IVF embryo culture supernatants and in sperm culture supernatants were similar, and the activity in IVF embryo cultures might be sperm derived.

3.4 ANALYSIS OF SPERM-ASSOCIATED SUPPRESSOR FACTORS

Seminal plasma of several species, including humans and mouse, is known to contain a variety of immunosuppressive molecules (James and Hargreave, 1984). The suppressive factors in sperm culture supernatants could have been related to immunosuppressive molecules in seminal plasma where the major contributor to suppression *in vitro* is due to the polyamine spermine. Spermine itself is non-suppressive, but when metabolized *in vitro* by amine oxidases present in FBS into acrolein or spermine dialdehyde, toxicity to a variety of cell types is observed (Allen et al., 1979).

As a first step in evaluating polyamines as putative suppressor factors in our supernatants, the effect of the three polyamines (spermine, spermidine, putrescine) upon the lymphocyte proliferation assay was tested. Spermine and spermidine

FIGURE 9:

Comparison of thin layer chromatographic analysis and suppression of lymphocyte proliferation in Con A stimulated cultures by different polyamines. 10 μ l of the μ M final concentration of polyamine shown (spermine, spermidine and putrescine) were plated and run as in Materials and Methods. The value of suppression of lymphocyte proliferation by a 25 μ l aliquot added to the lymphoproliferative assay is also shown.

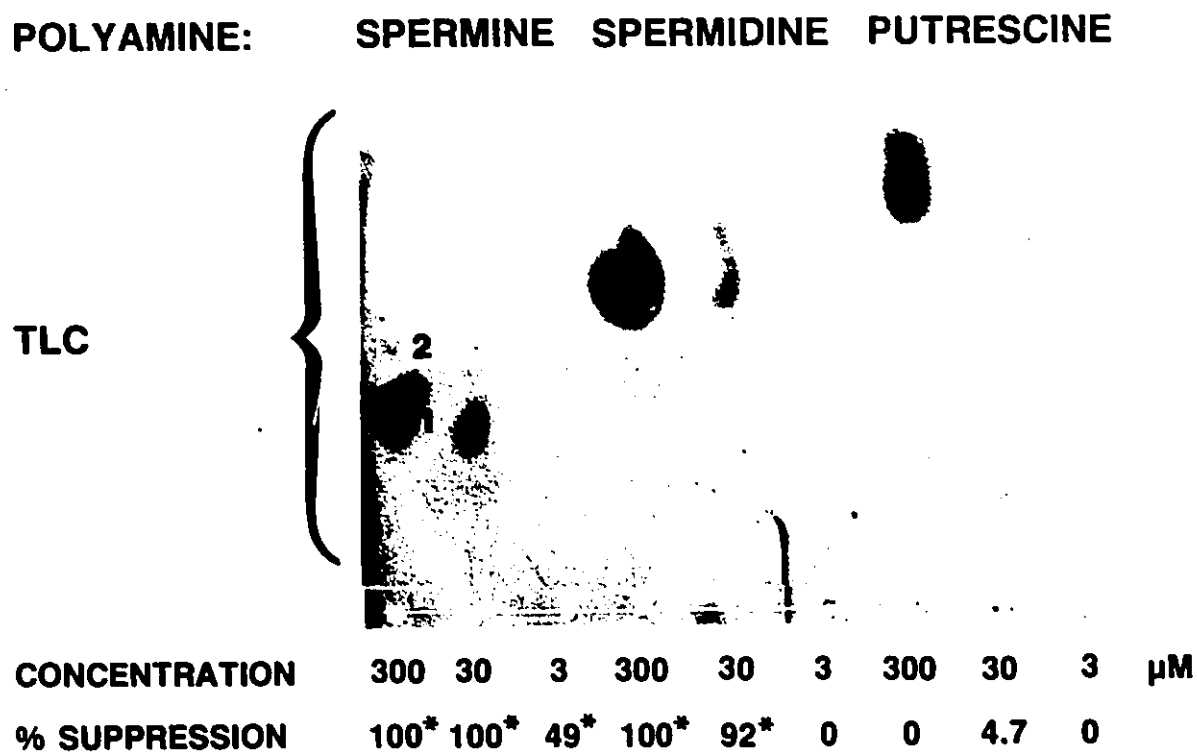


TABLE 2

SUPPRESSION OF LYMPHOCYTE PROLIFERATION BY
 CONCENTRATED SPERM CULTURE SUPERNATANTS
 FROM MICE OF DIFFERENT STRAINS (10% FBS)

Mouse Strain	MHC type	% Suppression ± 1 SEM (diluted 1:4)
DBA/2J	H-2 ^d	97.3 ± 1.9
Balb/CJ	H-2 ^d	97.4 ± 1.8
C.B-17	H-2 ^b	99.2 ± 0.9
SCID	H-2 ^b	98.9 ± 0.5

Tested on C3H/HEJ mouse lymphocytes stimulated by Concanavalin
 A as described in Materials and Methods.

proved to be suppressive but putrescine was not. On TLC, each polyamine migrated with a different R_f as expected. (R_f = distance travelled by solute / distance travelled by mobile phase). The TLC was less sensitive in detecting spermine and spermidine than the bioassay, particularly in the case of spermine. With spermine, a second spot of lesser intensity (numbered as 2), possibly a polyamine breakdown product, was also noted. Figure 9 demonstrates a relationship between suppressive activity of the polyamines and their detection by TLC - ninhydrin system.

It was of interest to know if the potent suppression seen with sperm culture supernatants was unique to DBA/2J sperm. Thus, experiments were conducted to determine if the suppression in sperm culture supernatants was related to genotype. Table 2 illustrates the suppression obtained with sperm culture supernatants from different donors. Sperm from all strains tested released suppressive activity into the medium. Sperm from male SCID mice, which lack mature T and B lymphocytes, was also active. There was no evidence the suppression was specific for the donor H-2 type. These data were compatible with a non-specific inhibitor associated with spermatozoa.

Polyamines may form complexes and attach to carriers (Allen et al., 1977) so that the HPLC suppressive peak "c" as well as peak "d" might be compatible with polyamines. Further experiments were done to test this hypothesis by blocking the activity of amine oxidase in the serum supplementing the media used in the

assay.

Both HPLC-fractionated sperm culture supernatants and IVF embryo culture supernatants were tested for loss of suppressive activity in lymphocyte proliferation assays when the FBS in the assay was replaced with human AB serum and aminoguanidine. It has been shown previously that human serum has much lower levels of amine oxidase than FBS. Human serum is not, however, totally devoid of amine oxidase. The enzyme can be blocked by aminoguanidine, and 100 $\mu\text{g}/\text{ml}$ proved sufficient to reduce amine oxidase in FBS by 90% (Allen et al., 1979; Illei and Morgan, 1979).

Figure 10 shows suppressive activity of HPLC fractions using concentrated sperm culture supernatants (top) tested with FBS without the amine oxidase inhibitor, aminoguanidine. The suppressive activity in eluted volumes from 26-38 ml (equivalent to all of the peaks in the range c - d in Figure 6) was completely abrogated when serum amine oxidase activity was minimized using human AB serum supplemented with aminoguanidine. Preliminary experiments indicated addition of 100 μM hydroxylamine to FBS-containing medium that abolished suppression of human lymphocyte growth by spermine and (Lea et al., 1990) was not effective with the mouse LNC. Human serum contains low but sufficient levels of amine oxidases to allow suppression to occur when murine lymphocytes are used as the target for suppression. Addition of 100 μM aminoguanidine, an amine

TABLE 3

EFFECT OF AMINOGUANIDINE AND SERUM ON
 SUPPRESSIVE ACTIVITY OF SPERM CULTURE
 SUPERNATANTS^a IN CON A STIMULATED LYMPHOCYTE ASSAY

Serum Source/Amount	% Suppression in serum alone (mean \pm 1 SEM)	% Suppression in serum + 0.05M AG (mean \pm 1 SEM)
10% AB	96.4 \pm 1.1	80.3 \pm 0.8
0.1%AB	71.0 \pm 9.3	24.1 \pm 6.7
10% FBS	98.6 \pm 0.3	94.7 \pm 0.7

a - Sperm were expressed from two vas deferens and two epididymes. A concentrated suspension was made and incubated at 37°C for 15 minutes in humidified atmosphere of 5% CO₂. Seventy five microliters of the suspension were diluted in 0.5 mL of medium and sperm concentration was adjusted to 1-2x10⁶ sperm/mL suspension, and incubated for 24 hours for generation of supernatants. Before assay, supernatants were spun 5 to 10 minutes at 12,000 rpm and were filter sterilized.

TABLE 4

POLYAMINE OXIDASE INHIBITION BY AMINOGUANIDINE:
EFFECTS ON SPERM CULTURE AND IVF EMBRYO CULTURE SUPERNATANTS
INHIBITORY ACTIVITY

TEST FACTOR	SERUM USED	% Suppression ± 1 SEM
1. pooled SPERM HPLC peak fractions		
	10% FBS serum	97.5 ± 5.6
	10% AB serum	40.2 ± 12.4 ^a
	10% AB + AG	-13.7 ± 3.1 ^b
2. pooled IVF HPLC peak fractions		
	10% FBS serum	85.8 ± 7.3
	10% AB serum	39.2 ± 8.2 ^a
	10% AB + AG	-25.5 ± 4.4 ^b

Significantly suppressed compared to control

a - Significant suppression (P<0.05)

b - Loss of suppression (P<0.05)

oxidase inhibitor, to human serum proved effective in reducing suppression by unseparated sperm culture supernatant (Table 3), and similar data were obtained using the HPLC purified suppressive "c" peak (Table 4). Where the suppressive activity of the test material was not too great, aminoguanidine completely abrogated the effect. The TLC of the HPLC fractions identified polyamine spots with an R_f typical of spermine in all of the fractions with suppressive activity but not in non-suppressive fractions.

Figure 11 shows the suppression by IVF embryo culture HPLC fractions and TLC analysis of the same fractions. It can be seen that in Figure 12 peak "d" is prominent. It is also seen that minimization of serum amine oxidase activity also abolished suppression detected in the Con A stimulated lymphocyte proliferation assay by HPLC fractions of other IVF supernatant where peak "c" was dominant. TLC analysis of these suggested the presence of faint spots compatible with spermidine associated with the low molecular weight suppressive peaks "d" (Figure 11) and "c" (Figure 12). However, the intensity of staining did not always correlate with the magnitude of suppression (Figure 12) and it can be seen that at maximal suppression in Figure 12 the putative spermidine spot was absent.

The suppressive activity of IVF embryo culture supernatants is in general lower than that found in sperm-conditioned supernatants. To determine if immunosuppressive activity in IVF embryo cultures might be due to spermine

present at levels below the limit of detectability on the TLC plate, the fractions from several HPLC-separated IVF young oocyte cultures were pooled and concentrated 3 fold as described in Materials and Methods. Figure 13 shows that spots with the R_f of both spermine, and in some cases spermidine, were present in association with the immunosuppressive peaks.

To determine if spermine elutes off the HPLC gel filtration column in a similar molecular weight range as the suppressive molecules, the HPLC profile was compared to that of "pure" spermine. Figure 14 shows the HPLC profile that was obtained following HPLC fractionation of 1 ml sample of 0.1 M spermine tetrahydrochloride, each fraction tested for suppressive activity in the murine Con A stimulated lymphocyte proliferation assay. Suppression was detected in fractions containing molecules between 5,000 and 500 daltons and this activity was reversed by testing the same fractions in medium supplemented with aminoguanidine treated human AB serum (data not shown).

FIGURE 10:

Analysis of an HPLC separated murine epididymal sperm culture supernatant. One hundred μ l of a concentrated DBA/2J was separated on a TSK G3000SW column at a flow rate of 1 ml/min. In the upper panel the fractions were tested in the lymphocyte proliferation assay in medium supplemented with human AB serum (\blacktriangle), or with human AB serum and 0.1 mM aminoguanidine (\blacktriangle). The human AB serum was incubated with 100 μ M aminoguanidine at 37°C for 30 minutes before use. Dotted line shows \pm 1 SEM of HPLC buffer control tested at the same concentration as each fraction. Spermine 300 μ M control is given in "a". In the lower panel 10 μ l of each fraction was separated on TLC plate as described in Materials and Methods.

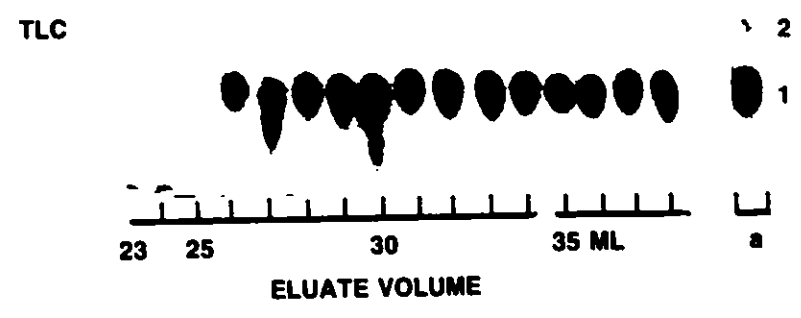
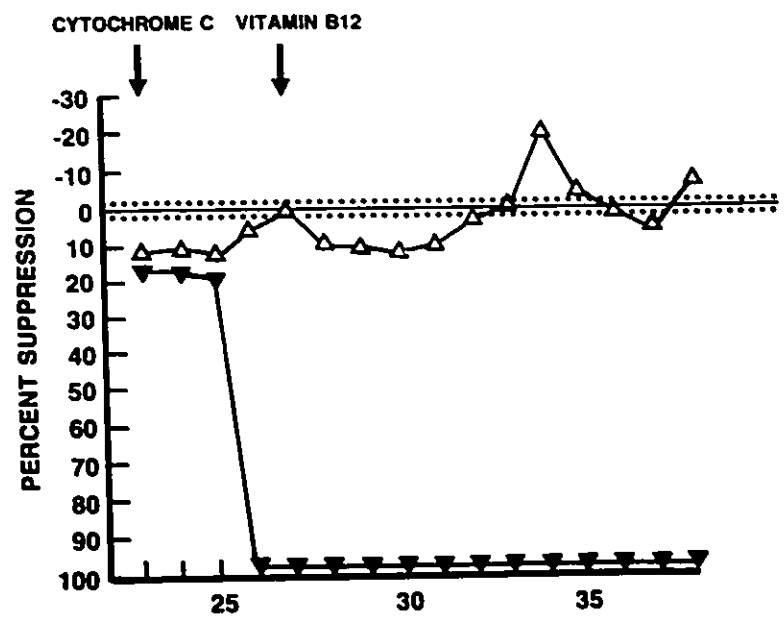


FIGURE 11:

Evaluation of suppression by murine IVF embryo culture supernatant with (■) or without (•) aminoguanidine in lymphocyte proliferation assay (upper panel). In the lower panel: TLC analysis of the same fractions is shown. "a" and "b" represent spermine controls at 300 μ M and 30 μ M respectively. "1" and "2" represent spermine and spermidine respectively, suggesting the presence of faint spots (marked by arrows) compatible with spermidine associated with the low molecular weight suppressive peak "d" (as shown in figure 6). Peak "c" (as shown in figure 6) is at approximate molecular weight 1 to 7 kD (vitamin B₁₂ marker), and peak "d" at 0.3 to 0.4 kD.

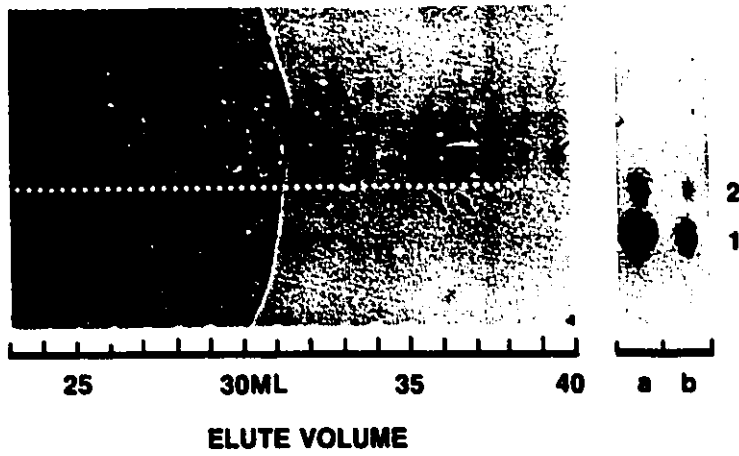
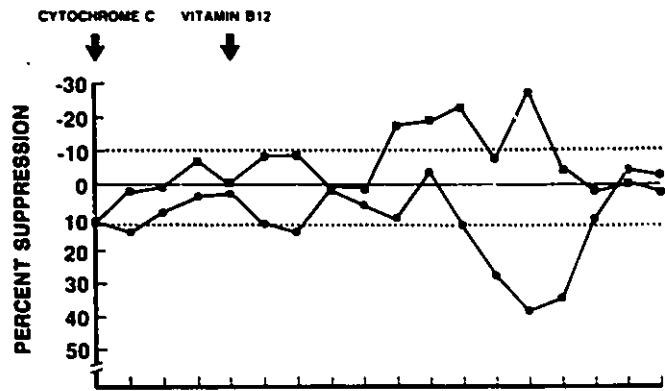


FIGURE 12:

Effect of reduced serum amine oxidase activity on suppression by murine IVF embryo culture supernatant. In the upper panel: Murine IVF embryo culture supernatant HPLC fractions were tested in assay treated with human AB serum (•). In the lower panel: TLC of the same fractions is shown. "a" and "b" represent spermine control at 300 μM and spermidine control at 300 μM . Peak "c" (as shown in figure 6) is at approximate molecular weight 1 to 7 kD (vitamin B₁₂ marker). Unlabelled spot represents putrescine control at 300 μM .

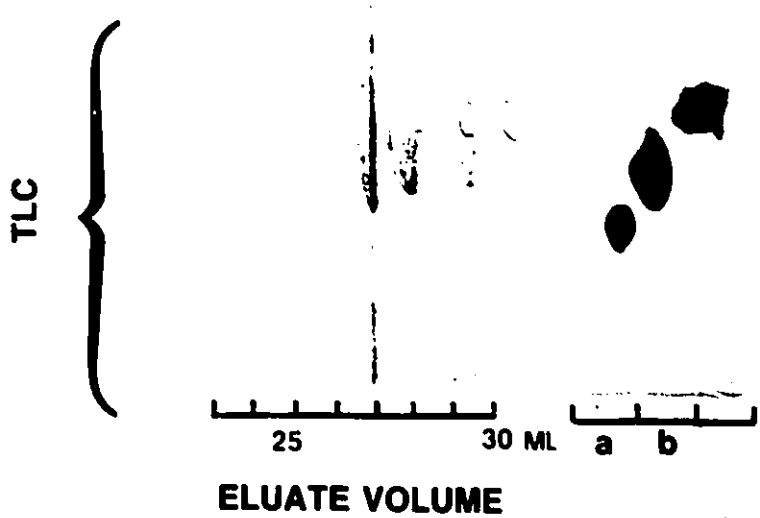
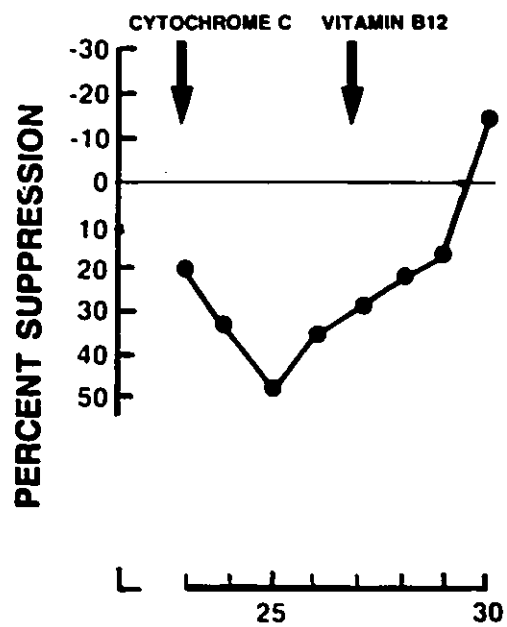


FIGURE 13:

TLC of HPLC separated immunosuppressive IVF embryo culture supernatants. A pool of supernatants was concentrated 3 fold to bring the concentration of polyamines up to a level that could be detected in the TLC. The HPLC fractions from the lower molecular weight range was tested by TLC. The TLC shows spots compatible with polyamines in the inhibitory fractions. Peak "c" (as shown in figure 6) is at approximate molecular weight 1 to 7 kD (vitamin B₁₂ marker), and peak "d" (as shown in figure 6) at 0.3 to 0.4 kD (PR marker). "a" and "b" represent spermine and spermidine controls at 30 μ M and 3 μ M respectively.

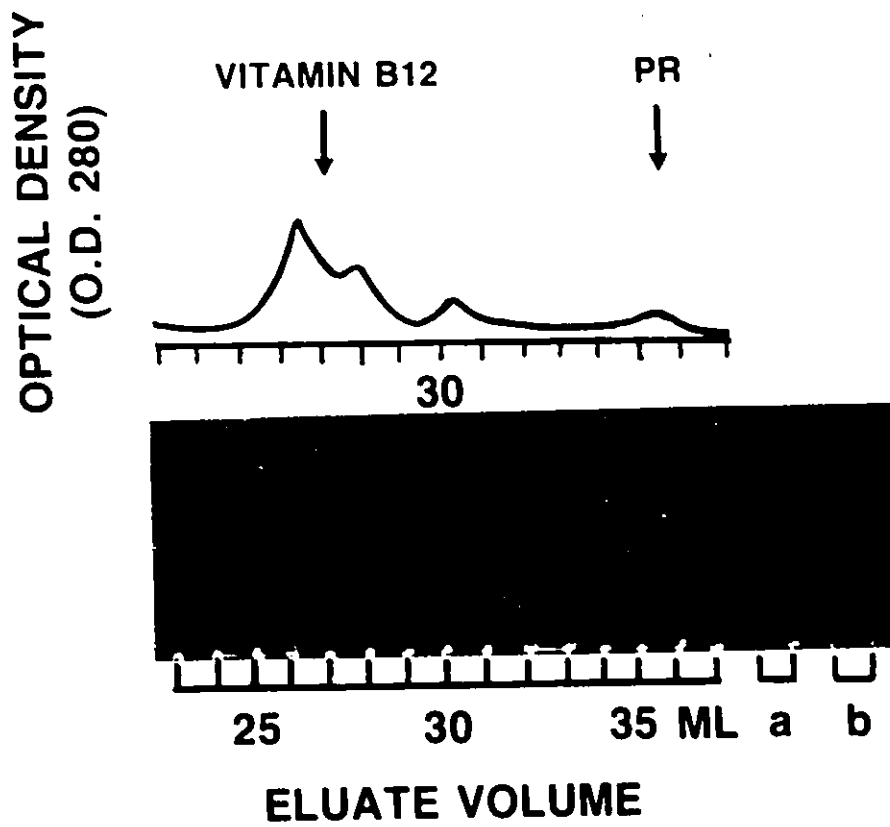
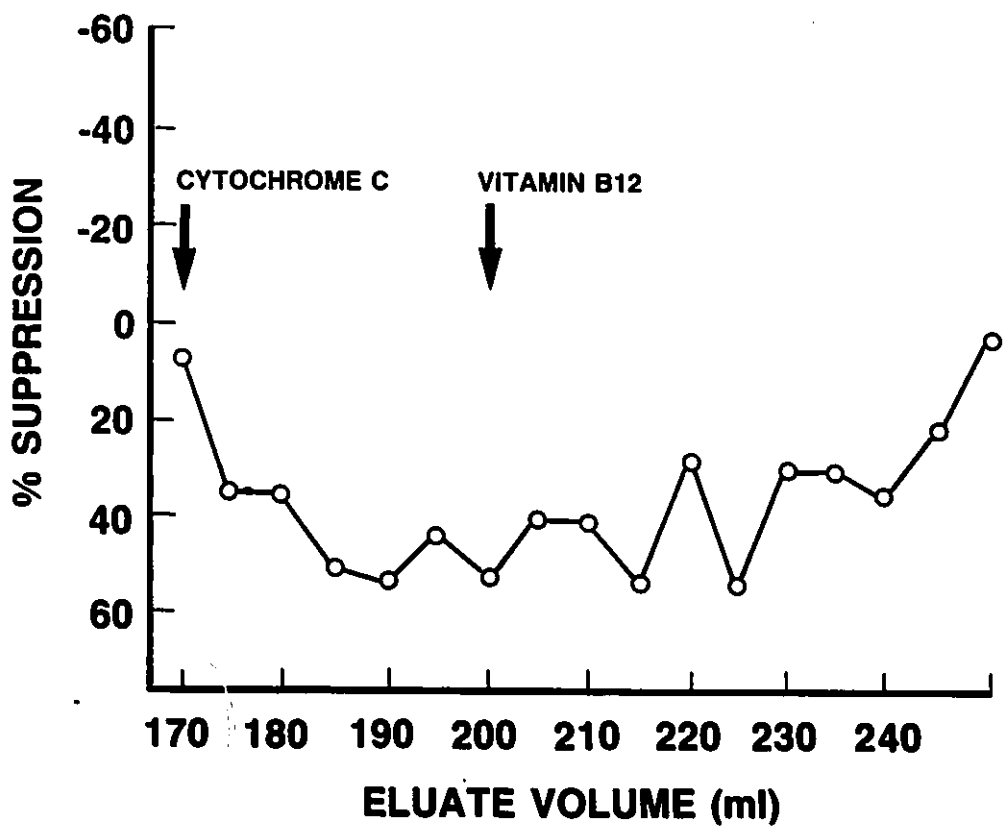


FIGURE 14:

Spermine HPLC analysis. One ml of 0.1 molar solution of spermine tetrahydrochloride was fractionated on a preparative TSK 2000SW column in Tris-buffered saline at flow rate of 5ml/min. Each fraction was tested as described under Materials and Methods in media supplemented by 10% FBS.



3.5 TOXICITY TESTS

Cytotoxicity tests were performed using murine spleen cells as described in Materials and Methods. Cells were incubated in the presence of IVF embryo culture suppressive HPLC fractions, in the presence of suppressive sperm HPLC fractions, HPLC buffer and spermine, spermidine and putrescine. Table 5 and Table 6 summarize the percent cytotoxicity at 48 hours and 72 hours of incubation determined by 0.4% Trypan blue exclusion.

To confirm the non-specificity of suppression by murine IVF embryo culture HPLC fractions and by sperm culture supernatants, the effect of HPLC fractions was assayed using P815 mouse mastocytoma cells in a proliferation assay (Table 7).

3.6 AGED FEMALE IVF OOCYTES CULTURE

It was noted in the experiment reported in Figure 4 , that old mouse oocytes fertilized with young mouse sperm failed to release suppressive factors, except for a very low level of suppression in one case. This is an important observation because it implies that *in vitro* fertilized oocytes may determine the level of production of the suppressive factors; the spermatozoa alone did not provide sufficient quantities of suppressive elements such as spermine.

TABLE 5

CYTOTOXICITY ON MURINE SPLENOCYTES INCUBATED 48 HRS. WITH
SPERM CULTURE AND IVF EMBRYO CULTURE SUPERNATANT
SUPPRESSIVE HPLC PEAKS

TEST SUBSTANCE	SERUM	% CYTOTOXICITY ^a ± 1 SEM	
		48 hours	72 hours
SPERM HPLC ^b	10% FBS	60.9 ± 8.1 ^e	89.6 ± 3.8 ^e
	10% AB serum + AG	5.5 ± 3.2	0.0
IVF HPLC ^c	10% FBS	0.0	6.6 ± 0.8 ^e
	10% AB serum + AG	0.0	0.0
BUFFER ^d	10% FBS	0.0	0.0
	10% AB serum + AG	0.0	0.0

(a) Compared to control as in Materials and Methods

(b) Sperm HPLC peak fraction

(c) IVF HPLC peak fraction

(d) HPLC buffer

(e) Significant toxicity

(f) Note disintegration of some of the dead cells present at 48 hours may explain improved viability at 72 hours where resistant or non-dividing cells are still present.

TABLE 6

CYTOTOXICITY ON MURINE SPLENCYTES INCUBATED 48 HRS. WITH
PUTRESCINE, SPERMIDINE AND SPERMINE

TEST SUBSTANCE	SERUM	% CYTOTOXICITY \pm 1 SEM 48 hours
Putrescine 300 μ M ^a		
	10% FBS	4.2 \pm 1.3 ^b
	10% AB + AG	0.0
Spermidine 300 μ M		
	10% FBS	17.0 \pm 0.9 ^b
	10% AB + AG	0.0
Spermine 300 μ M		
	10% FBS	23.5 \pm 4.0 ^b
	10% AB + AG	1.8 \pm 0.5

a - Final concentration

b - Significant suppression

TABLE 7

EFFECT OF IVF EMBRYO CULTURE SUPERNATANT HPLC FRACTIONS ON
 PROLIFERATION OF P815 MOUSE MASTOCYTOMA CELLS

TEST SUBSTANCE	CPM \pm 1 SEM	% Supression \pm 1 SEM ^a
Buffer Control	164,797 \pm 6,674	0.0
sample ^b 1	115,014 \pm 2,217	30.2 \pm 1.3
sample 2	118,562 \pm 5,169	28.1 \pm 3.1
sample 3	140,277 \pm 1,033	14.9 \pm 0.6

a - Average suppression by samples 1, 2, 3 was 24.4%
 Average suppression by samples 1, 2, 3 in the
 C3H LNC assay was 33.4%

b - HPLC fractions of IVF embryo culture
 supernatants

TABLE 8

EFFECT OF AGE AND EXOGENOUS SPERMINE ON
IVF OOCYTE DIVISION AT 48 HOURS

DONOR	SPERMINE TREATMENT	NUMBER <u>FERTILIZED</u> TOTAL	STAGE OF DEVELOPMENT			P value
			1-2 CELL	>4 CELL	%>4 CELL	
YOUNG CBA (10 wks)	-	75/89 (84.3%) ^a	16	59	(78%)	} P=2.3x10 ⁻⁹ }
OLD CBA (20-28 wks)	-	16/36 (44.4%) ^a	16	0	(0%)	
	+ ^b	31/87 (35.6%) ^a	23	8	(26%)	

- ^a Parentheses show that % of ovulated oocytes which fertilized (polar bodies). Young CBA provided an average of 5.1 oocytes/mouse and old CBA between 1.8-2.8 oocytes/mouse which ovulated. Fertilization rates were lower for old oocytes ($P= 1.4 \times 10^{-5}$) but spermine addition had no significant effect ($P= 0.238$). A separate set of 16 old CBA oocytes left uninseminated failed to show evidence of division.
- ^b 100 μ l of the polyamine was added to 900 μ l to give a final concentration 0.03 μ M (1/3 \geq 4 cell, 33%), 0.3 μ M (2/8 \geq 4 cell, 25%) or 3 μ M (5/18 \geq 4 cell, 28%).

IVF embryos from old females did not divide. Deficiency in polyamine-dependent suppression could have been the result of the failure of the embryos to divide. On the other hand, as outlined in the introduction, polyamines are essential for division. Lack of polyamine production could have been the factor responsible for the failure of the oocytes to divide.

To test the idea that failure of polyamine synthesis by old female oocytes might be a cause for division arrest, we added spermine to the IVF embryo cultures at the time of fertilization. Table 8 shows 16 of 16 of embryos from old females arrested at the 2-cell stage following IVF. The fertilization rate was slightly lower in older animals. A statistically significant proportion of IVF oocytes from old females progressed to the 4 to 8 cell stage by 48 hours in the presence of exogenous spermine.

3.7 STUDIES OF HUMAN IVF EMBRYO CULTURE AND SPERM CULTURE SUPERNATANTS

To determine the relevance of the findings with murine IVF embryo culture supernatants and sperm culture supernatants to the human situation, some parallel studies were performed using human material. Figure 15 shows the results from a number of HPLC studies of human IVF embryo culture and sperm culture

supernatants. Figure 15A shows the pattern of suppression obtained separating a suppressive supernatant tested within 4 weeks of an IVF procedure in human serum-supplemented medium (Clark et al., 1989b). Several peaks of immunosuppressive activity were seen as well as a major peak of stimulatory activity in the 40 to 80 kD range. The dotted line shows the pattern of suppression using supernatants from IVF embryo cultures carried out with cord serum. The first difference that can be seen is that the IVF embryo cultures containing human serum showed inhibitory activity associated with molecules of more than 100 kD. In the lower molecular weight range, suppression was seen at approximately 3700 and 1200 daltons, bracketing the vitamin B₁₂ marker, as well as in those fractions containing molecules smaller than 1200 daltons. To determine whether or not the high molecular weight factor might be contributed by adult serum, a pool of control medium containing adult serum was subjected to analysis. The pool proved to be suppressive and Figure 15B shows that by HPLC analysis, the serum associated suppressive activity occurred at a molecular weight higher than 100 kD. Supernatants with suppressive activity from patients achieving pregnancy were also pooled and separated by HPLC. Lymphocyte proliferation assay of these HPLC fractions showed the expected high molecular weight peak of suppression, and activity in fractions 23 and 27, similar to the suppression seen in Figure 15A. In contrast, a pool of suppressive supernatants from patients not achieving pregnancy did not appear to contain appreciable quantities of this low molecular weight activity.

Supernatants from culture of human sperm alone were similarly found to be suppressive. HPLC analysis of human sperm culture supernatant is illustrated in Figure 15C. A peak of suppression occurred in the high molecular weight range, (200,000 to 300,000 kD) and there was also suppressive activity in the lower molecular weight range, with some of the peaks corresponding rather closely to the low molecular weight suppressive activity seen in the human IVF embryo culture supernatants.

To determine if the suppressive activity in human IVF embryo culture supernatants could be due to the polyamines spermine and spermidine, a pool of suppressive IVF embryo culture supernatant was concentrated by lyophilization to 5 x and subject to HPLC at pH 7.4 . A potent suppressive peak was seen in association with the vitamin B₁₂ marker (Figure 16). Selected fractions were then subjected to TLC separation. It can be seen in the lower half of Figure 16 that only the suppressive peak contained polyamines with an R_f compatible with spermine and spermidine.

FIGURE 15:

HPLC analysis of human IVF embryo culture and sperm culture supernatants using TSK 2000SW column. (A) (•) A single suppressive supernatant was separated and tested using human serum as described under Materials and Methods. (.....) human IVF embryo culture with fetal cord serum suppressive supernatants. (B) Pooled supernatant from suppressive serum control (o), suppressive supernatant from IVF embryo culture of patients who became pregnant (•), and suppressive IVF embryo culture supernatant from patients not achieving pregnancy (Δ) were separated and tested. (C) Supernatant from 24 hrs incubation of 10^6 human sperm/ml was separated by HPLC, and each fraction tested as described under Materials and Methods. The molecular weight markers apply to all three panels.

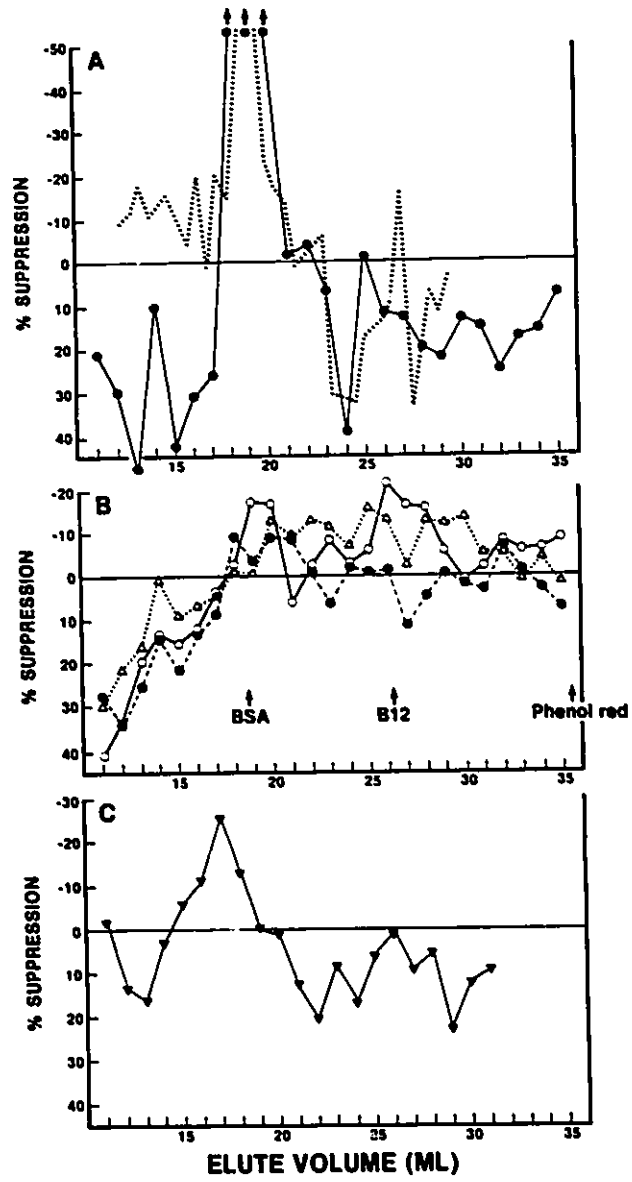
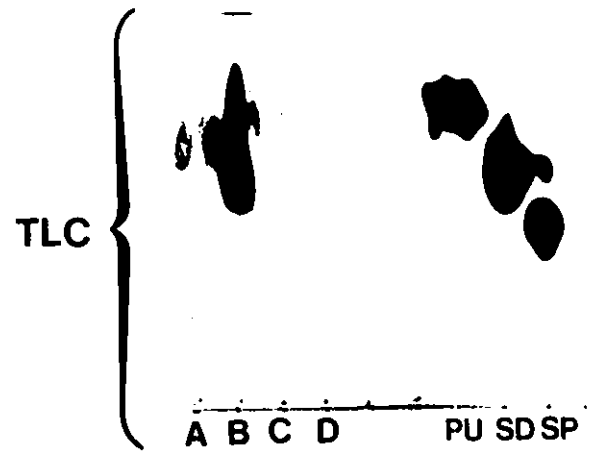
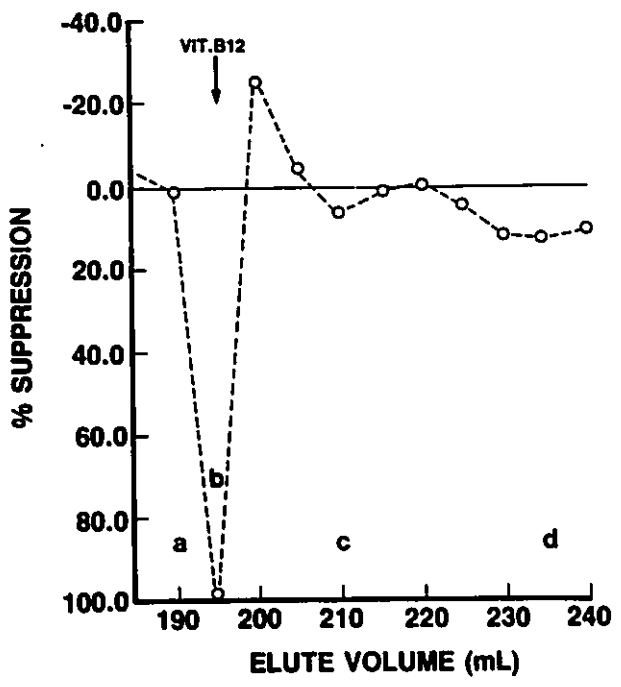


FIGURE 16:

HPLC-TLC of supernatants of human IVF embryo cultures (growth phase). FBS supplemented RPMI media was used for the Con A stimulated lymphocyte proliferation assay in the upper panel. In the lower panel TLC of the fractions marked "a-d" and polyamine markers putrescine (PU), spermidine (SD) and spermine (SP) 30 μ M is shown.



4. DISCUSSION AND CONCLUSIONS

The data in this thesis show that supernatants from mouse IVF embryo cultures suppress *in vitro* lymphocyte proliferation stimulated by concanavalin A. HPLC analysis of the IVF embryo culture supernatants showed several peaks correlating with suppressive activity in the range of 200 to 7000 daltons, similar to the activity found in human IVF embryo culture supernatants. Supernatants conditioned by 24 hour incubation with mouse epididymal sperm alone were also highly suppressive, and the active molecules appeared to have a similar molecular weight by HPLC analysis. Supernatants from unfertilized oocytes did not display suppressive activity. Both the IVF and the sperm-derived suppressive molecules appeared to be polyamines based on reversal of activity when the serum in the assay was treated with amine oxidase inhibitors, aminoguanidine, and by TLC studies of the suppressive HPLC fractions.

Suppressive activity indicated by low molecular weight molecules in both IVF embryo culture supernatants and sperm culture supernatants was eliminated by replacing FBS in the assay, with human serum, which is lower in amine oxidase content, together with aminoguanidine (Allen et al., 1979). Aminoguanidine is an

inhibitor of the copper-containing amine oxidases, the enzymes responsible for terminal catabolism of polyamines (products of the reaction cannot be reconverted into polyamines) (Seiler et al., 1985). The data suggest that polyamines oxidized by amine oxidases to toxic metabolites, are responsible for this suppression.

The TLC was less sensitive in detecting spermine and spermidine than the bioassay, particularly in the case of spermine. The suppressive factors in sperm culture supernatants could be related to immunosuppressive molecules present in seminal plasma, where the major contributor to suppression *in vitro* is due to the polyamine spermine (Tabor and Tabor, 1984). Spermine itself is non-suppressive, but when metabolized *in vitro* by amine oxidases present in FBS into acrolein or spermine dialdehyde, toxicity to a variety of cell types is observed (Allen et al., 1979). In agreement with such a mechanism, it was found that exposure to suppressive IVF embryo culture supernatants or sperm culture supernatants nonspecifically inhibited the growth of mouse mastocytoma cells and reduced the viability of lymphocytes.

Molecules present in IVF embryo cultures, especially those in the low molecular weight range, exhibited suppression of lymphocyte proliferation. In HPLC profiles, several peaks of suppressive activity were determined, and repeatedly, there was a suppressive peak at the vitamin B₁₂ marker and at lower molecular weights. When the IVF embryo culture HPLC peak suppressive molecules were tested for cytotoxicity, only 6.6% specific killing was detected in 72 hour cultures. The same IVF HPLC peak suppressive fractions displayed 39.2% suppression when tested in

the lymphocyte proliferation assay with 10% AB serum in the incubation media (Table 4). It is possible that it is only the proliferating lymphocytes that are killed, and thus the percentage of suppression is greater than the percentage of killing.

Murine epididymal sperm culture supernatants display a much higher suppressive activity than was observed for IVF embryo culture supernatants. In sperm culture supernatant HPLC profiles, several peaks of suppressive activity were determined, and there was a suppressive peak at the vitamin B₁₂ marker, as well as at lower molecular weights. When the sperm culture supernatants HPLC peak suppressive molecules were tested for cytotoxicity, 29.6% killing was detected by 72 hour cultures. The same sperm culture supernatants HPLC peak suppressive fractions displayed 40.2% suppression when tested for lymphocyte proliferation. The latter result may also be explained by the fact that in lymphocyte cultures only a small fraction of the cells are dividing: it would appear that the dividing cells are those most affected.

Polyamines, as positively charged molecules which form complexes and polymers, and can attach to carriers such as proteins (Pegg, 1986; Heby and Persson, 1990). This may explain why the peaks of suppression were usually detected by HPLC at a molecular weight greater than 202 daltons (Figure 2) occurred. Indeed, human IVF embryo culture supernatants (Figure 16) and spermine have been found to produce a dominant peak at 1500 daltons with suppression of human lymphocyte proliferation extending to the cytochrome C marker (Lea et al., 1990). Spermine HPLC fractions tested in the lymphocyte

proliferation assay in this thesis (Figure 14), however, displayed peaks ranging from 5000-500 daltons and below. The HPLC of murine IVF embryo culture supernatants also showed activity at molecular weights smaller than 1000. In the Lea et al. (1990) experiments approximately 10 times the concentration of spermine was loaded on the same column, and the activity of the concentrated IVF supernatants was much greater. Since the HPLC buffer was similar (unpublished data) and protein was present in separated human IVF supernatants, the dominant 1500-1000 dalton peak seen by Lea et al (1990) may be explained by a physical-chemical complexing of spermine-spermidine when at high concentrations.

TLC analysis of murine IVF embryo cultures suggested both spermine and spermidine were present in IVF embryo culture supernatants, and correlated with polyamines responsible for suppressive activity in the lymphocyte proliferation assays. Putrescine was not suppressive (Figure 9). In human IVF embryo culture supernatants, a dominant TLC spot of spermidine with a small amount of spermine has been found in the low molecular weight peak "c". This result was also obtained, however, using a supernatant that was concentrated before performing the HPLC (Lea et al., 1990), and the HPLC peak was as potent as concentrated sperm culture supernatant fractions (Figure 7). We have shown human sperm culture supernatants to be suppressive (Figure 15C) (Schechter et al., 1988; Clark et al., 1989b). TLC analysis of the murine sperm culture HPLC fractions suggested the suppressive molecule in sperm culture supernatants could be spermine (Porat and Clark, 1990a).

Although a strong correlation was obtained suggesting spermine in sperm and spermine together with spermidine in embryo culture supernatants were responsible for suppression, it has not been formally proven that the polyamine-like suppressive molecules are identical to spermine/spermidine. An attempt to solve this problem was made by using mass spectroscopy. Some peaks at the expected molecular weight were seen when the HPLC fraction with suppressive activity were tested, however, the high salt concentration produced many background spikes that made definitive conclusion impossible. The suppressive activity in the human IVF HPLC peak (Figure 16) has recently been successfully neutralized using specific rabbit anti-spermine/spermidine antibodies (Lea et al., 1991). However, even this does not exclude the possibility that a spermine-like molecule is actually responsible for suppression (Kerr and Atkins, 1989).

A high polyamine content in an extracellular compartment is a rare occurrence (Pegg and McCann, 1982). Seminal fluid of a number of species (including that of man and mouse) contain very high concentrations of spermine (Mann, 1964; Tabor and Tabor, 1964; Quinn et al., 1965; Janne et al., 1973). The prostate gland of sexually mature men is almost the exclusive source of the exceptionally high concentrations of spermine in human seminal plasma, in which spermine can be present in concentrations of even more than 10 mM (Mann, 1964; Tabor and Tabor, 1964). The ratio of putrescine, spermidine and spermine in human seminal plasma is 2:1:30, respectively (Janne et al., 1973). As well, spermine

is present in association with human and other mammalian spermatozoa (Levy and Fair, 1973; Pulkkinen et al., 1975), but has not been shown to be produced by the sperm cell. Spermine molecules found on sperm cells may be carried over from the seminal fluid to the spermatozoa by being internalized and/or by attaching to the spermatozoal plasma membrane. However, whether spermine or spermidine are really present intracellularly inside spermatozoa of any mammalian species, rather than being tightly associated with spermatozoal plasma membranes has not been definitively demonstrated to date.

The formation of spermatozoa is a continuous and dynamic process, which is regulated hormonally and non-hormonally. Testicular polyamine concentrations are closely associated with spermatogenesis and testicular maturation (MacIndoe and Turkington, 1973; Oliva et al., 1982; Shubhada et al., 1989). Spermatozoa formed in the testes are matured and stored in the epididymis and mix with other semen components upon ejaculation. Polyamine-metabolizing enzymes such as S-adenosyl-L-methionine decarboxylase (SAM-D), spermine synthase, spermidine synthase and DAO (Figure 3) are present in human semen, and are likely derived from the prostate gland. DAO activity has been associated with spermine in human spermatozoa (Pulkkinen et al., 1975), and has been detected in the sperm cells (Janne et al., 1973).

Complex processes likely occur once the spermatozoa are exposed to prostatic secretions and to other seminal fluid components in some species including humans. Contact of spermine from the seminal plasma with spermatozoa, and

perhaps its incorporation, would be expected to result in a potentially toxic effect, as oxidized spermine and spermidine are extremely cytotoxic to spermatozoa. Yet, under normal circumstances, spermatozoa escape the cytotoxic effect of oxidized polyamines (Rosenthal and Tabor, 1956; Mann, 1964; Bachrach, 1973). In fact, polyamines may be necessary for the spermatozoa - if they are to survive and function. For example, in bovine sperm, polyamines stimulate protein phosphorylation of sperm cytosolic and plasma membrane proteins (Chaudhry and Casillas, 1989).

In man, an increase in putrescine secretion caused by frequent ejaculation was reported by Rui et al., (1989). This is surprising, because other prostatic secretory products and seminal plasma constituents decrease in quantity under the same condition. As well, eight years after vasectomy, no reduction in putrescine contents in human seminal plasma was demonstrated, while other polyamines were greatly reduced (Jakobsen et al., 1989). It has been suggested that high putrescine secretion may reflect its involvement in intracellular biochemical mechanisms, such as RNA and protein synthesis (Pohjanpelto, 1976; Pegg and McCann, 1982; Tabor and Tabor, 1984).

Following copulation spermatozoa have to survive in the female genital tract if they are to fertilize the oocyte. It is conceivable that, in some species, sperm survival immediately after ejaculation is regulated by polyamines or polyamine products present in the seminal plasma. Transglutaminases (EC 2.3.2.13) catalyze covalent attachment of polyamines to proteins (Folk, 1980). Seminal plasma

transglutaminase could cross-link polyamines to spermatozoa, or form protein-polyamine complexes (Mann, 1964; Mann and Lutwak-Mann, 1981). In certain rodents, cross-linking of polyamines by transglutaminases forms the copulation plug in the vagina after coitus (to aid spermatozoa passage to the uterine cervix) (Williams-Ashman, 1965). On the other hand, the high concentrations of spermine derived from prostatic secretions may be responsible for inhibition of rapid coagulation of semen by transglutaminase (Williams-Ashman and Canellakis, 1980).

There are several reports on the relationship between the immunosuppressive and cytotoxic effects of human seminal plasma, and the role of spermine and spermine oxidation in immunosuppression *in vitro* (Marcus et al., 1978; James and Hargreave, 1984; Allen and Roberts, 1986, 1987, 1988; Rees et al., 1986; Szymaniec et al., 1987). Valley and Rees (1986) reported seminal plasma suppression of human lymphocyte responses *in vitro* requires the presence of ruminant serum factors. On the other hand, Szymaniec et al., (1987) reported human seminal plasma suppresses lymphocyte responses *in vitro* in serum-free medium. It is conceivable that a polyamine, or a polyamine metabolite, acts as an immunosuppressor in association with the presence of other seminal plasma elements within the reproductive tract. Inhibition of lymphocyte DNA-synthesis by spermine or spermine-derived polycations may be responsible in some species for blocking immune reactions in male and in female genital tract, without causing harm to spermatozoa (Patt et al., 1982). At the same time, the non-functioning spermatozoa are removed by the female tract by degradation and phagocytosis

(Austin, 1957; Olive et al., 1987). Factors present in seminal plasma are also responsible for suppression of natural killer cell activity (Vallely et al., 1988).

Strzemienski (1989) reported that affinity of spermatozoa for certain immunoglobulins is reduced by seminal plasma. In the rat, suppression of epididymal sperm antigenicity was achieved *in vitro* by uteroglobin and transglutaminase (Mukherjee et al., 1983). Transglutaminases have been suggested to modulate immunologic functions by enzymatic alterations of antigens and cell receptors (Alexander and Anderson, 1987). If polyamine metabolites are important as immunosuppressive factors, and as in the case of bovine serum polyamine oxidases the polyamines form cytotoxic and immunosuppressive products, different forms of polyamine oxidases (other than spermine oxidase) and more specific factors may be involved in these reactions.

It is possible that at least some of the spermine detected in IVF embryo cultures may be sperm-derived. In human IVF, "sperm washing" is a standard procedure whereby the seminal plasma is removed prior to insemination and the sperm cells are suspended in insemination fluid which is defined. Culture media of washed human sperm following 24 hour incubation highly suppress lymphocyte proliferation. However, epididymal sperm, used in the mouse IVF system does not come in contact with seminal fluid. Since polyamines are present in association with human and other mammalian spermatozoa, it is likely that sperm-contributed polyamines would be present in the culture (Levy and Fair, 1973; Pulkkinen et al.,

1975). Normally, at fertilization a single spermatozoon penetrates the oocyte, while hundreds of sperm cells remain attached to the outer oocyte coating - the zona pellucida. Spermatozoa tightly bound to the zona pellucida can be carried over to the second 24 hour culture medium and thus could contribute to the polyamines detected in these IVF embryo cultures. However, the data in Figure 4 show little if any suppression when oocytes obtained from old females were fertilized with sperm obtained from young male donors. Supernatants conditioned by young female oocytes that had been fertilized with sperm from young males were suppressive. Therefore most of the murine polyamine-derived suppressive activity found in the IVF embryo culture supernatants is likely produced by the embryo rather than by the spermatozoa.

Since spermidine can be an interconversion product of spermine, the presence of spermidine as well as spermine in 48 hour IVF embryo culture supernatants does not exclude sperm as the origin of polyamines in IVF embryo culture supernatant activity. The presence of polyamines contributed by dividing embryos may explain why in some human IVF embryo cultures, the peak suppressive activity occurs between 20 to 40 hours after fertilization when the majority of sperm had been removed from the incubation medium (Clark et al., 1989b).

The lack of suppression in old female oocyte IVF culture supernatants is interesting given that absence of suppression has been associated in the human IVF system with failure to implant (Daya and Clark, 1988) and the fact that CBA/J is

a strain of mouse that develops premature infertility by 6 to 8 months (Jones and Krohn, 1962; Finch, 1978). Figure 17 illustrates the relationship between CBA female age and CBA litter size (Jones and Krohn, 1962). On average, there is a rise in litter size until the female is 16 weeks, a sharp downslope in litter size follows until the age of about 28 weeks. A possible explanation for each supernatant suppression could be that increased release of stimulating factors (Figure 4B) from the old IVF oocytes antagonized the suppressive effect likely due to polyamines in the lymphocyte proliferation assay. Alternately, dividing embryos like any dividing cells produce and contribute polyamines to the surrounding culture medium. This could be the sole contribution of the dividing embryos or could add to the contribution of sperm-derived suppressor activity. Since the old IVF oocytes did not divide, they would also fail to synthesize polyamines after being fertilized.

Ageing has been defined as a time-dependent process which results in the organism failing to cope with environmental factors and failing to change in a manner comparable to its initial capacity to do so. An aged female would not perform reproductively as efficiently as a young female, because of a variety of mechanisms, including ageing of gametes (loss of zygotes due to aneuploidy) and of the uterus, changes in the endocrine system and in the general physiology of the animal (cardiovascular, etc.). Although ovarian activity and endocrine functions appear to be generally well maintained in the ageing mammal, there is evidence that oocyte quality does deteriorate (Thung, 1961). Incidence of aneuploidy in first-cleavage mouse embryos is shown to correlate with maternal age (Maudlin and

Fraser, 1978). It is known that blastocysts obtained from old mice fail to implant and develop when transferred to old uterine environments. However, when transferred to the uteri of young mice, just as many blastocysts develop as from reproductively active females (Rugh, 1967). Some researchers postulate that the reduction in litter size with increasing maternal age is probably due to an increasingly less favourable uterine environment rather to a decline in the viability of the ova. A combination of factors should be considered as affecting the uterine environment at the time of implantation. In aged animals the preimplantation component may assume increased significance, and uterine failure is more crucial as it tends to occur earlier in the embryo's life. As a result, with advancing age prenatal mortality occurs progressively earlier in pregnancy (Talbert, 1968).

Epidemiologic analysis of the observations of karyotyped spontaneous abortions in humans revealed a certain amount of information on the influence of various factors related to the ageing of the gametes participating in fertilization (Fabricant and Schneider, 1978; Gosden, 1973; Mikamo and Hamaguchi, 1975). The role of maternal age was shown to be significant in trisomies (Hook, 1983). An effect of ageing on the nucleolus and disturbances in the timing of fertilization might account for the occurrence of polyploidy (Boue et al., 1973). Since polyamines have been implicated in nucleolar organization, their lack of involvement in cellular and fertilization processes may be the cause of aberrations.

In humans, recent studies have shown oocytes from young donors to be highly successful in older females suffering from infertility, suggesting it is not the

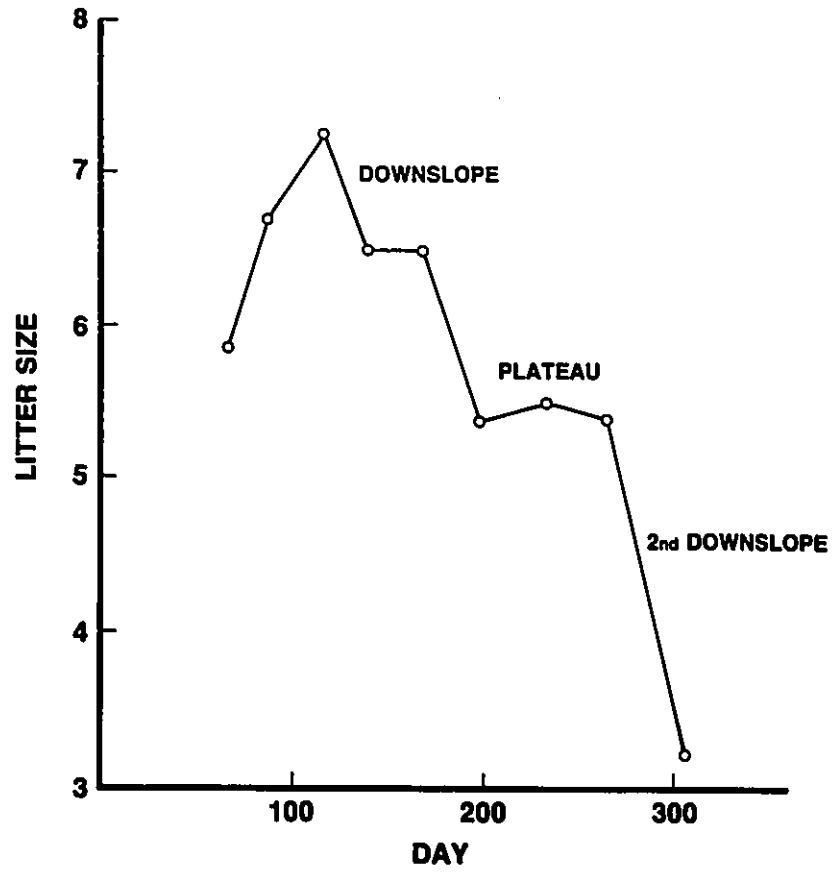
recipient's aged uterus that is at fault (Serhal and Craft, 1989). I speculate that adequate polyamine production by young fertilizable oocytes could, in part, be responsible for their higher success rate. Oocytes which progress to the blastocyst stage, are known to have a higher rate of success in implanting following ET than embryos of earlier stage (Bustillo et al., 1986). Since there may be an association between polyamine deficiency in IVF tissue culture supernatants (spermine/spermidine) and age-onset infertility, adding spermine to old female polyamine-deficient oocytes in IVF cultures, could stimulate division by a proportion of them, and enhance the percentage of these embryos reaching blastocyst stage. Whether an increase in the levels of certain polyamines present in IVF medium can increase *in vitro* cleavage rates, and improve implantation rates with young IVF oocytes remains to be tested in rodent systems.

FIGURE 17:

The relationship between CBA female age and litter size

(data from Jones and Krohn, 1962)

**CBA FEMALE AGE &
LITTER SIZE RELATIONSHIP**



The mechanism of action of polyamines in mammalian cells is still not completely understood. According to Heby and Persson (1990), spermine charged with four positive charges at physiological pH may neutralize the negative charge on the DNA backbone and thus stabilize DNA by binding the two helix strands together. Polyamine intracellular concentration is known to be regulated by the cells. Studies with inhibitors of polyamine biosynthesis point to the importance of polyamines in cell growth, regulation of cell proliferation, division and differentiation (Janne et al., 1973; Williams-Ashman and Canellakis, 1979; Heby, 1981; Heby and Emanuelsson, 1981; Tabor and Tabor, 1984; Rees et al., 1986; Pegg, 1988). Conversions of spermine into spermidine and of spermidine into putrescine have been shown to occur *in vivo*.

Several studies of mouse preimplantation embryo development in the presence of polyamine synthesis inhibitors point to the importance of polyamines in embryo division. *In vitro* cultures of mouse preimplantation embryos in the presence of methylglyoxal- bis-(guanylhydrazone) (MGBG), an inhibitor of SAM-D, demonstrated arrest of embryo development at the 8-cell or morula stage. In addition, *in vitro* embryo DNA synthetic rate, as measured by ^3H thymidine incorporation, was strongly inhibited (Zwierzchowski et al., 1986).

In another study, polyamines have been implicated in cavitation of mouse preimplantation embryos cultured *in vitro* (Alexandre, 1979). The inhibition of blastocyst formation and DNA synthesis by MGBG was noted to be readily reversible by an exogenous supply of spermine and/or spermidine to the culture

medium. In contrast, D,L- α -methylornithine or D,L- α -difluoromethylornithine (α -DFMO), highly selective enzyme-activated inhibitors of putrescine biosynthesis, had no effect on embryos cultured for 1 or 2 days. On the third day, embryo DNA synthesis in the presence of α -DFMO was significantly depressed. These observations suggest that during early development of mouse preimplantation embryos, spermine and spermidine are involved in regulating embryo growth and DNA synthesis. A role for putrescine at a later stage of mouse embryo development may also be indicated.

In a different study, addition of spermine to nuclei isolated from decidual tissue, caused the nuclei rate of RNA synthesis to increase (Hoshiai et al., 1981). Spermidine in these nuclei is likely involved in elongation of RNA chains, yet it does not initiate the synthesis of new RNA chains (Hoshiai et al., 1981). There are basic difficulties in comparing early embryonic development with cell growth in culture. It is difficult to carry out experiments where the polyamine content of early embryo cells is first limited and then relieved by supplying the specific polyamines, as the compromise of the developmental capacities of these embryos is likely to be irreversible.

Polyamines are also necessary for lymphocyte function. In eukaryotic cells L-ornithine decarboxylase (ODC), catalyses the initial and usually key step in the synthesis of polyamines (McCann, 1980; Pegg, 1988). The induction of increased ODC activity to produce putrescine, is an integral event regulating lymphocyte differentiation, proliferation and function (Fillingame et al., 1975; Klimpel et al.,

1979; Pasquali et al., 1984). Mitogen stimulated lymphocytes can synthesize 0.1 - 1 μ mol polyamines per cell, spermine being the most abundant (Kay and Lindsay, 1973b; Fillingame and Morris, 1973). By utilizing α -DFMO and methylacetylenic putrescine (MAP), polyamines have been shown to be required for mitogen-induced T-cell activation, lymphokine-dependant cell growth, and T-cell dependant antibody production (Pasquali et al., 1984; Bowlin et al., 1988).

The reduced capacity of mammalian aged organs for polyamine biosynthesis may be one of the factors responsible for the lower efficiency of immune response in tissues of aged mammals. Reduced polyamine biosynthetic capacity of aged mammals might account for the slower course of some tumors in elderly patients (Scalabrino and Ferioli, 1984). As well, tumor promoters have typical stimulatory effects on polyamine biosynthesis in target tissues.

Development of mouse and human fertilized oocytes to at least the blastocyst stage requires only simple culture media based on Krebs's supplemented by pyruvate, lactate and glucose. IVF culture medium containing factors, including polyamines produced by dividing embryos is discarded at the time of ET to the recipient uterus. Supplementation of the ET medium with polyamines at this stage could prove to be advantageous to embryo implantation in two possible ways. The first in embryo division, as already been discussed, the second in suppression of lymphocyte proliferation.

Accordingly, two possible types of molecules may be lost on transfer of the

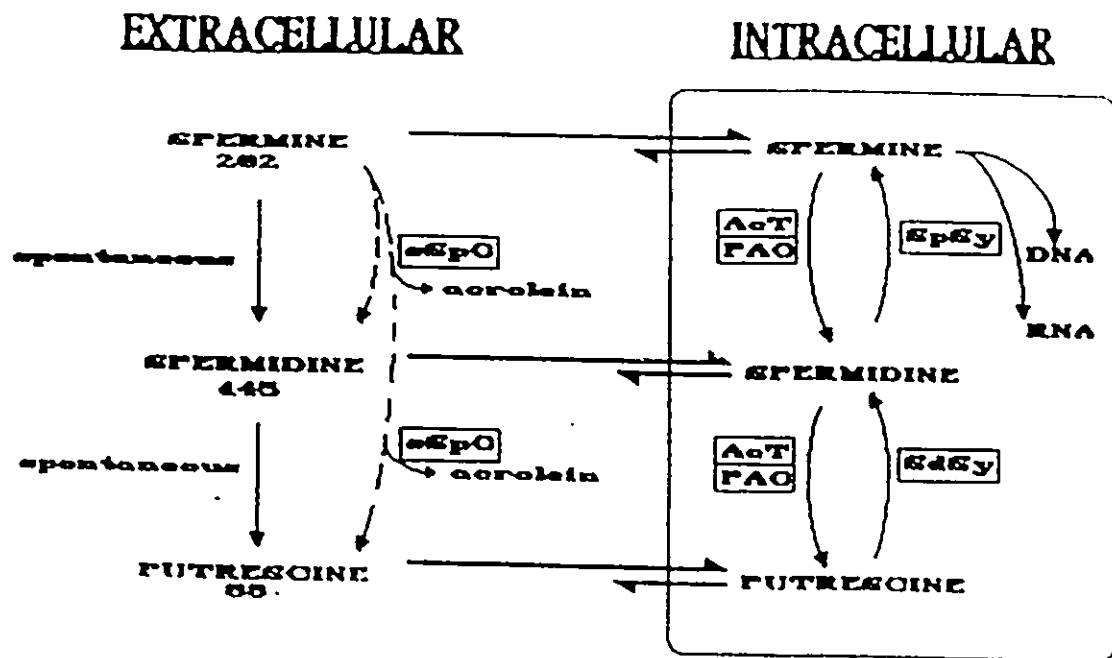
IVF embryo to the uterus without the culture medium: (a) immunosuppressive factors, (b) growth and implantation factors. Beside their role as growth factors polyamines, especially spermine, which is a known bactericide (Levy and Fair, 1973; Tabor and Tabor, 1984) may also have a role as local immunosuppressants in the uterus.

Extracellular oxidation of polyamines by serum polyamine oxidases yields aldehyde polyamine derivatives that are unstable and can liberate the appropriate polyamine and acrolein (Figure 3). These derivatives can also be taken up by the cell and metabolized as illustrated in Figure 18 (Pegg, 1986). Conversion of mono- or bis-terminally acetylated spermine to spermidine, and of N¹-acetylspermidine to putrescine by an intracellular polyamine oxidase that acts on secondary amine groups, does not produce acrolein as one of the reaction products (Pegg and McCann, 1982).

In the lower insert of Figure 3 the utilization of free spermine or spermidine is described. These reactions are catalyzed by "terminal" polyamine oxidases, which are found in the serum of adult males and non-pregnant females in a restricted number of species only. The physiological significance *in vivo* of these reactions is questionable. However, one must take into account that low levels of amine oxidase may be present in human sera used to supplement IVF embryo culture media. It could be hazardous to supplement such IVF media with extra spermine or spermidine due to a potential risk of toxicity to the embryo.

FIGURE 18:

Polyamines in the extracellular and intracellular compartments



Non-specific reactions of polyamine oxidation products with cell membrane proteins can cause major disturbances to normal growth and development (Kimes and Morris, 1971b; Holttä, 1977; Allen et al., 1979). Gaugas and Dewey (1979) noted that cells arrest in the G₁ phase of the cell cycle when incubated in the presence of FBS and polyamines. However, a latent interval of 30-36 hours (more than one cell cycle) is obligatory before morphological manifestations of cell death are evident (Blewitt et al., 1983), implying toxic products are accumulated, or some vital substances are depleted. Polyamine oxidase (PAO) inhibitors may completely abolish polyamine toxic effects, implying that it is the oxidation products that are cytotoxic and not the polyamines (Labib and Tomasi, 1981; Abbott and Bird, 1983). Compartmenting the reaction sequences to intracellular and extracellular probably protects the tissues from reactive exogenous polyamines.

If PAO and polyamines are involved in lymphocyte suppression, how does the pre- and post-implantation embryo escape this cytotoxicity? One cannot exclude toxicity resulting from polyamine oxidation as an explanation for the putative beneficial effect of polyamines in improving cleavage rates (Table 8) being only partial. Alternately, failure of all of the old IVF oocytes to divide in spite of exposure to spermine may reflect a variety of age-associated oocyte defects. In only a subset may the polyamine deficiency represent remediable pathogenic situation. Indeed, as CBA/J females age, a substantial proportion of the ovulated oocytes from old mice appear to degenerate *in vitro* and do not fertilize. *In vivo*, the

extracellular polyamine content is normally low; the cellular interconversions may be of primary importance in preventing higher than required levels of spermine and spermidine from accumulating in the cells. Since there is metabolic interconversion of the polyamines, addition of putrescine, which appears non-toxic to lymphocytes (Table 6), to IVF culture media might effectively boost intracellular spermine/spermidine levels in the fertilized oocyte without risk of toxicity and achieve a higher percentage of proliferating oocytes.

Several polyamine complexes have been identified in human extracellular fluids. A high-molecular weight polyamine conjugate in which spermine is bound to an immunoglobulin has been identified in human serum (Roch et al., 1978). In human amniotic fluid most of the spermidine and putrescine are present in the form of polyamine-polypeptide conjugates (Chan et al., 1979). The spermidine-containing peptides weigh between 10,000 and 30,000 daltons. The putrescine conjugate has a molecular weight of 5000 (Seale et al., 1979b). A similar putrescine complex has been isolated and characterized from human plasma (Seale et al., 1979a). One must take into consideration that maternal serum levels do not necessarily reflect endometrial synthesis and secretion. As well, amniotic fluid and *in vitro* cultures may only partly reflect the ongoing production and secretion of pregnancy- or other *in vivo* associated polyamines, complexes and enzymes.

Human PAO activity has been determined in the fetomaternal compartments in pregnancy (Illei and Morgan, 1979) and in spontaneous abortion (Illei and

Morgan, 1982). A rapid increase in PAO activity was reported by Morgan et al., (1983) in pregnancy serum from 8 to 20 weeks of gestation, leading to an increase in polyamine oxidation products.

Maternal decidua has been considered the source of plasma DAO in pregnancy (reviewed by Bell, 1986). The activity of DAO increases after 5-6 weeks of pregnancy and rapidly reaches up to 1000 times normal values, to which it returns after parturition (Buffoni, 1966). DAO activity was also localized in the cytoplasm of the maternal decidual cells of the placenta (Weisburger, 1978). It is possible that regulation of decidual DAO activity is induced by fetally derived polyamines or their derivatives (paracrine regulation by embryo-derived putrescine and other polyamines).

It is possible that amine oxidases present in amniotic fluid are of different specificities to amine oxidases present in FBS. Spermine oxidase in mouse amniotic fluid produced a non-cytotoxic immune inhibitor from spermine and from spermidine (Labib and Tomasi, 1981). Gaugas and Curzen (1978) described human pregnancy serum as strongly immunosuppressive in the presence of spermine and putrescine, while fetal cord serum and normal human sera were not inhibitory in mitogen stimulated lymphocyte proliferation assays.

Injection of interleukin 2 (IL-2) which may activate cytotoxic immune effector cells such as lymphokine activated killer (LAK) cells, appears able to inhibit embryo implantation (Tezabwala et al., 1989). As macrophages present at the implantation site may also bear IL-2 receptors, IL-2 activation of uterine macrophages could

also be involved in inhibition of embryo implantation (Tachi and Tachi, 1989) unless expression is suppressed. Maternal decidua contains amine oxidases (Illei and Morgan, 1979) and therefore local activation of polyamines could, in theory, inhibit maternal effector cell activation by lowering levels of cytokines such as IL-2, lowering IL-2 receptor formation or interfering with signal transduction.

Whether acting as growth factors, as immunosuppressants or both, spermine and spermidine generated by spermatozoa in or near the uterus appear to increase embryo implantation success. This may be the reason why gamete intra-fallopian transfer (GIFT) in humans, (where sperm and their associated polyamines are retained within the environment of the oocyte in the oviduct *in vivo*), results in a higher success rate than that attained by IVF (Asch et al., 1988). Further, semen deposited in the vaginal vault has been reported to increase the success rates of human IVF transfers (Bellinge et al., 1986; Marconi et al., 1989). Improved blastocyst implantation rates have been seen in rodent models after uterine exposure to sperm (Carp et al., 1984).

The advantageous effect of sperm presence in the female reproductive tract was demonstrated by Carp et al. (1984), who reported that sperm penetration into the uterus may be a factor contributing to decidualization. Forty-five percent of the blastocysts transferred to the uteri of pseudopregnant rats implanted, while a 90% rate of implantation resulted when pseudopregnant rats were inseminated prior to the transfer of blastocysts. More data is needed to determine if such effects are

mediated by polyamines, and if so, by which one(s) and by what mechanism(s).

One must consider species differences in the fate of extracellular polyamines that are deposited in the female reproductive tract during copulation. In many species, including man, in which insemination is intravaginal, it has not been shown unequivocally that substantial amounts of spermine or spermidine derived from semen ever enter into the lumen of the uterus or the oviducts. For those species in which insemination is essentially intrauterine (e.g. mouse and rat), there is not enough information about the quantities of polyamines present in semen that actually infiltrate uterine and oviductal fluids.

The data in this thesis provide new insights into the basic biology of embryo-maternal interactions and have potential to enhance clinical outcome of assisted reproductive techniques. An increase human IVF success rate might be attained by altering polyamine levels in IVF culture media. It is necessary to first test whether the loss of factors produced either by the sperm or by the embryo is the cause for the low implantation rate evident in IVF programmes. Current methods for *in vitro* fertilization of mouse ova have been refined to the point where they can be used for analysis of the parameters which govern mammalian fertilization and can reflect on human fertilization. Using the murine system, it is possible to test the effect of spermine on an IVF system. As well, it might be very important to test a combinations of growth factors, including polyamines. Supplementation of IVF culture media and of ET media with particular polyamines might enhance survival

rates and successful implantation of IVF embryos.

In IVF-ET programmes, assay of metabolic performance of embryos using quantitative microanalytic procedures which are non-invasive could be helpful in assessing the embryonic potential to implant. Polyamines and polyamine metabolite presence in media conditioned by the incubating embryo may serve as a tool in such assessment. This has to be further investigated, fully validated and simplified for routine prognostic purposes and for improving the results of IVF treatments by selecting the embryos most likely to succeed. Application of polyamines or polyamine biosynthesis inhibitors in diagnosis and treatment of infertility (or in reducing undesired fertility) is completing the circuit from basic understanding of the role polyamines play in cell growth and differentiation to therapy.

5. SUMMARY

Supernatants from mouse IVF embryo cultures suppress *in vitro* lymphocyte proliferation stimulated by concanavalin A. HPLC analysis showed several peaks of suppression in lymphocyte proliferation assays, including activity at 1-7 kD, similar to the activity found in human IVF embryo culture supernatants. Supernatants conditioned by incubation with mouse epididymal sperm alone were even more inhibitory. Unfertilized oocyte culture supernatants were either suppressive or stimulatory. HPLC analysis of sperm-conditioned medium showed several peaks of inhibitory activity, many of which were similar to those in the IVF embryo culture supernatants. Suppressive activity of the low molecular weight inhibitory molecules was eliminated by replacing fetal bovine serum in the assay, with human serum, which is lower in amine oxidase content, together with aminoguanidine (an amine oxidase inhibitor). These data suggest that a polyamine, oxidized by amine oxidases to toxic metabolites, was responsible for this suppression. TLC analysis suggested spermine in sperm culture supernatants and spermidine in IVF embryo culture supernatants were the polyamines responsible. Putrescine was not suppressive.

Further, *in vitro* fertilized oocytes from aged (20-30 weeks) CBA/J females (a mouse strain that develops early infertility), had supernatants that were stimulatory *in vitro* (i.e non-suppressive) even though sperm from young males had been used. Loss of suppression could be due to stimulatory factors liberated by the oocyte, by absence of polyamine release by sperm, or by failure of the old oocytes to synthesize polyamines after being fertilized. Inhibition of spermine synthesis is known to cause delayed division arrest of fertilized oocytes. Addition of spermine to oocytes from old mice, which had lost cleavage ability and which had division arrest, enabled a proportion of them to proceed dividing. The failure of IVF oocytes to produce adequate quantities of polyamines could lead to failure of implantation due to division arrest. The potential *in vivo* roles of spermine/ spermidine as an immunosuppressant are discussed. New insights into the basic biology of embryo-maternal interactions presented may have potential to enhance clinical outcome of assisted reproduction techniques.

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