CYTOKINES AND THE HUMAN OVARY

LING JIA WANG. MD.

A thesis submitted for the Degree of Doctor
of Philosophy at the University of Adelaide

Reproductive Medicine Unit,
Department of Obstetrics and Gynaecology,
The Queen Elizabeth Hospital,
The University of Adelaide
Australia

September 1992

TO

KE-HUI

TABLE OF CONTENTS

ABSTRACT

Studies reported in this thesis examined aspects of the distribution of leukocyte subpopulation in human corpus luteum, cytokine determination in human preovulatory follicular fluid, as well as the effects of cytokines on human granulosa–lutein cells with the aim of investigating one of the ovarian regulatory systems, which may be controlled by immune cell–derived cytokines.

In the first series of experiments, distribution of leukocyte subpopulation in human corpus luteum was demonstrated by immunocytochemistry. Ten intact corpora lutea were collected from female patients who had no apparent ovarian disease. The mean age of these patients was 37 years (range 23–55 years). Frozen and paraffin sections were subjected to analysis using monoclonal antibodies which were specific to leukocyte marker antigens. The results showed that there are macrophages, cells positive for leukocyte common antigen (LCA), T lymphocytes including T helper/inducer (T4) cells, T cytotoxic/suppressor (T8) cells and activated T (Ta) cells (interleukin–2 receptor–positive cells), monocytes and natural killer (NK) cells but B lymphocytes were not found to be present in the human corpus luteum. The distribution of leukocytes present in the different parts of the corpus luteum was found to be in the order of: theca–luteal area > loose connective tissue area > granulosa–luteal area. Macrophages and T lymphocyte subsets comprised the main components of the total leukocytes in the human corpus luteum. Ta cells were only localized in the loose connective tissue of the corpus luteum. In most cases, macrophages, LCA cells and T4 cells tended to be situated in a single cell layer on the edge of the theca–luteal area and surrounding the granulosa–luteal area. These results suggest that the leukocytes may act to a greater extent in the theca–luteal area than in the granulosa–luteal area. A large concentration of leukocytes in the corpus luteum reinforces the view that cytokines may play a significant role in ovarian physiology.

In the second series of experiments, the presence of immunoreactive interleukin (IL)−1, IL−2 and tumor necrosis factor α (TNFα) in human follicular fluid obtained at the time of oocyte collection for in−vitro fertilization was ascertained by radioimmunoassay. Results obtained from group I (20 fluids of 20 patients) showed that the concentrations of IL−1 were 9±0.6pmol/l and 19±0.4pmol/l in follicular fluid and plasma respectively. A positive correlation existed between IL−1 levels in follicular fluid and plasma ($r = 0.56$, $p<0.01$). Concentrations of IL−2 were 35±2pmol/l and 61±3 pmol/l in follicular fluid and plasma respectively. A positive correlation of IL−2 levels was also found between follicular fluid and plasma ($r = 0.65$, $p<0.01$). There was no association between IL−1, IL−2 and steroid levels, regardless of whether they were compared in follicular fluid or plasma. Group II was composed of a series of fluids (2 to 7 samples for each of seven patients) in which the follicular concentrations of IL−1 and IL−2 did not show a positive correlation with the volume of follicular fluid or the concentrations of follicular fluid steroids. For measurement of immunoreactive TNFα levels, a total of 32 fluids were selected from 9 patients (2−7 samples from each patient). The concentration of TNFα in follicular fluid was 20±1 pmol/l and there were no significant correlations between levels of TNFα and of steroids or follicular volume. However, bioactivity detected by bioassay for TNFα in human follicular fluid was considerably less. These results suggested that human follicular fluid contains immunoreactive IL−1, IL−2 and TNFα at the preovulatory stage.

The following series of experiments examined the effects of cytokines on the human ovary, where the human granulosa−lutein cell culture was used as a model. These cells were collected from follicular fluid obtained from women undergoing in vitro fertilization procedures and cultured with or without conditioned media, cytokines or human chorionic gonadotrophin (hCG) etc., up to 7 days before supernatant measurement of progesterone, cAMP or prostaglandins (PGs).

Lymphokine-rich conditioned media was prepared from cultured human peripheral blood leukocytes (HPL-CM) or from the culture of a Gibbon T cell line MLA-144 (MLA-CM). The influence of HPL-CM and MLA-CM_1 stimulated by mitogens on granulosa-luteal cell progesterone production was inhibitory. In contrast, MLA-CM_1, without mitogen stimulation, stimulated basal progesterone secretion. Human recombinant IL-2 (from 0.26 to 2600pmol/l) alone did not change progesterone levels, compared to control values, after 24h of cell culture. However, 26, 260 and 2600pmol IL-2 significantly inhibited progesterone secretion from cells stimulated by 5iu hCG ($p<0.01$). The enhanced progesterone levels stimulated by forskolin were also significantly inhibited by 260pmol IL-2 ($p=0.01$). This effect was not mediated through decreased cAMP, since the forskolin-enhanced cAMP level was not influenced by IL-2. Human recombinant (from 0.06 to 60pmol/l, equal to 0.001-1μg/l) IL-1β, with or without hCG, did not show any effect on progesterone production during either 24 or 48h of cell culture. It is concluded that IL-2 significantly inhibits progesterone production stimulated by hCG in human granulosa-luteal cells; IL-2 also had a marked inhibitory effect on forskolin-induced progesterone release, but did not influence the increased cAMP level stimulated by forskolin; the inhibitory influence of IL-2 on progesterone synthesis may be down stream in the signal transduction pathway from cAMP activation and HPL-CM and MLA-CM produced inhibitory and stimulatory effects, respectively, on both basal and hCG-stimulated progesterone levels as well as on granulosa-luteal cell proliferation. These activities cannot be completely attributed to IL-2, and other mediators of leukocyte origin may therefore exist.

Human recombinant TNFα (from 6 to 6000pmol/l) showed a dose-dependantly stimulated proliferation of cultured granulosa-lutein cells as measured by incorporation of ^{3}H-thymidine, but did not have any effect on the basal level of hCG stimulated progesterone accumulation during culture periods of up to 72h in either serum containing or serum-free medium. However, the accumulation of both PGE_2

and PGF2α was dose-dependantly increased by TNFα during a 48h incubation period. Time course studies revealed that maximal levels of both PGE2 and PGF2α were reached within 12h of culture. It is concluded that TNFα may have a physiological role in stimulating proliferation of follicular cells and PG production at the time of ovulation and formation of the corpus luteum.

The cellular composition in primary culture of human granulosa-lutein cells with or without IL-2 and TNFα was examined by immunocytochemistry with monoclonal or polyclonal antibodies. Cells collected from follicular fluid were washed and separated by a Percoll gradient followed by nylon mesh filtration to obtain single cells. Cultures were performed in glass-slide chambers for periods up to 144h. After 48h of culture the majority of the cells were shown to be granulosa-lutein cells exhibiting positive staining against 3β-hydroxysteroid dehydrogenase (3β-HSD), oestradiol and vimentin. About 35% of the total cell population stained positive with monoclonal antibodies against specific antigen sites on cells of bone marrow lineage. The majority of these cells were leukocyte common antigen (LCA) positive (about 20% of the total population). In the group of LCA positive cells, the major cellular components were lymphocytes ($^3/_4$ helper T-cells and $^1/_4$ cytotoxic T cells) > natural killer (NK) cells. Most of the T-lymphocytes were activated as judged by expression of the IL-2 receptor. There were also a considerable number of monocytes/macrophages (about 15% of the total cell population). After 48h in culture, the cytokines IL-2 (2600pmol) and TNFα (6000pmol) significantly ($p<0.05$) reduced the proportion of granulosa-lutein cells and in a dose-dependent manner (IL-2 from 26 to 2600pmol/l and TNFα from 60 to 6000pmol/l) increased the proportion of LCA positive cells and monocytes. At the end of 144h of culture, LCA positive cells and monocytes were to be found, but with decreasing numbers. These results indicate that white blood cells are present in primary cultures of human granulosa-lutein cells. The main target for the proliferative effects of IL-2 and TNFα in this culture system during the period of 48h seems to be the leukocytes rather than

the steroid–producing cells. Thus, any observed effects by added cytokines in this culture system may be due to a direct or indirect effect from cytokine–activated resident leukocytes on granulosa–lutein cells.

DECLARATION

The experimental work described in this thesis was conducted under the supervision of Senior Lecturer Dr. Robert J. Norman in the Department of Obstetrics and Gynaecology, The Queen Elizabeth Hospital, The University of Adelaide, Woodville, South Australia from September 1988 to September 1992. This dissertation has not previously been submitted, wholly or in part, to any other University for any degree or diploma. To the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due references are made in the text of the thesis and any input from colleagues is duly acknowledged. I consent to this thesis being made available for photocopying and loan if accepted for the award of the degree.

Ling Jia Wang

1992

ACKNOWLEDGEMENTS

I wish to express my sincere gratitude to my supervisor, Dr. Robert John Norman for his constructive advice and unfailing assistance. His continual support and encouragement contributed greatly to the completion of my research.

I am very grateful to Professor Colin Douglas Matthews for providing the excellent research conditions and financial support in the Reproductive Medicine Unit, Department of Obstetrics and Gynaecology, The Queen Elizabeth Hospital and to Dr. Robert Frederick Seamark for his enlightened discussions. My thanks also go to Professor Jeffrey Robinson, Department of Obstetrics and Gynaecology, the University of Adelaide for granting a Reproductive Medicine Postgraduate Scholarship.

I would like to express my appreciation to all the staff of the Reproductive Medicine Unit, Department of Obstetrics and Gynaecology, The Queen Elizabeth Hospital for supporting this study, in particular, Mr. Fred Amato for assistance in high performance liquid chromatography and all aspects of experimental requirements; Mr. Alan Gilmore for providing the knowledge and intellectual advice in radioimmunoassay; Dr. Mats Brännström for discussions and co-operation particularly with the section on TNFα and prostaglandins; Ms. Jenny McLoughlin, Tracey Lloyd, Anne Wight and Jenny Bowman for helping me with part of the radioimmunoassays; Ms. Regan Jeffrey, Leanne Hourigan, Dianna Payne, Tina Kopsaftis and Monica Briffa for collection of human follicular fluid; Drs. Ossie Petrucco, David Leadbetter and Susan Evans for collection of human corpus luteum; Dr. Sean Flaherty for photography; Dr. Jim Wang for statistical advice and Mrs. Helen Holmes for her highly efficient and skillful secretarial assistance as well as her friendship. I would also like to express my appreciation to Ms. Kathy Govas for her careful proof reading of this thesis.

Many thanks also to Ms. Vivian Pascoe, Maria Bellis and Cheryl Battams in immunopathology, The Queen Elizabeth Hospital for technical assistance in tissue processing and immunocytochemistry; to all staff in Clinical Photography for developing the photographs contained in this thesis; to Professor A.S-Y. Leong, Institute of Medical Sciences, Adelaide for expert advice in the immunology; Dr. R. Rodgers, Flinders Medical Centre, for stimulating discussions and Dr. J. I. Mason, University of Texas, U.S.A. for kindly supplying 3β-HSD polyclonal antibody.

Deep gratitude is also bestowed on my parents for their continued concern and love and to my two daughters, Jing and Yan, for understanding "their busy mum". This thesis is dedicated to my husband, Ke-hui whose support was invaluable throughout this study.

PUBLICATIONS ARISING FROM EXPERIMENTS PRESENTED IN THIS THESIS

ARTICLES:

1. **Wang. L.J., Robertson, S., Seamark, R.F. and Norman, R.J.** (1991). Lymphokines, including interleukin–2, alter gonadotropin–stimulated progesterone production and proliferation of human granulosa–luteal cells in vitro. J. Clin. Endocrinol. Metab. 72:824–831.

2. **Wang, L.J., Pascoe, V., Petrucco, O.M. and Norman, R.J.** (1992). Distribution of leukocyte subpopulations in the human corpus luteum. Hum. Reprod. 7:197–202.

3. **Wang, L.J. and Norman, R.J.** (1992). Concentrations of immunoreactive interleukin–1 and interleukin–2 in human preovulatory follicular fluid. Hum. Reprod. 7:147–150.

4. **Wang, L.J., Brännström, M., Robertson,S.A. and Norman R.J.** (1992). Tumor necrosis factor α in the human ovary: presence in follicular fluid and effects on cell proliferation and prostaglandin production. Fertil. Steril. 58:934–940.

5. **Wang, L.J., Brännström, M., Pascoe, V. and Norman, R.J.** (1992). Cellular composition of primary cultures of human granulosa–lutein cells and the effect of cytokines on cell proliferation. Submitted.

6. **Norman, R.J., Robertson, S., Wang, L.J., Pascoe, V. and Seamark, R.F. (1991).** The role of cytokines in implantation and function of corpus luteum. Proceedings of 13th World Congress of Gynaecology and Obstetrics. FIGO. Singapore, Sept. 1991 Vol. 2. Fetal Physiology and Pathology, p17.1–11.

ABSTRACTS:

7. **Norman, R., Wang, L., Robertson, S. and Seamark, R.** (1990). Interleukin 2, but not interleukin 1, inhibits the human chorionic gonadotrophin (hCG) induced stimulation of progesterone in human luteinized granulosa cells. Proceedings 33rd Annual Meeting, The Endocrine Society of Australia, Perth, Vol 33. Abstact 34.

8. **Norman, R.J., Wang, L. and Shand, K.** (1990). Concentrations of oestradiol, progesterone, 17 hydroxyprogesterone and interleukin 2 in peripheral blood of patients with normal and ectopic pregnancy. Proceedings 9th Annual Scientific Meeting, The Fertility Society of Australia. Perth, p4.

9. **Brännström, M., Wang, L.J., McLoughlin, J. and Norman, R.J.** (1991). Tumor necrosis factor α (TNFα) stimulates prostaglandin production in human granulosa–lutein cells in vitro. Proceedings 10th Annual Scientific Meeting, Fertility Society of Australia, Lorne, Victoria, p44.

10. **Norman, R.J. and Wang, L.J.** (1991). Tumor necrosis factor immunoactivity in human follicular fluid and its effects on human granulosa–lutein cells. Proceedings 34th Annual Meeting of the Endocrine Society of Australia, Sydney, Abstract 102.

11. **Wang, L.J., Pascoe, V., Petrucco, O.M. and Norman, R.J.** (1991). Leucocyte distribution in human corpus luteum. Proceedings 34th Annual Meeting of the Endocrine Society of Australia, Sydney, Abstract 103.

12. **Brännström, M., Robertson, S., Wang, L.J., Seamark, R.F. and Norman, R.J.** (1992). Cytokine production and action in the ovary. 9th International Congress of Endocrinology, Paris, France, Abstract 14.

13. **Wang, L.J., Brännström, M., Pascoe V. and Norman, R.J.** Cellular composition of primary cultures of human granulosa–lutein cells and the effect of cytokines on cell proliferation. Proceedings 35th Annual Meeting of Endocrine Society of Australia, Adelaide, Abstract 125.

ABBREVIATIONS

ABC	avidin–biotin–peroxidase complex
B cells	B (bone marrow) lymphocytes
B–SA	biotin–streptavin amplified (detection system)
cAMP	cyclic adenosine monophosphate
DHEA	dehydroepiandrosterone
DNA	deoxyribonuclease
E_2	oestradiol
EGF	epidermal growth factor
FGF	fibroblast growth factor
FK	forskolin
FSH	follicle stimulating hormone
GM–CSF	granulocyte–macrophage colony–stimulating factor
hCG	human chorionic gonadotrophin
HMG	human menopausal gonadotrophin
HSD	3β–hydroxysteroid dehydrogenase
IFNs	interferons
IGF–I	insulin–like growth factor
IL	interleukin
IVF–ET	fertilization–embryo transfer programme
LH	luteinizing hormone
LHRH	luteinizing hormone releasing hormone
NADH	nicotinamide adenine dinucleotide phosphate
NADPH	nicotinamide adenine dinucleotide phosphate
NGF	nerve growth factor
OMI	oocyte maturation inhibitor
P_4	progesterone
PA	plasminogen activator

PDGF	platelet–derive growth factor
PGs	prostaglandins
PKC	protein kinase C
RIA	radioimmunoassay
RNA	ribonucleic acid
T cells	T (thymus–derived) lymphocytes
TGF–α and β	transforming growth factor α and β
TNF	tumor necrosis factor

CHAPTER ONE

LITERATURE REVIEW

1.1 Introduction

The control of ovarian growth, development, maturity and performance of its function has been traditionally considered under the regulation of the hypothalamic–pituitary–ovarian axis. In the last few decades, evidence has accumulated to show an important intra–ovarian regulatory activity by several substances including ovarian steroids and a number of nonsteroid products (Hsueh *et al*, 1984). The role of the immune system in the regulation of ovarian function has also been of interest. Intact thymic function during the pre– or postnatal stage is essential to maintain normal ovarian differentiation throughout the life of a mouse, as indicated by abnormal ovarian morphology and infertility observed in congenitally athymic or post–thymectomy mice (Shire and Pantelouris, 1974; Allen *et al*, 1984; Scalzo and Michael, 1988). Ovarian function is restored and the abnormal morphological changes are prevented in the neonatally thymectomized or congenitally athymic mice who were treated postnatally by the grafting of spleen lymph nodes, or thymic tissue (Sakakura and Nishizuka, 1972; Shire and Pantelouris, 1974; Rebar *et al*, 1979). Aberration of ovarian function related to abnormal immune function is also seen in other species. Thus, induction of lymphopenia in cattle causes luteal dysfunction (Alila and Hansel, 1984) and a selective defect of T lymphocytes is also found in many patients with primary or premature ovarian failure (Mathur *et al*, 1980; Ho *et al*, 1988). These studies may be one of the reasons leading to the investigation of relationships between immune–cell derived cytokines and ovarian function carried out during the last five years. Although studies in this area are very preliminary, Adashi (1989) has proposed that cytokines may consist of a third kind regulator of ovarian function, at least, in several animal species. The literature review in this thesis will discuss the following

points: a brief overview of the immune system particularly of the cytokines, a review of ovarian function and an introduction to investigations between immune cell–derived cytokines and reproductive systems, in particular, the ovary.

1.2 Overview of the Immune System

1.2.1 The major components and functions of the immune system

The immune system is very important in maintaining homeostasis and health. The multiple functions of the immune system include recognition of self versus nonself, elimination of foreign pathogens, neutralization of toxins and the killing of tumour cells. The function of the immune system is based on its specialized cells and molecules.

The cellular components of the immune system include lymphocytes, macrophages and a series of related cells. The lymphocytes are classified into several types. T (thymus–derived) lymphocytes are responsible for cell–mediated immune reactions as well as for coordinating the functional activation of various other cell types. B (bone marrow) lymphocytes are effector cells involved in the production and secretion of antigen–specific immunoglobulin molecules (antibodies) that mediate soluble immunity. Null lymphocytes (non–T, non–B, or "third population") include two major cell types which are lymphocytes with antibody–dependent cytotoxic capacity (ADCC) and natural killer (NK) activity. Macrophages originally derived from bone marrow are phagocytic cells in tissues and in the circulation are called monocytes. Macrophages play key roles in concentrating and presenting antigens to lymphocytes and in antigen processing. Other cells related to the immune system are the dendritic cells of the spleen, the epidermal Langerhans cells and specialized epithelial cells, such as those found in the thymus, bone marrow, and other lymphoid tissues.

The molecular components of the immune system are immunoglobulin (Ig) molecules and the major histocompatibility complex (MHC). Ig molecules are the products of antibody–secreting cells (B lymphocytes) and are the prototypes of the large group of proteins that are members of the immunoglobulin supergene family. These proteins include the T cell receptor and the associated CD3 proteins (the third component of complement), MHC antigens, and a variety of other molecules (not limited to the immune system) many of which are involved in the function of recognition function. The MHC molecules are a group of membrane glycoproteins. Class I MHC molecules are expressed on virtually all cells and class II MHC molecules are found mainly on cells of the immune system including B cells, macrophages, epidermal cells, Langerhans' cells, dendritic cells, thymic epithelium, and in the human, activated T cells. The MHC plays a central role in many aspects of immunity both in encoding histocompatibility (or transplantation) antigens and in the regulation of immune responses against conventional antigens.

1.2.2 Cytokines

Cytokines represent a broad class of proteins and polypeptides that regulate intercellular communication in both normal and pathophysiological conditions. The nomination of cytokines covers the previous designation of interleukins (i.e., products of white cells that affect the growth and differentiation of lymphoid or myelomonocytes), monokines (i.e., products of mononuclear cells), lymphokines (i.e., products of lymphocytes), interferons, colony–stimulating factors, and a variety of growth factors. Cytokines are produced throughout the body. In localized areas cytokines can act on other types of cells in the immediate vicinity (exerting a paracrine effect), or on the same cell that produces them (exerting an autocrine effect). Cytokines that are produced in large amounts and gain access to the circulation act in a hormonal fashion (exerting an endocrine effect) and have profound systemic effects (Dinarello and Mier, 1987). Cytokines also include another group of polypeptide growth factors and differentiation factors such as epidermal growth factor (EGF),

nerve growth factor (NGF), platelet-derived growth factor (PDGF), transforming growth factor α and β (TGF-α and β), which will not be discussed in the present study. Some characteristics of cytokines related to this thesis are listed in Table 1.1.

1.2.2.1 Functions of macrophage-derived cytokines

Cytokines derived from macrophages include interleukin (IL) 1, tumour necrosis factor (TNF), IL-6 and interferons (IFNs). The general functions of these cytokines are summarized in Table 1.2.

1.2.2.2 Function of T lymphocyte-derived cytokines

Cytokines derived from T lymphocytes are IFNγ, IL-2, IL-3, granulocyte macrophage colony-stimulating factor (GM-CSF), IL-4, IL-5, IL-6 and IL-7. The general functions of these cytokines are summarized in Table 1.3.

1.3 Review of Ovarian Function

The ovaries have two primary functions: the production of female germ cells (ova or oocytes) and the production of hormones and other secretory substances. Ovarian functions are carried out by its basic and principal functional units, follicles and the corpora lutea. The female germ cell develops inside the follicle and attains its maximum potential – that of uniting with a male germ cell (spermatozoan), to produce an embryo capable of developing and leading to the birth of a normal viable offspring. Secretory substances including hormones produced by the follicle and corpus luteum act on either the reproductive system or several other organs and systems by endocrine, autocrine or paracrine mechanisms.

Table 1.1 **Cytokines[a]**

Name	Molecular Mass (Kd[b])	Amino acids	Cell Source	Target cells mass (Kd)	Receptor molecular
Interleukins					
IL-1α and β	17.5	271(159)[c] 269(153)	Mo/Ma, Ke, Ki-me, Co-ep, Tc, la-gr, As, En, Lan, Neu, Mu, Many	Tc,Bc,Fi, Ma, En, CNS, Bo, Ca, many	80
IL-2	15-17	153(133)	Tc	Tc, Bc, NKc, LAK	55,75
IL-3	20-26	152(134, or 140)	Tc	BM, PreBc	72.4
IL-4	20	153(129)	Tc, Mast	aT, aB, St, Mo/Ma, Mast	139
IL-5	50-60	134(115)	Tc	aB, Eo	92.5
IL-6	26	212(184)	Mo/Ma, Tc, Fi, En, Many	Tc, Bc, Neu Many	80
IL-7	25	177(152)	BSC	Bc.Tc.	
Interferons					
IFN-α	16-27	α 189(166) α2188(165)	Mo	Many	100-120
IFN-β	20	187(166)	Fi	Many	100-120
IFN-γ	20-25	166(143)	Tc, NKc.	Mo/Ma	
Others					
GM-CSF	22	144(127)	Tc, Fi, En	Mo/Ma, Eo, Neu BM	150
TNFα	17	233(157)	Mo, Fi, T	Many	80

a. The references for this table are from Gillis, 1989; Durum and Oppenheim, 1989; Arai *et al*, 1990; Banks *et al* 1991.
b. Kd. Kilodaltons.
c. The numbers in parenthesis denote the number of amino acid residues of processed, mature cytokines.

aB: activated B cells; **As**: astrocytes; **aT**: activated T cells; **Bc:** B cells; **BM**: bone marrow cells; **B:o** bone; **BSC**: bone marrow stromal cells; **Ca:** Cartilage; **CNS**: Central nervous system; **Co-ep**: corneal epitheliuem; **En**: endomethelial cells; **Eo**: eosinophils; **Fi**: fibroblast; **Ke**: keratinocytes; **Ki-me**: kidney mesangial cells; **La-gr**: large granular lymphocytes **LAK**: lymphokine activated killer cells; **Lan**: Langerhan's cells; **Ma**: macrophages; **Many**: many cell types; **Mast**: mast cells; **Mo**: monocytes; **Mu**: Smooth muscle cells; **NKc**: natural killer cells; **Neu**: neutrophils; **St**: stromal cells; **Tc :** T cells.

Table 1.2 **Functions of macrophage–derived cytokines**

Name of Cytokine	General Functions	Authors	Year
IL–1	Induces secondary mediators:		
	PAF;	Braquet and Pola	1977
	IL–6, TNF;	Durum *et al*	1985
	CSF;	Fibbe *et al*	1988
	IL–1 itself	Tartakovsky *et al*	1986
	Increases Tc IL–2R and	Mannel *et al*	1985
	development and function of Bc	Hoffman *et al*	1987
	Stimulates activities of Ma, APCs and NKc	Durum *et al*	1985
TNFα	Kills certain tumors and	Carswell *et al*	1975
	tumor cell lines	Wang *et al*	1985
	Induces inflammatory events	Dinarello *et al*	1986
	Stimulates aT, Bc and	Scheurich *et al*	1987
	MHC class I and II antigens	Kehrl *et al*	1987
		Collins *et al*	1986
		Chang and Lee	1986
IL–6	Stimulates hybri	Aarden *et al*	1985
	and plasmac growth	Van Damme *et al*	1987a.b.
	Stimulates hepato and Bc	Gauldie *et al*	1987
	Resemble activities of	Rosenwaser *et al*	1979
	IL–1 and TNFα	Garman *et al*	1987
INFα, β	Antipro activities on	Chen and Najor	1987
	normal and tumor cell types	Vogal *et al*	1982
	Antiviral activities	Friedman	1988
	Promotes functions of APCs and Ma	Chen and Najor	1987
	Enhances NK activities of LGCL	Ortaldo and Herberman	1986

Antipro: Antiproliferative; **APCs:** Antigen–Presenting Cells; **aT:** activated T cells; **Bc:** B cells; **CSF:** Colony–Stimulating Factor; **hepato:** hepatocyte; **hybri:** hybridoma; **IL–2R:** IL–2 receptor; **LGCL:** Large Granule Containing Lymphocytes; **Ma:** macrophages; **NKc:** natural killer cells; **PAF:** Platelet Activating Factor; **Plasmac:** plasmacytoma; **Tc:** T cells.

Table 1.3 **General Functions of T Lymphocyte–derived Cytokines**

Name of Cytokine	General Functions	Authors	Year
IFNγ	Anti–viral activity	Wheelock	1965
	Activates Ma	Virelizier *et al*	1984
IL–2	Enhances the generation of cT	Smith	1980
	Induces NKc activity	Kuribayashi *et al*	1981
	Generates ASPFc	Robb *et al*	1881
	Elevates NK responses of animals	Hefeneider *et al*	1982
	Eliminates tumor burden in animals	Mule *et al*	1985
		Rosenberg *et al*	1985
GM–CSF	Activates Ma	Grabstein *et al*	1986
	Increases circulating numbers of GN, esino and Ma	Herrmann *et al*	1988
		Rifkin *et al*	1988
IL–4	Proliferates activated Bc		
	Induces Bc function	Kishimoto	1985
	Activates Tc	Grabstein *et al*	1987
	Enhances the generation of cells to kill tumor cells	Mule *et al*	1987
IL–5	Proliferate Bc and	Azuma *et al*	1987
	Generates esino	Warren and Moore	1988
		Sanderson *et al*	1988
IL–6	See Table 1.2		
IL–7	Promotes growth of pre–Bc and pre–Tc	Lee *et al*	1989
		Conlon *et al*	1989

ASPFc: antigen specific plague–forming cells; **Bc:** B cells; **cT:** cytotoxic T cells; **esino:** esinophils; **GN**: granulocytes neutrophils; **Ma:** macrophages; **NK:** natural killer; **NKc:** natural killer cells; **Tc:** T cells.

1.3.1 Evolution of follicles and formation of corpus luteum

The evolution of the follicle involves several stages including growth, development, maturity, ovulation or atresia. Follicular classification includes primordial, primary (preantral), mature (antral or Graafian) and atresia follicles (Fig 1.1).

Primordial follicle. Primordial follicles consists of an ovum inconsistently and incompletely surrounded by a single layer of flattened follicular cells which are columnar in early life and cuboidal in the adult. About 400,000 primitive follicles have been estimated to be present in the ovaries of a newborn infant. All together some 400 eggs may actually reach maturity during the period of the active sexual life of a woman. All of the others ultimately degenerate, and from birth on, the follicles progressively diminish in number (Copenhaver, 1964).

Primary follicle. The first phase of follicular development is the transition to a primary follicle. The granulosa layer proliferates to become a multiple (preantral) cell layer surrounding an enlarged oocyte while mucopolysaccharide is secreted to surround the oocyte and form the zona pellucida. Stromal fibroblasts adjacent to the follicle become arranged in concentric layers to form the theca cell layer.

Mature (antral or Graafian) follicle. Continued granulosa cell proliferation results in an enlarged follicle in which the granulosa cells are the largest components. Within the granulosa layers follicular fluid collects, then coalesces to form the antrum, the presence of which marks the transition to the mature (antral or Graafian) follicle. As the antrum enlarges the granulosa cells adjacent to the basal lumina become separated from those adjacent to the oocyte. These two complements of granulosa cells become the membrane granulosum and the cumulus oophorus respectively. Antrum formation in the granulosa layer is accompanied by the formation of the theca interna in which thecal fibroblasts accumulate cytoplasm and exhibit cellular

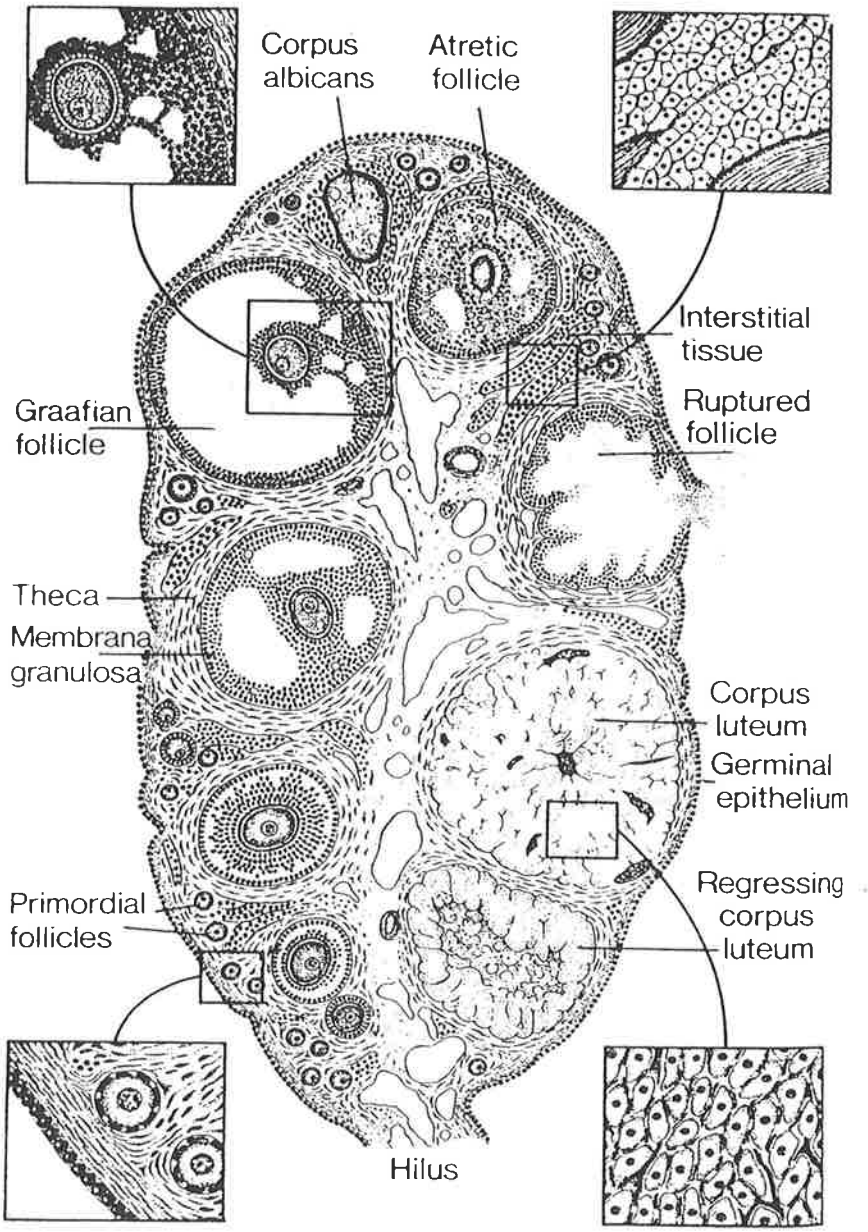


Fig. 1.1 Presentation of follicle and corpus luteum development with the ovary (Reproduced from Turner 1971 and obtained from Odell 1979).

organelles characteristic of steroid–secreting cells, and theca layer blood vessels and lymphatics proliferate. The less closely associated theca cells retain their fibroblast appearance to become the theca externa.

Ovulation. The maturing follicle is prepared for ovulation in which the oocyte and cumulus oophorus are released.

Atresia. Follicles which do not mature to ovulation exhibit major changes in all follicular components as atresia develops. There is degeneration of the oocyte, eventual replacement of granulosa cells by fibroblasts and return of the theca cells to the stromal fibroblasts and return of the theca cells to the stromal fibroblast–like pattern.

Corpus luteum. The corpus luteum as a transient endocrine organ from cells of the follicle forms following ovulation. During this stage, follicular cells exhibit cytoplasmic lipid droplets, extensive smooth endoplasmic reticulum and free ribosomes. Angiogenesis leads to penetration of the basal lamina and vascularization of the granulosa cells, giving an intermingling of theca lutein cells, new blood vessels and granulosa lutein cells. The corpus luteum will function for 10–12 days supported mainly by the luteinizing hormone (LH) but if pregnancy has not occurred luteolysis is inevitable with structural degeneration which is characterized as cells of corpus luteum gradually decrease in size, show increasing vacuolization, and finally reabsorption. The connective tissue between the luteal cells increases in amount and the whole corpus luteum becomes progressively smaller (Tindall, 1987).

1.3.2 Ovarian Secretory Products

1.3.2.1 Steroid Hormones

Ovarian steroids can be classified on the basis of their principal biological function into one of the three major classes: oestrogens, progestins and androgens. The biosynthetic pathways of these hormones are summarized in Fig 1.2.

1.3.2.1a Oestrogens

The oestrogens, oestrone and oestradiol–17β, are the most important of the follicular steroids. Oestradiol is about 10 times as potent as oestrone in most biological assays and, on a molar basis, is the most active of all steroids produced by the ovary (Gore–Langton and Armstrong, 1988). Oestrogens are converted from the androgen precursors, androstenedione and testosterone. Androgen conversion to oestrogen involves reduction of carbon number (C_{19} – C_{18}) and further ring A dehydrogenation. Both events occur during a series of enzyme regulated steps called aromatization. They occur in the cytosol and require molecular oxygen and nicotinamide adenine dinucleotide phosphate (NADH) cofactor (Fishman, 1982). The participation of both cytochrome P–450 and nicotinamide adenine dinucleotide phosphate (NADPH) – cytochrome C – reductase enzymes are required to maintain the above irreversible reactions (Thompson and Siiteri, 1976). The conversion of androstenedione to testosterone, or oestrone to oestradiol is mediated in the reversible reaction by the enzyme 17β–hydroxy steroid dehydrogenase (Bjersing, 1967 and 1977). This reaction is much more efficient in granulosa cells than in thecal cells (Bjersing and Carstensen, 1967). Although theca cells may also aromatize androgens and contribute directly to ovarian oestrogen secretion, evidence from both *in vivo* and *in vitro* studies indicates that the granulosa cells of large antral and preovulatory follicles are the principal site in all species of ovarian aromatase activity and oestrogen biosynthesis (Hillier, 1981; Hillier, *et al*; 1987). Oestrogens are inactivated by the liver and their metabolites are excreted in the urine and faeces.

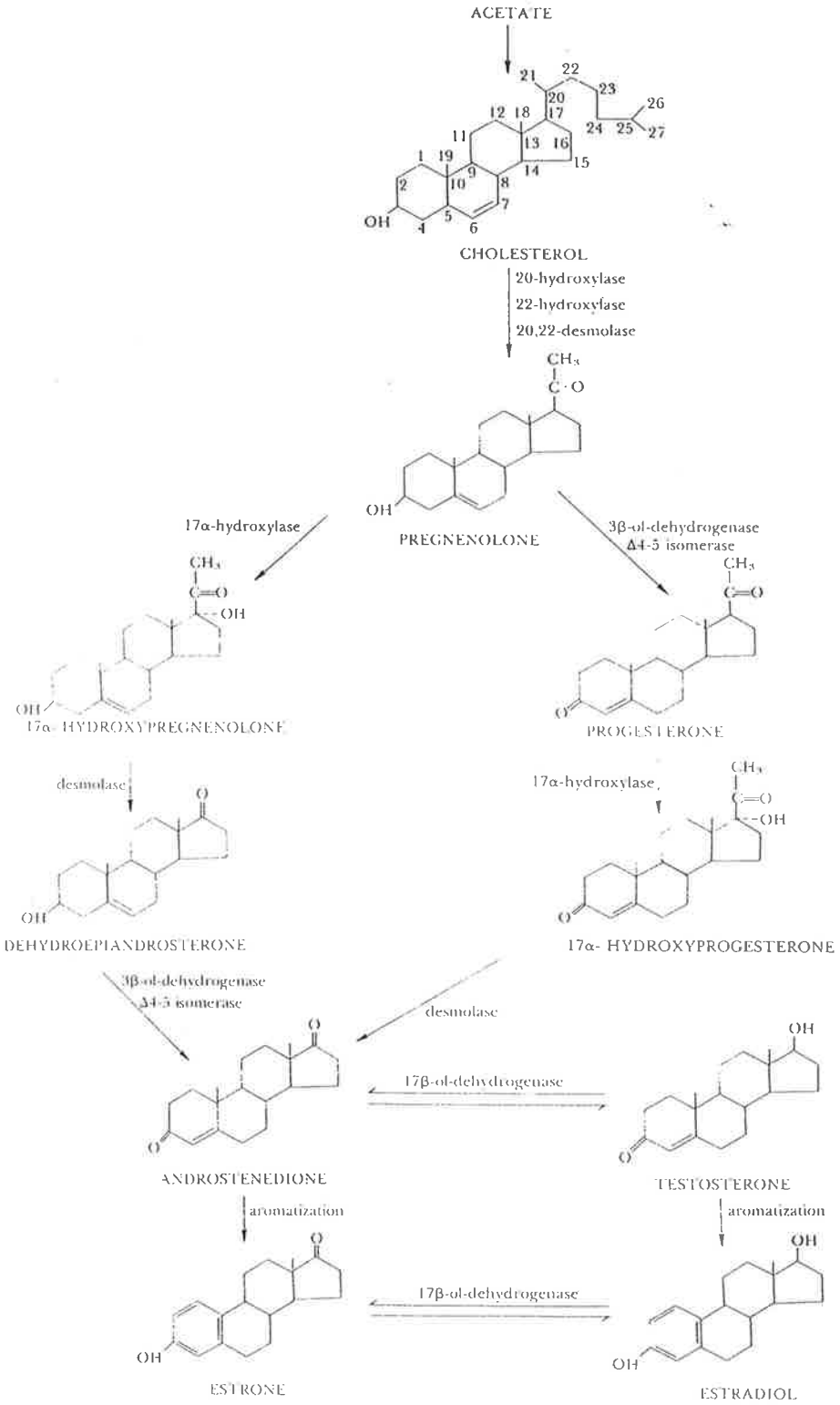


Fig. 1.2 The ovarian biosynthetic pathways from acetate via cholesterol to progesterone, androgens and estrogens (Reproduced from Kase 1990).

1.3.2.1b Progestins

Pregnenolone is the most important progestin produced by the follicle because of its key position as the precursor of all the steroid hormones. The most abundant C_{21} product in the follicle is progesterone, produced as a biosynthetic intermediate by follicles at all growing stages of development and as a secretory end product in the pre– and postovulatory (corpus luteum) periods (Zmigrod *et al*, 1972; Karakawa *et al*, 1976; Tsuji *et al*, 1982). The ovarian progesterone biosynthetic pathway involves conversion of cholesterol to pregnenolone and then to progesterone.

Conversion of cholesterol to pregnenolone. Cholesterol derives from one of three possible sources: (a) preformed cholesterol taken up from the blood, primarily in the form of circulating lipoproteins; (b) preformed cholesterol stored within the ovarian cell, a constituent of cell membranes and (c) cholesterol synthesized *de novo* in the ovarian cell from C_2 components derived from metabolism of carbohydrate, fat, or protein within the cell (Strauss *et al*, 1981). The generation of pregnenolone (21 carbons) from cholesterol (27 carbons) proceeds by the elimination of a large portion (6 carbons) of the cholesterol side chain. This step was thought to be the rate–limiting step in progestin biosynthesis. This involves introduction of hydroxyl groups at C_{20} and C_{22} and cleavage of the 20–22 bond. Both the hydroxylase and desmolase reactions occur within mitochondria and require molecular O_2, reduced NADPH, and the mitochondrial cytochrome P450. LH accelerates the conversion of cholesterol to pregnenolone in the ovary by generation of cyclic adenosine monophosphate (cAMP).

Conversion of pregnenolone to progesterone. This step proceeds by oxidation of the hydroxyl group at the 3β–position (removal of hydrogen) and by an isomerization reactions in which the double bond between C_5 and C_6 is shifted to the 4–5 position. These reactions are controlled by the enzyme 3β–hydroxy steroid dehydrogenase and the Δ^{5-4} isomerase. Once formed, progesterone may undergo

reversible enzymatic reactions (addition of hydrogen) to form 17α or 20α hydroxyprogesterone (Peters and McNatty, 1981).

Granulosa cells are the main cellular source of progesterone. Progesterone is rapidly metabolized by the liver and excreted by the urine.

1.3.2.1c Androgens

It has been recognized there are a series of androgens as either intermediate or end products of steroidogenesis in human ovary. Ovarian interstitial cells located in the loose connective tissue of both the cortex and medulla were thought to be androgen producing cells. Androstenedione and testosterone are probably the most important androgens produced by the ovary (Erickson *et al*, 1985). Progestins can be looked at as the precursors of androgens.

The conversion of progesterone to Δ^4–androstene–3, 17–dione is accomplished by the introduction of a hydroxyl group at 17α–position by means of the enzyme 17α–hydroxylase, reduction of the ketone at C_{20}, and subsequent cleavage of the C_{17}–C_{20} bond. The three carbon side chain is removed by this step, and the 19–carbon, 17–ketosteroid androgen androstenedione is formed. Androstenedione can be reduced reversibly to the 17β–hydroxyl steroid testosterone by the enzyme 17β–hydroxy steroid dehydrogenase. Another progestin, pregnenolone could be directly converted to the Δ^5–3β–hydroxyl 19–carbon androgen dehydroepiandrosterone (DHEA). This is accomplished by 17α–hydroxylation followed by desmolase cleavage of the pregnenolone side chain. DHEA is further converted to androstenedione by means of the $^3\beta$–ol–dehydrogenase Δ^{5-4} isomerease reaction.

1.3.2.2 Nonsteroidal products

Ovarian nonsteroidal products include; 1) protein products; (including inhibin, plasminogen activator, oocyte maturation inhibitor and relaxin); 2) prostaglandins; 3) proteoglycans and 4) growth factors. The cell sources and main functions of ovarian nonsteroidal products are listed in Table 1.4.

1.3.3 Receptors in the ovary

Receptors in the ovary permit ovarian cells to distinguish a specific hormone or regulator message from the diversity of circulating signals that impinge on all cells and to relay that information to sites within the cell to induce an appropriate response involved in the differentiation of ovarian cells.

1.3.3.1 FSH receptor

Follicle stimulating hormone (FSH) receptors are present on the granulosa cells from follicles of all sizes (Nimrod *et al*, 1976). The content of FSH receptor in the granulosa cells is regulated by FSH itself. This regulation includes "up or down regulation", that is FSH induces FSH receptors in immature granulosa cells (White and Ojeda, 1981), or inhibits FSH receptors when granulosa cells are continuously exposed to FSH (Nimrod and Lamprecht, 1980; Knecht *et al*, 1984). The number of FSH receptors is also increased by oestrogens synergistically with FSH (Richards *et al*, 1976; Ireland and Richards, 1978a).

1.3.3.2 LH receptor

Luteinizing hormone (LH) receptors are present on the theca cells at all follicular stages (Henderson *et al*, 1984; Channing and Kammerman, 1974) and on granulosa cells of large preovulatory follicles (Amsterdam *et al*, 1975). Studies *in vivo* and *in vitro* have demonstrated that FSH is able to increase LH receptor numbers in whole ovarian tissue, isolated follicular units, or cultured granulosa cells (Zeleznik *et al*, 1974; Richards *et al*, 1976; Hillier *et al*, 1978; Erickson *et al*, 1979). The

Table 1.4 **Ovarian nonsteroid products**

Name	Cell Source	Main function	Author (year)
Inhibin	granulosa	inhibits FSH secretion modulates folliculo–genesis	Schwartz and Channing 1977; Findlay *et al*, 1991
Plasminogen activator (PA)	granulosa	transforms plasminogen to plasmin which decreases follicle wall strength related to ovulation	Beer, 1975
Proteoglycans		increases in intrafollicular pressure	Jensen and Zachariae 1958; Zachariae, 1959
Prostaglandins (PGs)	granulosa	stimulates production of PA and proteoglycans	Strickland and Bear, 1976 Wang and Leung, 1982
Relaxin	pregnant corpus luteum	participates in the parturition	Sherwood and Downing, 1983
Oocyte maturation inhibitor (OMI)	granulosa	arrests development of oocyte	Sato *et al* 1982 Tsafriri and Bar–Ami, 1982
Insulin–like growth factor (IGF–I)	granulosa	interacts with gonadotropin and regulates cell growth and differentiation in ovary	Adashi *et al*, 1989a
Fibroblast growth factor (FGF)	granulosa	same as IGF–1	Baird *et a*,l 1989
Transforming growth factor (FGF)	theca	same as IGF–1	Skinner *et al*, 1987
Epidermal growth factor (EGF)	theca/ interstitial	same as IGF–1	Lobb *et al*, 1989 Skinner *et a,l* 1987

augmentation of LH receptor content induced by FSH has been found in the concomitant presence of oestrogen (Richards *et al*, 1976), progestins, androgens (Rani *et al*, 1981), insulin (May *et al*, 1980), platelet–derived growth factor (Mondschein and Schomberg, 1981a) and LH/ human chorionic gonadotrophin (hCG) itself (Ireland and Richards, 1978b). In contrast, FSH action is inhibited by EGF (Mondschein and Schomberg, 1981b; Knecht and Catt, 1983) and glucocorticoids (Erickson *et al*, 1983). Once the LH receptor is induced, the maintenance of the LH receptor of the granulosa cells requires the presence of FSH and prolactin (PRL) (Casper and Erickson, 1981; Richards, 1979).

1.3.3.3 PRL receptor

The PRL receptor is located in granulosa cells with minimal numbers at the immature stage prior to priming with FSH (Wang *et al*, 1979; Richards and Williams, 1976) and in increased numbers at both stages of large preantral follicles (Midgley, 1973; Rolland and Hammond, 1975) and corpora lutea (Davis *et al*, 1980; Koppelman and Dafau, 1982; Ben–David and Schenker, 1982). The appearance and maintenance of the PRL receptor requires the stimulation of FSH, LH or hCG (Navickis *et al*, 1982).

1.3.3.4 Steroid receptors

Oestrogen receptors (ER) have been identified in follicles and corpora lutea of laboratory and domestic animals by classical radioligand binding techniques (Lee *et al*, 1971; Richards, 1975; Glass *et al*, 1984). Likewise, progesterone receptors (PR) are detected in the cytosol of whole ovaries of rats (Schreiber and Hsueh, 1979) and human (Jacobs *et al*, 1980). Specific androgen binding sites have been identified in the cytoplasm of granulosa cells and translocated to the nuclei after androgen binding (Hsueh, et al 1984). These findings support the hypothesis that oestrogens and progestins can act locally within the ovary through receptor mediated pathways.

1.3.3.5 Growth factor receptors

IGF–1 receptors are detected in both thecal and granulosa cells of porcine follicles (Hylka *et al*, 1989; Caubo and Tonetta, 1989). EGF receptors are also found in granulosa cells and bovine corpora luteum of pregnancy. These findings suggest a possible role of growth factors in ovarian cell differentiation (Jones *et al*, 1982; Chegini *et al*, 1989).

1.3.4 Regulation of ovarian function

1.3.4.1 Mechanisms of hormone action

Mechanisms of action of luteinizing hormone releasing hormone (LHRH) and gonadotrophins. LHRH and gonadotrophins (LH, FSH, PRL, and hCG) do not enter the cell to stimulate physiological events but unite with the corresponding receptors on the surface of the cell. Uniting with the receptor gonadotrophins activate the adenylate cyclase enzyme within the membrane wall leading to the conversion of adenosine 5'–triphosphate (ATP) within the cell to cyclic adenosine monophosphate (cAMP), the so called second messenger. The cAMP released is specifically bound to a cytoplasmic receptor protein, and this cAMP receptor protein complex activates a protein kinase. The later catalyses the phosphorylation of cellular proteins. The physiological event follows this cAMP mediated energy producing event. cAMP is then degraded by the enzyme phosphodiesterase to the inactive compound, 5'–AMP. Acute responses such as increased steroidogenesis do not operate through gene transcription but rather through phosphorylation. Long term effects of these hormones such as differentiation and growth do operate through nuclear activity, and cAMP may exert an effect on ribonucleic acid (RNA) polymerase activity (transcription) as well as on translation (Catt *et al*, 1979, Pollet and Levey, 1980; Richardson *et al*, 1984).

Mechanism of action of steroid hormones. The specificity of the reaction of tissues to steroid hormone is due to the presence of steroid–specific intracellular receptor protein. This mechanism includes 1) diffusion of hormone across the cell

membrane; 2) transfer across the nuclear membrane to the nucleus and binding to receptor protein; 3) interaction of a hormone receptor complex with nuclear deoxyribonuclease (DNA); 4) synthesis of messenger RNA (mRNA); 5) transport of the mRNA to the ribosomes; and finally 6) protein synthesis in the cytoplasm, which results in specific cellular activity (Grody *et al*, 1982).

1.3.4.2 Regulation of the follicular development and ovulation

The phase of follicular development may be summarized as the recruitment of early primary (antral) follicle, selection of a dominant follicle, followed by preparation for ovulation. Ovulation as the last step of follicular development has the characteristics of follicular rupture and ovum expulsion. The regulation of follicular development and ovulation is dependent on the control of the hypothalamic–pituitary–ovarian axis (H–P–O axis) and ovarian paracrine function.

1.3.4.2a Hypothalamic–pituitary–ovarian (H–P–O) axis

Pulsatile stimulation of hypothalamic releasing hormone (LHRH) is essential to stimulate the production of FSH and LH from pituitary gonadotropic cells. An appropriate gonadotrophin stimulus is essential to full follicular development. Moreover, ovarian feedback is a major determinant of the changes in gonadotrophin secretion and much of this feedback acts at the level of the pituitary (Knobil *et al*, 1980). Ovarian follicles are exposed to pulses of FSH and LH throughout the cycle (with the exception of the mid cycle surge), and the FSH levels exceed those of LH. In the follicular phase FSH secretion is influenced by both oestrogen negative feedback and inhibin (Hsueh *et al*, 1987). After ovulation the combined negative feedback of oestradiol and progesterone results in a low level of gonadotrophin output in which pulse frequency and amplitude are reduced (Yen *et al*, 1972). As the dominant follicle approaches ovulation the sustained high oestradiol levels produce an accumulation of LH at the gonadotrope cells and increase gonadotrope sensitivity to LHRH (Yen and Lei, 1976). As the follicle approaches maturity there is a detectable

rise in circulating progesterone and 17α hydroxy progesterone, closely associated with the start of the massive release of gonadotrophin (Hoff *et al*, 1983) and which may further sensitize the gonadotrope cells to facilitate the LH surge (Chang and Jaffe, 1978).

1.3.4.2b Intraovarian paracrine regulation

The similarity between the processes of ovulation and the inflammatory response has been related by Espey in 1980. Both events involve release of proteolytic enzymes and prostaglandins, hyperaemia, increased capillary permeability and fibroblast proliferation.

Proteolytic enzymes. The influence of proteolytic digestion of the follicle wall and the action of histamine and prostaglandins (PGs) induces the formation of the raised hyperaemic stigma preceding follicle rupture. Follicular fluid contains a variety of proteolytic enzyme activators and inhibitors (Reichert, 1962; Espey and Rondell, 1967). The principal activity of these factors is to stimulate plasminogen activator (PA) secretion by granulosa cells, mediated by relaxin and PGs at the gonadotrophin surge (Canipari and Strickland, 1986). The PA converts blood borne plasminogen to plasmin which digests the follicle wall (Espey and Lipner, 1965) and activates latent collagenase secreted by fibroblasts (Espey, 1971) and granulosa cells (Too *et al*, 1984). The activated collagenase achieves a dissociation of the collagen of the follicle wall, thus permitting considerable thinning of the follicular apex leading to ovulation (Espey, 1967).

Prostaglandins. The gonadotrophin stimulation of the theca and granulosa cells result in considerable stimulation of PGE_2 and $PGF_{2\alpha}$ biosynthesis in the preovulatory follicle (Plunkett *et al*, 1975). The principal effects of the increased prostaglandin activity are on proteolytic enzyme activation (predominantly PGE_2), smooth muscle contraction (predominantly $PGF_{2\alpha}$) and periovulatory vascular

changes (predominantly prostacyclin), (Koos and Clark, 1982).

Relaxin. Relaxin is found in the follicular fluid (Bryant–Greenwood, 1982). The suggested role for relaxin is the stimulation release of the proteolytic enzymes, plasminogen activator, collagenase and proteoglycanase, by the granulosa cell layer (Too *et al*, 1984).

1.3.4.3 Regulation of steroidogenesis

1.3.4.3a FSH

FSH responsive cells consist exclusively of granulosa cells, which only acquire the ability to respond to LH later in the follicular maturation. The steroidogenic pathways in the granulosa cells are organized principally for the metabolism of C_{19}–steroids (i.e. androgen) to estrogens and for the *de novo* synthesis of progesterone and its C_{21} metabolites.

Effects on oestrogen biosynthesis. The regulation of androgen aromatization and, in turn, oestrogen biosynthesis in granulosa cells appears to be by the action of FSH, which induces its receptor (Hillier *et al*, 1981); stimulates the activities of aromatase cytochrome P450 (Mendelson *et al*, 1985) and NADPH–cytochrome P450 reductase (Durham *et al*, 1985) and requires RNA and protein synthesis for expressing its action (Wang *et al*, 1982a).

In addition to this action of FSH to induce aromatase enzyme in granulosa cells, oestrogen biosynthesis requires the co–operation of the theca cells in supplying the oestrogen substrates for aromatization. This important aspect has led to the formation of a modern two cell–type, two gonadotrophin theory in which theca interna cells (and perhaps also interstitial cells) are stimulated by LH to produce androgens, which in turn transverse the follicular basement membrane to be utilized for oestrogen biosynthesis in an FSH– stimulated reaction within the granulosa cells (Gore–Langton

and Armstrong, 1988).

Effects on progestin biosynthesis. The production of progesterone and its metabolites (i.e. progestins) is one of the major biosynthetic activities of granulosa cells in large antral and preovulatory follicles. Progesterone biosynthesis occurs in granulosa cells initially in response to FSH stimulation, but this action is later augmented by LH after its receptor has differentiated (Gore–Langton and Armstrong, 1988). In culture, FSH stimulates progesterone biosynthesis in undifferentiated granulosa cells from immature rats (Nimrod, 1977; Dorrington and Armstrong, 1979) and granulosa cells from human (Moon *et al*, 1978) and porcine (Veldhuis *et al*, 1982). FSH influence on progestin biosynthesis is related to the following steps; increasing LH receptor formation (Zeleznik *et al*, 1974; Richards *et al*, 1976) and increasing the activities of cholesterol side chain cleavage (Jones and Hsueh, 1982), Δ^5–3β–hydroxysteroid dehydrogenase (Zeleznik *et al*, 1974) and 20α–hydroxysteroid dehydrogenase (Jones and Hsueh, 1981).

1.3.4.3b LH

LH responsive secretory cells comprise theca interna cells of the follicular envelope and the interstitial cells of the ovarian stroma. Granulosa cells, from mature follicles develop the ability to respond to LH. The most abundant steroid products of mature theca cells of all species are C_{19}– compounds, including the 4–ene–3β–hydroxysteroids and 5–reduced androgens, which are produced from the catabolism of cholesterol. LH action via specific receptors present on the theca cells at all follicular stages (Uilenbroek and van der Linden, 1983; Henderson *et al*, 1984) and consequent production of cAMP (Weiss *et al*, 1978; Tsang *et al*, 1979), provides the principal stimulus for steroidogenic activities.

Effects on androgen biosynthesis. LH apparently increases the thecal activities of 17α–hydroxylase, $C_{-17,\ 20}$–lyase in ovaries or follicles of rats (Fukuda *et*

al, 1979; Aono *et al*, 1981; Bogovich and Richards, 1982) and hamster (Makris and Ryan, 1980). These enzyme activities are rate-limiting and appear to be the site at which LH stimulates C_{19}-steroid production by theca cells, as follicles progress from small antral stages to early preovulatory follicles in the rat (Bogovich and Richards, 1982) and where ovarian androgen production is substantially restricted in late preovulatory follicles (Lieberman *et al*, 1975). Androgens induced by LH, as the precursor of oestrogens, further participate in the oestrogen biosythetic pathway as described by the two-cell-type, two gonadotrophin theory (see 1.3.4.3a).

Effects on progestin biosynthesis. Progestin (progesterone or pregnenolone) accumulation is stimulated by hCG or LH in theca cells, interstitial cells, (Magoffin and Erickson, 1982a), mature granulosa cells from antral follicles (Hillier *et al*, 1987; Wang *et al*, 1981) and corpus luteum (Schomberg *et al*, 1967). Consistent with the LH stimulation of progestin production, LH is found to stimulate the activities of steroidogenic enzymes, which are the rate limiting 3-hydroxy-3-methylglutaryl coenzyme A reductase (Schuler *et al*, 1981), cholesterol esterase (Behrman and Armstrong, 1969; Caffrey *et al*, 1979), cholesterol side-chain cleavage and 3β-hydroxysteroid dehydrogenase (Rubin *et al*, 1963; Boyd *et al*, 1975; Madej, 1980).

1.3.4.3c PRL

The regulatory actions of PRL on steroidogenesis are both luteotrophic and luteolytic. Luteotrophic action known particularly in rodents is involved in initiating luteinization of granulosa cells, in maintaining their level of progesterone synthesis as luteal cells and in inhibiting the activity of the progesterone catabolizing enzyme, 20α-hydroxy steroid dehydrogenase (Crisp, 1977; Rothchild, 1981). PRL also enhances progesterone production in cultured granulosa cells obtained from preovulatory rat follicles (Crisp, 1977) and porcine follicles (Veldhuis *et al*, 1980). There is also evidence for a role of PRL in induction and maintenance of LH receptors on luteal cells at late gestational stages in the rat (Gibori and Richards, 1978).

In contrast to luteotrophic roles, PRL may also act as a luteolytic agent in the rat (Wang *et al*, 1980; Wang and Chan, 1982), porcine (Veldhuis *et al*, 1980) and human (Demura *et al*, 1982), by inhibiting productions of progesterone, estrogen and androgen from granulosa cell culture or perfused ovaries. The site of luteolytic action of PRL appears to be distal to adenylate cyclase, as PRL also inhibited the stimulatory action of cAMP. The inhibitory effect of PRL on rat follicle aromatase activity has also been demonstrated by *in vivo* exposure of intact rats to the hormone (Tsai–Morris *et al*, 1983).

1.3.4.3d Ovarian steroids

Steroids appear to have a direct function in the intraovarian regulation of ovarian steroidogenesis in addition to their feedback effects on the H–P–O axis.

Oestrogens. Studies *in vivo* or *in vitro* have demonstrated that oestrogen suppresses androgen production from the ovaries (Leung *et al*, 1978; Leung and Armstrong, 1979). This inhibitory effect acts directly on the theca cells (Leung and Armstrong, 1980; Hunter and Armstrong, 1986) and is independent of the regulation of gonadotrophin (Leung *et al*, 1978) and cAMP (Leung and Armstrong, 1979). All investigations showed that oestrogen inhibits ovarian androgen synthesis probably at the enzymatic steps in the steroidogenic pathway between androgens and their C_{21} precursors. Some important activities of enzymes, 17α–hydroxylase, C–17, 20–lyase (Magoffin and Erickson, 1981, 1982b) and 5α reductase (Eckstein and Nimrod, 1977) in the androgen metabolism are reduced by oestrogens.

Ovarian oestrogen production is enhanced by oestrogens through direct actions on granulosa cells (Engels *et al*, 1968; Zhuang *et al*, 1982; Adashi and Hsueh, 1982; Welsh *et al*, 1984). This stimulatory effect is correlated to receptor binding affinity and not accounted for by increased granulosa cell viability or protein mass (Adashi

and Hsueh, 1982). It is therefore estimated that oestrogens may function within the ovary or individual follicles as end product amplifiers to enhance FSH induced aromatase (Gore–Langton and Armstrong, 1988).

Oestrogens decrease progesterone secretion in granulosa cells or large follicles (Thanki and Channing, 1978; Fortune and Hansel, 1979; Adashi and Hsueh, 1982). This action appears to limit the conversion of pregnenolone to progesterone, resulting in enhanced pregnenolone accumulation in culture and increased cholesterol side–chain cleavage activity (Toaff *et al*, 1983; Veldhuis, 1985) and mitochondrial content of P–450 (Toaff *et al*, 1983). Stimulatory effects of oestrogens on progesterone secretion were observed in long term culture of porcine granulosa cells which were from small but not large follicles. This effect was found to be due to increased activities of Δ^{5}–3β–hydroxysteroid dehydrogenase, Δ^{5-4}–isomerase, and cholesterol side chain cleavage and hydrolysis of endogenous cholesterol esters (Veldhuis, 1985)

Androgens. Androgens have been shown to enhance basal or FSH stimulated progesterone biosynthesis by intact follicles, granulosa cells and thecal tissue *in vitro* (Shemesh and Ailenberg 1977; Hillier *et al*, 1977; Batta *et al*, 1980; Bieszczad *et al*, 1982; Welsh *et al*, 1982). This action occurs at both pre– and post– cAMP sites in rat granulosa cell culture. Androstenedione enhances stimulation of progestin production by the cAMP analog dibutryl cAMP (Nimrod, 1977), FSH–responsiveness, as measured by the FSH stimulation of cAMP production (Goff *et al*, 1979; Hillier and deZwart, 1982; Daniel and Armstrong, 1984), [^{125}I] FSH binding (Knecht *et al*, 1984) while suppressing cAMP metabolism (Hiller and deZwart, 1982). Androgens and FSH act synergistically to enhance lipoprotein utilization (Schrieber *et al*, 1983). Both FSH and testosterone independently enhance conversion of cholesterol to pregnenolone, indicating a stimulatory action on cholesterol side chain cleavage. Combined treatment results in synergism (Nimrod, 1981; Jones and Hsueh, 1982; Welsh *et al*, 1982). In contrast to the studies using rat tissue, aromatizable androgens

have a negative influence on progesterone production by human granulosa cells (Batta *et al*, 1980) and on FSH stimulated progesterone accumulation by granulosa cells isolated from porcine ovaries (Lischinsky *et al*, 1983; Evans *et al*, 1984). This action appears to be due to restricted conversion of pregnenolone to progesterone through induction of Δ^{5}–3β–hydroxysteroid dehydrogenase: Δ^{5-4} isomerase activity (Tan and Armstrong, 1984).

Androgens have a regulatory role in ovarian oestrogen secretion. They act as substrates for FSH stimulated aromatase and enhance FSH induction of enzyme activity (Daniel and Armstrong, 1980). They influence granulosa cell aromatase activity by acting at a site before cAMP production. Since androgens have no effect on cAMP–induced oestrogen synthesis, they appear to enhance the responsiveness of cultured granulosa cells to FSH by the production of cAMP, as well as in stimulating cellular [^{125}I] FSH binding (Daniel and Armstrong, 1984).

Progestins. In rat granulosa cell culture, progestins (progesterone or R5020) increase FSH stimulated production of cAMP (Goff *et al*, 1979), progesterone 20α–dihydroprogesterone, pregnenolone and LH stimulated progesterone secretion (Fanjul *et al*, 1983). In contrast to the effect on progestin synthesis, progestins (progesterone, 20α–dihydroprogesterone or R5020) reduce FSH enhanced oestrogen secretion and aromatase activity (Schreiber *et al*, 1980; 1981).

1.3.4.3e Regulatory peptides

In recent years, the interest in the intraovarian regulation of steroidogenesis has greatly increased because of the discovery of groups of regulating peptides which may have independent roles in or may modify the effects of gonadotrophins on follicular differentiation. They include 1) growth factors, IGF–I, FGF, TGFα and β, and platelet–derived growth factor (PDGF); 2) Inhibin and related peptides; and 3) Cytokines (see 1.5). Their ovarian function, in particular in the regulation of steroidogenesis will be discussed in brief as follows:

IGF–I. Higher levels of IGF–I gene expression have been found in adult rat ovary compared to other tissues (Hamond *et al*, 1981) and are located in the granulosa cells (Adashi *et al*, 1989a). IGF–I binding is also confirmed to be present on FSH primed rat granulosa cells (Adashi *et al*, 1988a). This binding can be upregulated by FSH and LH (Adashi *et al*, 1986; 1988b). In addition to the biological roles of IGF–I in augmentation of differentiation of the developing granulosa cells and FSH supported proteoglycan biosynthesis (Adashi *et al*, 1985), IGF–I is capable of increasing FSH supported biosynthesis of progesterone, oestrogen (Adashi *et al*, 1985) and inhibin (Zhang *et al*, 1987) as well as FSH mediated acquisition of LH receptors (Adashi *et al*, 1985). In the theca–interstitial cells, IGF–I increases ovarian androgen biosynthesis (Hernandez *et al*, 1988) which may provide the substrate for the aromatizing process and further promote oestrogen biosynthesis in the granulosa cells.

FGF. Basic FGF is present in ovarian tissue. Granulosa cells and possibly luteal cells are among the cell types producing FGF in ovarian tissue (Neufeld *et al*, 1987) while the receptors for FGF are also identified on rat granulosa cells (Neufeld and Gospodarowicz, 1985; Moscatelli, 1987). In granulosa cells, the capacity of basic FGF is to modify (by mainly inhibiting) aromatase activity (Baird *et al*, 1989).

TGFβ. The bioactivity of TGFβ, with a molecular weight of approximately

25,000 Da has been observed in conditioned medium of bovine thecal cell culture. The bioactivities of TGFβ showed an inhibitory effect on [^{3}H] thymidine incorporation into bovine granulosa cell DNA in the presence of FSH and a stimulatory effect on FSH–induced aromatase activity (Lobb and Dorrington, 1989).

EGF. High–affinity, low capacity EGF receptors have been found on rat granulosa cells (Jones *et al*, 1982). EGF inhibits FSH–stimulated oestrogen biosynthesis (i.e., aromatase activity), augments FSH–stimulated progestin biosynthesis and independently stimulates production of pregnenolone, progesterone, and 20α–dihydroprogesterone (Hsueh *et al*, 1981; Jones, *et al*, 1982).

PDGF. PDGF is derived from the platelet during clot formation (Ross and Vogel, 1978) and increases FSH–stimulated progestin production, which may involve an enhanced sensitivity to FSH and cAMP (Knecht and Catt, 1983).

Inhibin. Inhibin, a glycoprotein, has been purified from the ovary of several species (Ling *et al*, 1985; Miyamoto *et al*, 1985) including human (Robertson *et al*, 1990) and is found mainly to be derived from granulosa cells (de Jong and Sharpe, 1976). The amino acid and nucleotide sequence of inhibin subunits have been identified (Mason *et al*, 1985; Forage *et al*, 1986). In addition to the negative regulatory effect on pituitary FSH secretion, inhibin has significantly stimulatory effects on LH–induced androgen production from theca cells (Hillier *et al*, 1991).

1.4 Cytokines and the Reproductive System

1.4.1 Cytokines and endometrium–decidua and placenta

The realization that cytokines are produced by, and may play a number of roles in human endometrium–decidua and placenta has been one of the exciting advances in the physiology of maternal immunological recognition of the developing fetal semiallograft in the last decade. The interaction between leukocytes and surrounding tissues is a model for the role of these cells in the endometrium–decidua and placenta.

1.4.1.1 Leukocyte subpopulation in endometrium–decidua and placenta

The presence of leukocytes with the capacity to produce cytokines in endometrium–decidua and placenta provides the basic evidence to further investigate the physiological significance of cytokines in this area.

The macrophage is the major cellular component of the leukocyte population in the human endometrium (Bulmer and Ritson, 1988). The number of macrophages increase markedly in the secretory phase of the menstrual cycle (Tabibzadeh, 1990). These cells are scattered throughout the gestational endometrium and aggregate around the spiral arteries, which are adjacent to the endometrial glands (Bulmer and Johnson, 1984) and have been observed in association with extravillous trophoblasts in the decidual basalis (Bulmer and Ritson, 1988). Many macrophages in normal uteroplacental tissues are in an activated state as suggested by their expression of class II major histocompatibility antigen (Hunt, 1989).

There are also large granular lymphocytes in the human endometrium. These effector cells have an unusual antigenic phenotype (CD 56 positive) and possess natural killer activity. They are prominent in late secretory endometrium (Tabibzadeh, 1990) and first trimester decidua (Bulmer and Sunderland, 1984; King *et al*, 1989).

The presence and activation of T lymphocytes have been observed in human endometrium (Tabibzadeh, 1990). The T cytotoxic suppressor (T c/s) cell is the main component of the T cell population inside the human endometrium. The number of T c/s cells increases during ovulation and the late luteal phase, as compared to the early luteal phase (Bohme and Donat, 1989).

Human early pregnancy decidua also contains NK cells with a high affinity to the IL–2 receptor. These cells express an early activation antigen, CD69, *in vivo* (Nishikawa *et al*, 1991). Approximately 75% of the decidual cells of early pregnancy are derived from bone marrow (45% large granular lymphocytes; 19% macrophages; 8% T cells) (Starkey *et al*, 1988). These cells with an immunological capacity may limit trophoblastic invasion by cytotoxic activity.

1.4.1.2 Cytokine production from endometrium–decidua and placenta

Cytokines, IL–1α, β, TNFα and IL–2–like material, have been detected in human amniotic fluid, even though the original tissue sources of these cytokines could be from the decidua, placenta or fetus (Soubiran *et al*, 1987; Tamatani *et al*, 1988; Jäättelä *et al*, 1988). Placental phagocytes spontaneously produce IL–1 and can be stimulated to release greater quantities of IL–1 (Flynn *et al*, 1982). Stimulated decidual lymphocytes produce more GM–CSF than stimulated peripheral lymphocytes (Dudley *et al*, 1990). IL–6 production has been found in decidual tissue explants in response to bacterial endotoxin (Romero *et al*, 1990).

Immune cells are not the only cytokine producing cells inside decidual and placental tissue, as epithelial and decidual cells also produce cytokines. The expression of cytokines IFN–α, GM–CSF, IL–1α and TNFα in Hofbauer cells were found in first, second, and third trimesters in human placenta (Berkowitz *et al*, 1990). Using immunohistochemical analysis, IL–6 has been located in syncytiotrophoblasts rather than in cytotrophoblasts of human placenta (Kameda *et al*, 1990). Molecular

biological studies have showed that mRNAs encoding IL–1, TNF, GM–CSF and colony– stimulating factor 1 (CSF–1) were detected in murine placenta (Azoulay *et al*, 1987; Crainie *et al*, 1990; Yelavarthi *et al*, 1991). The mRNA of the IL–1, IL–2 gene, cDNA of GM–CSF receptor, mRNAs of macrophage–colony stimulating factor (M–CSF) and its receptor were all isolated from human placenta (Boehm *et al*, 1989; Gearing *et al*, 1989; Kauma *et al*, 1990; Saji *et al*, 1990).

1.4.1.3 Effects of cytokines on endometrium–decidua and placenta

Although immune cells have been identified, along with the expression of cytokines in endometrium–decidua and placenta, the function of cytokines in this area is poorly understood.

Synthesis and secretion of cytokines from endometrium–decidua and placenta can be regulated by the cytokine itself. Incubated with PHA or IL–2, human placental cells synthesized significant quantities of IFN–γ which, in turn, develop considerable lymphocyte–activated killer (LAK cell) cytotoxicity on placental cells (Chin *et al*, 1988). This suggests that placental cells may have a primary role in fetal defence. Endometrial stromal cells respond to the actions of IL–1 and TNFα These cells synthesize both IL–1 and IL–6 after stimulation by the addition of IL–1 and TNFα (Semer *et al*, 1991). The possibility exists that the synthesis and action of cytokines may be involved in the mechanisms that serve to regulate the mesenchymal–epithelial interactions between endometrial stromal and glandular components, and the formation and action of cytokines in decidua may serve to modulate immunological and infectious challenges encountered by this tissue in pregnancy.

Cytokines may play a role in the regulation of maternal secretion and human embryonic signals which facilitate the successful establishment of pregnancy. Endometrial glandular epithelial cells cultured with IL–1α consistently secrete increasing levels of PGE_2 (Tabibzadeh *et al*, 1990[a]) which induces vascular changes,

stromal oedema and the decidual cell reaction in the mouse (Kennedy, 1987). Il–1 regulates PGE_2 synthesis by binding to a receptor which has been found to have a high affinity for IL–1 on the membranes prepared from human endometrial epithelium (Tabibzadeh *et al*, 1990[a]). Accordingly, PGE_2 secretion by first trimester in human decidual cells blocks the activation of maternal leukocytes in the decidua and potential anti–trophoblast killer function by, inhibiting IL–2 receptor generation and IL–2 production in situ (Parhar *et al*, 1989). The series of reactions between IL–1 and PGE_2, or between PGE_2 and IL–2 as well as, IL–2 receptor may be very important for embryo implantation and for protection of the feto–placental unit from rejection. Trophoblast–derived IL–1 and TNFα, dose–dependently stimulate hCG released from trophoblasts. However, this effect is totally dependent on IL–6 and IL–6 receptor since the stimulated trophoblasts release IL–6 prior to hCG release and PM_1, a monoclonal antibody specific for IL–6 receptor, has been shown to completely block hCG release (Masuhiro *et al*, 1991; Shimoya *et al*, 1991). IL–6, derived from trophoblast, stimulates hCG secretion by trophoblasts to a level similar to that stimulated by GnRH analogue. The analogue, however, released hCG by an IL–6–independent mechanism because PM_1 failed to block GnRH–mediated responses, suggesting the existence of two distinct regulatory pathways for hCG release (Nishino *et al*, 1990). IL–1β increases the release of CRF and ACTH from cultured placental cells. This effect is associated with increased intracellular cyclic nucleotide concentrations and is in part reversed by a prostaglandin synthesis inhibitor (Petraglia *et al*, 1990). Another cytokine IFN–γ inhibits the growth of an endometrial cell line in a dose– and time–dependent manner (Tabibzadeh *et al*, 1990[b]). G–CSF promotes chorionic cell proliferation and has been effective in preventing abortion in the murine model (Miyama *et al*, 1991).

1.4.1.4 Effects of endometrium–decidua and placenta on lymphocyte proliferation

The activation of resting lymphocytes is critical to most immune responses since cellular activation allows these cells to exert their regulatory or effector activities. The activation of lymphocytes is a complex process which results in cell growth and differentiation (Weiss, 1989). The observation on the effects of endometrium–decidua and placenta on lymphocyte proliferation partly explains one of the mechanisms of lymphocyte activation.

A common inhibitory effect on lymphocyte proliferation has been exerted by placental secretory products such as PGE_2, progesterone and oestradiol (Low and Hansen, 1988), the cells of the human first trimester pregnancy endometrium (Nakayama *et al*, 1985), syncytiotrophoblast plasma membranes prepared from full–term human placenta (Thibault *et al*, 1991), cultured human choriocarcinoma TEG–3 cells (Wolf *et al*, 1983) and uromodulin, an 85 KDa glycoprotein isolated from pregnancy urine (Brown *et al*, 1986). The mechanism of the inhibitory effect is not understood. However, PGE_2–mediated immunosuppressor function of early gestational human decidual cells is accomplished by an afferent blockade of the early events in T lymphocyte activation which was confirmed by a down–regulation of IL–2 receptor development on lymphocytes and an inhibition of IL–2 production in mixed lymphocyte culture (Lala *et al*, 1988). An opposite report described that both human trophoblasts and their culture supernatants stimulate lymphocyte proliferation and trophoblast culture supernatant inducing the expression of the IL–2 receptor on lymphocytes. The proliferative effect can be suppressed by anti–human chorionic gonadotrophin antibody (Yagel *et al*, 1989). This data suggests that the trophoblast–derived human chorionic gonadotrophin has a lymphocytotrophic function.

1.4.2 Cytokines and the embryo

Human embryo–derived cytokines have been reported recently. IL–1, IL–6, CSF and TNFα are present in oocytes before fertilization and increase in quantity from one to eight cell embryos (Zolti *et al*, 1991), however inhibitory effects have been observed on embryo growth. IL–1 and TNF significantly inhibited while CSF and IFNα significantly arrested embryo development (Hill *et al*, 1987a; Sueldo *et al*, 1990).

1.4.3 Cytokines and the uterine cervix

Uterine cervical explants in culture from pregnant rabbits at term spontaneously synthesized and secreted three species of IL–1–like factors in culture medium. This was not observed with explants from nonpregnant cervices (Ito *et al*, 1988). These factors might be involved in the cervical ripening and dilatation at term by the mechanisms to accelerate the productions of both specific collagenase and gelatinolytic metalloproteinase (Ito *et al*, 1987).

1.5 Cytokines and the Ovary

1.5.1 Distribution and action of leukocyte subpopulations in the ovary

To better understand the production and effects of immune cell derived cytokines in the ovary and on ovarian function, it is important to firstly broaden our understanding of the distribution and action of leukocyte subpopulations in the ovary.

1.5.1.1 Macrophages

Macrophages, as a predominant blood cell type in the ovary, have been observed within the preantral follicle and different stages of the corpus luteum of several species by light microscopy (rat: Bulmer, 1964; guinea pig: Paavola, 1979; pig: Standaert *et al*, 1991; human: Gillim *et al*, 1969) and by immunocytochemical studies (mouse: Kirsch *et al*, 1981; rabbit: Bagavandoss *et al*, 1990; human: Katabuchi

et al, 1989; Louikides *et al*, 1990; Lei *et al*, 1991). In the human ovary, macrophages are not found in the ovarian cortex and medulla at the resting stage of the ovarian follicle. With the development of follicles a few macrophages appear in the stroma (Katabuchi *et al*, 1989). The number of macrophages increase in the preantral follicle, and subsequently decrease in the developing and mature corpus luteum and then increase again in the regressing corpus luteum (Standaert *et al*, 1991; Lei *et al*, 1991). The presence of macrophages in the ovary has been primarily associated with the heterophagy of dead or dying cells (Paavola, 1979), and with tissue remodelling (Anderson and Hill, 1988). Further investigation has shown the ovarian or peritoneal macrophages significantly stimulate progesterone production and the effect is obtained by the proximity of macrophages and luteal cells (Kirsch *et al*, 1981). It is therefore inferred that the increasing number of macrophages with development of the follicle and regressing corpus luteum may have certain roles in follicle development, ovulation and luteolysis.

1.5.1.2 Lymphocytes

Lymphocytes have also been observed in human follicular fluid collected from IVF cycles (Hill *et al*, 1987b; Droesch *et al*, 1988; Castilla *et al*, 1990) and rabbit corpus luteum (Bagavandoss *et al*, 1990). Compared to the ratio of T4/T8 cells in peripheral blood, follicles contain more T4 (helper/inducer) than T8 cells (suppressor/cytotoxic). The mean concentrations of T4 and T8 lymphocytes decrease with increased oocyte maturation (Droesch *et al*, 1988). The T8 population in peripheral blood contains oestradiol receptors but the T4 population do not (Cohen *et al*, 1983) indicating that the increased level of oestradiol in the mature follicles may alter T8 cell number. The number of T cells in the corpus luteum remains consistent between different days of rabbit pseudopregnancy, pregnancy and post-partum (Bagavandoss *et al*, 1990). While there is no information on T cells in the corpus luteum of women, the populations of peripheral blood lymphocyte subtypes do not vary during the menstrual cycle (Eichler and Keiling, 1988). Peripheral blood

lymphocytes co-cultured with human granulosa cells have been reported to have a significantly stimulatory effect on the basal, and hCG induced progesterone production (Emi *et al*, 1991).

1.5.1.3 Other leukocytes

Mast cells have been located in the ovaries of several species (rat: Jones *et al*, 1980; hamster: Krishna and Terranova, 1985; bovine: Cupps *et al*, 1959; man, rhesus monkey, pig and mouse: Krishna *et al*, 1989). The distribution of mast cells is in the stromal, hilar, luteal and external thecal regions of the human ovary (Jaiswal *et al*, 1987). Histamine, the important product of mast cells, participates in the regulation of LH-influenced capillary permeability and blood flow in the ovary (Varga *et al*, 1967; Lipner, 1971; Piacsek and Huth, 1971) while stimulating ovarian contractility (Wallach *et al*, 1978), ovulation (Schmidt *et al*, 1986, 1988) and follicular progesterone secretion (Schmidt *et al*, 1987) *in vitro*.

Eosinophils have their classical roles in allergic inflammation and in the defense of the host against parasitic infection (Gleich and Adolphson, 1986). Eosinophils infiltrate the ovaries of sheep in a cyclic fashion. Preovulatory follicles and regressing corpora lutea secrete leukotactic substances quite adept at attracting eosinophils (Murdoch 1987; Cavender and Murdoch, 1988; Murdoch and McCormick, 1989). However, the role played by eosinophils in the mechanisms of ovulation and luteal regression is unclear since drug (prednisolone) induced ovarian eosinopenia could not block the follicular rupture or effect the ability of $PGF2\alpha$, which caused luteolysis in sheep (Murdoch and Steadman, 1991).

1.5.2 Expression and Action of Cytokines in Ovary

1.5.2.1 IL-1

IL-1-like activity was first reported in porcine and human follicular fluid in 1989 (Takakura *et al*, 1989). The expression of both IL-1α and IL-1β and type I IL-1 receptor gene was also found in human preovulatory follicular aspirates and whole ovarian tissue by solution hybridization/RNase protection assay (Hurwitz *et al*, 1991). This study provides very important evidence to support the possibility that IL-1 acts on the ovary via a paracrine mechanism, firstly by the local production of IL-1 and secondly by ovarian reception, as demonstrated by the existence of IL-1 receptor. However, the precise cellular origin of IL-1 remains unknown despite depletion of macrophages from the tested cell samples in this study because macrophages are not the only source of IL-1. IL-1 has been reported to be secreted also by epithelial, fibroblasts, and endothelial cells (Kupper, 1991) which are present in the ovary.

At the preantral follicle stage, IL-1 has been observed to suppress the steroidogenic function of cultured murine and porcine granulosa cells which were in a undifferentiated state (Gottschall *et al*, 1987; Kasson and Gorospe, 1989; Fukuoka *et al* 1989; Zhou and Galway, 1991). These findings suggest that the effect of IL-1 on undifferentiated granulosa cells is antigonadotrophic. The mechanism may involve the reduction of binding capacity of the LH receptor and the inhibition of cAMP accumulation by the sites of action both proximal and distal to cAMP generation (Gottschall *et al*, 1988a; Fukuoka *et al*, 1989; Yasuda *et al*, 1990). At the preovulatory follicle stage, progesterone and testosterone secretion from cultured hamster thecal cells were significantly increased by IL-1 in the presence of hCG (Nakamura *et al*, 1990). Furthermore, in response to IL-1, fully differentiated, highly

luteinized granulosa cells from hamster, bovine and human ovary show increased production of PGs including PGE_2, $PGF_{1\alpha}$ and $PGF_{2\alpha}$ and no change in steroidogenesis (Polan *et al*, 1988; Nakamura *et al*, 1990; Nothnick and Pate, 1990). Although the studies of IL–1 function in ovarian physiology are preliminary, the above research suggests that IL–1, as a putative modualtor of gonadotrophins, could be a suppressor of premature follicles and a promoter of mature follicles.

1.5.2.2 TNFα

There is conflicting evidence to support the ovarian origin of TNFα. The identification of ovarian TNFα shows contradictory results by immunocytochemistry. In the earlier reports, immunoreactive TNFα has been demonstrated by polyclonal anti–TNFα antibody in the ovaries of several species including rat, rabbit, cow and human and is mainly localized in the mature follicle and the corpus luteum (Roby and Terranova, 1989, 1990; Bagavandoss *et al*, 1990). A later study did not detect any TNFα reactivity in the human ovary with anti–TNFα monoclonal antibody (Bukovsky and Caudle, 1992). It is not clear whether the polyclonal anti–TNFα antibody has a different specificity from the monoclonal antibody or represents an alterative type of circulating TNFα. Although the mRNA for TNFα has been found in the rat and human ovary (Sancho–Tello *et al*, 1991; Clinton *et al*, 1991), the results were obtained by reverse transcribed polymerase chain reaction in which the amplified TNFα mRNA may not represent TNFα of ovarian origin but TNFα from peripheral blood circulation.

The ovarian action of TNFα, like that of IL–1 function in ovary (1.5.2.1), is also biphasic. The anti–gonadotrophic role played by TNFα in the preantral follicle is demonstrated by the inhibition of FSH induced estrogen and progesterone production in undifferentiated rat and porcine granulosa cells (Emoto and Baird, 1988; Adashi *et al*, 1989b; Darbon *et al*, 1989; Andreani *et al*, 1991).

Conversly, in the preovulatory follicle TNFα stimulates progestin production in the theca cells of rat and appears to have a gonadotrophin-like activity (Roby and Terranova, 1990). Although TNFα is capable of inducing tumour necrosis *in vivo* and of exerting cytolytic or cytostatic effects on a broad range of transformed cell line *in vitro* (1.2.2.1), the inhibitory action of TNFα on ovary could not be accounted for by a decrease in cellular viability or plating efficiency, nor by a decrease in the number of cells or their DNA content (Adashi *et al* 1989b, 1990). Instead, TNFα inhibits gonadotrophin action at the level of LH receptor formation, cAMP accumulation and the key biosynthetic steroidogenic enzyme production (Adashi *et al* 1989b, 1990). The action of TNFα on the preovulatory follicle is not directly related to cAMP but rather to promote the function of protein kinase C which phosphorylates key proteins involved in ovarian cell differentiation (Sancho-Tello and Terranova, 1991). In the fully differentiated human granulosa-lutein cells, the secretion of both PGF2α and progesterone were stimulated by TNFα (Zolti *et al*, 1990). TNFα, as a potential ovarian regulator, is being explored. It is speculated that TNFα may play a role in the still enigmatic processes of atresia and/or luteolysis. However, the above evidence does not support this hypothesis because TNFα has stimulatory effects only in preovulatory follicles and luteinized granulosa cells.

1.5.2.3 Other cytokines

T-lymphocyte-derived cytokines IL-2 and IFNγ may participate in the regulation of ovarian function. *In vitro* studies show that IL-2 enhances FSH induced progestin produciton (Kasson and Gorospe 1989) and IFNγ in the presence of FSH, inhibits secretion of estrogen, progestins and inhibin (Gorospe *et al* 1988; Xiao and Findlay, 1991) in undifferentiated rat granulosa cells. In contrast to these observations, Adashi and his colleagues (1989b) could not find any effect of IL-2 or other cytokines including IL-3, G-CSF and IFNγ on rat granulosa cells.

The current status of intraovarian function of cytokines have been summarized in Table 1.5, During the preantral follicle stage, the predominant effect of cytokines is inhibitory and gonadotrophin–dependent. The main target cells of cytokines at this stage seem to be undifferentiated granulosa cells. During the preovulatory follicle stage, the theca cell is a major site for cytokine reception with increasing secretory activity, whereas luteal cells in response to cytokines appear to produce increased PGs and less response in steroidogenesis.

In order for the role of cytokine in ovarian physiology to be clarified, their in vivo origins must be determined. Immune cell, as a confirmed origin of cytokines, have been identified in the ovary of several specials (1.5.1). However, a systematic cyclical observation has not been available on the distribution of immune cells at the different stages of follicle or corpus luteum which prevents the evaluation of their real function in the ovary. Inconsistent findings of immunoreactive cytokines in the ovary (1.5.2.2) could not confirm whether the cytokine exists in the ovary or not. The detection of cytokine mRNA (1.5.2.1 and 1.5.2.2) from the extraction of ovarian tissue has proved the existence of cytokine production within the ovary but which cell population responds to cytokinesis unclear. Furthermore, the identification of cytokine receptors in the ovary should be investigated too. Although many questions are waiting to be explored, the available observations (1.5.2), taken together, indicate the intraovarian action of cytokines corresponds with physiological secretory activity of preantral and preovulatory follicles. Thus, it can be inferred that cytokines may play local roles in the control of follicular development, ovulation and secretory activities.

1.5.3 Interaction between Gonadotrophins, Ovarian Steroids and Immune cells

Menstrual cyclic variation of IL–1 has been observed in human subjects. Increased plasma IL–1 during the luteal phase of the menstrual cycle is consistent with

Table 1.5 Summary of cytokines and their intraovarian function

Stage of follicle	Cells	IL-1	TNF	IL-2	IFNγ
Preantral follicle	Undiff G.C	Inhibit: FSH-induced P4. E_2 and LHr (rat)	Inhibit: FSH-induced aromatase E_2 and P4 (rat and porcine) Stimulate PGs (porcine)	Increase: FSH-induced P4 (rat)	Inhibit: FSH-induced aromatase, E_2, P4 and Inhibin (rat)
	Diff G.C.	Inhibit: basal and LH-induced P4; basal and FSH-induced aromatase, E_2 and P4 (porcine)	Inhibit FSH-induced P4 (rat)		
	Theca-inter		Inhibit: hCG-induced androgens (rat)		
Preovulatory follicle	Granulosa-Lutein	Increase: PGs (bovine) Inhibit: FSH-induced E_2 (human)	Inhibit: basal and FSH-induced P4 (rat) Increase PGs (human) Inhibit: FSH-induced E_2 (human)	Inhibit: hCG-induced P4 (human)	Inhibit: hCG-induced P4 and FSH-induced E2 (human)
	Theca	Increase: hCG-induced P4 and T (hamster)	Increase: progestins, androgens and E2 (rat)		

Legends

aromatase: aromatase activity. **Diff G.C:** differentiated granulosa. **E_2:** estradiol. **Lutei GC:** luteinized granulosa cells: **LHr:** LH Receptor. **granulosa-lutein:** granulosa-lutein cells. **PG:** prostaglandin. **P4:** progesterone. **T:** testosterone. **Theca:** theca cells. **Theca-inter:** theca interstitial cells. **Undiff G.C:** undifferentiated granulosa cells.

the raised body temperature (0.2–0.6°C) at that stage (Cannon and Dinarello, 1985). Human peripheral monocytes cultured in the luteal phase of the cycle secrete significantly more IL–1 bioactivity compared to that in the late luteal phase (Polan *et al*, 1990). These increased levels of IL–1 *in vivo* or *in vitro* during the luteal phase may indicate the possible influence from menstrual cyclic release of hormones, which include LHRH, LH and steroids, on the immune cells function. A brief discussion of this aspect will be presented.

1.5.3.1 LHRH

Immune cells have been demonstrated to be the sites of LHRH reception, production and action. Rat thymocytes and splenocytes contain LHRH receptors (Marchetti *et al*, 1988; 1989). LHRH mRNA has been detected in rat spleen lymphocytes (Azad *et al*, 1991). LHRH and its agonist also show the direct stimulatory effects, both *in vitro* and *in vivo*, on lymphocyte proliferation (Marchetti *et al*, 1988, 1989), ornithine decarboxylase activity (Marchetti *et al*, 1989a), IL–2 receptor expression (Batticane *et al*, 1991) and thymus morphology and function (Marchetti *et al*, 1989; Morale *et al*, 1991).

1.5.3.2 LH

IL–1 acts acutely and directly to release preformed stores of LH and PRL from perfused rat pituitaries and pituitary cell culture (Rettori *et al*, 1987, Beach *et al*, 1989). IL–6 stimulates LH and PRL release from anterior pituitary (AP) (Spangelo *et al*, 1989). In response to LHRH or one mitogen, Concanavalin A (Con A), lymphocytes from human, porcine, mouse and rat can produce a LH or LH–like molecule (Ebaugh and Smith, 1988; Gorospe and Kasson, 1989; Costa *et al*, 1990;

Standaret *et al*, 1991). LH, probably in an endocrine or autocrine manner, is able to stimulate cell proliferation and the release of IL–1 and IL–2 in cultured mouse spleen cells (Rouabhia *et al*, 1991). A possibility therefore exists in the ovary that resident white cells with immune function may have their paracrine action on the steroid–producing cells by secreting locally produced LH, or by being influenced by LH.

1.5.3.3 Sex Steroids

In human, oestrogen receptors have been found in peripheral blood mononuclear cells and thymocytes (Danel *et al*, 1983; Carbone *et al*, 1986; Weusten *et al*, 1986; Scambina *et al*, 1990) and in T–lymphocytes of the cytotoxic/suppressor CD8 cell subset (Cohen *et al*, 1983; Stimson, 1988). Human monocytes respond in culture, to both oestradiol and progesterone, by stimulation at low concentrations and by inhibition at high concentrations (Polan *et al*, 1988). IL–1β mRNA levels are decreased in cultured human peripheral monocytes by increased levels of both oestradiol and progesterone (Polan *et al*, 1989). Progesterone is also able to block T cell activation *in vitro* (Stites *et al*, 1983).

1.6 Aims of this thesis

The literature review presented has summarized the investigations of the relationship between the immune cell derived cytokines and the female reproductive system. It indicates that immune cell derived cytokines can be locally produced from, and have functions on any part of the axis of the hypothalamus–anterior pituitary–ovary and the rest of the reproductive organs. Furthermore, the secretory products from the reproductive organs can also act on these immune cells and their cytokine production. The ovary, as one of the organs of both endocrine and reproductive systems, is a site of active cell turnover during the reproductive phase of life with follicular growth, ovulation and formation of the corpus luteum requiring regular tissue remodelling. It is therefore not surprising that a considerable number of blood

leukocytes may traffic and reside in the ovary following ovulation. However, the real significance of the residence of these leukocytes with immune function inside the ovary is not clear, especially since few studies were conducted using human material to investigate the relationship between immune cell–derived cytokine and ovary. This thesis is thus designed to extend the above knowledge to the human ovary mainly using granulosa–lutein cells and corpus luteum as studied models. The aims of this thesis are to study the following aspects.

1) localization of the presence and distribution of leukocytes with immune function in the human ovary, with particular reference to the corpus luteum;

2) measurement of immunoreactive cytokines in the human ovary, particularly the follicular fluid;

3) study of the effects of cytokines on steroid production and cell proliferation in human granulosa cell culture.

Because it is difficult to obtain sufficient tissue from the human ovary before ovulation, these questions were studied using follicular fluid, luteinizing granulosa cells and corpus luteum excised at the time of gynaecological operation.

CHAPTER TWO

MATERIALS AND METHODS

All materials including instruments, chemicals, reagents and individual experimental methods used in this thesis are introduced in this section:

2.1 Equipment

- Milli Q water (M.Q water) system and filter unit (0.22μm); Millipore Corporation, MA, U.S.A.
- Nylon mesh (37–400μ pore size); Schweiz, Seidengazefabrik, Switzerland.
- Centrifuge tubes (15ml, propylene) with yellow cap (gamma sterile), Disposable Products Pty. Ltd., South Australia.
- Centrifuge tube (50ml); Becton Dickinson Labware., NJ, U.S.A.
- Disposable plastic culture vessels (25ml and 200ml); Inter Med Nunc, Denmark.
- 8–well tissue culture chamber slide; Nunc Inc., Naperville, IL, U.S.A.
- 24–well and 96–well tissue culture plates; Flow Laboratories Inc., Sydney, NSW, Australia.
- Disposable Pasteur pipettes (glass); Chase Instruments., Glens Falls, NY, U.S.A.
- Disposable plastic pipettes (1, 5 and 10ml); Becton Dickinson Labware., NJ, U.S.A.
- Microscope slides, 25.4 x 76.2mm, C.N.M.C., Shanghai, P.R. China.
- Cellulose tubing seamless (dialysis tube, MW cut–off 3.5KDa); Illinois, U.S.A.
- Mixing cannulae; Indoplas., Sydney, Australia.
- Glass–fibre filter; Enzo Diagnostics., New York, U.S.A.

- Centrifuges, Beckman models J-6B and GPR; Beckman Instruments Pty., Gladesville, NSW, Australia.
- Cytospin; Shandon, Cheshire, U.K.
- CO_2 incubator (model 3029); Forma Scientific Division of Mallinckrod Inc., Marietta, Ohio, U.S.A.
- Activon pH meter (model 209); Activon Scientific Products Company Pty., Bright VC Australia.
- Magnetic stirrer; Stansen Scientific Pty., Adelaide, Australia.
- Multi-Tube Vortexer; American Dade., Miami, FL, U.S.A.
- Cryostat, Tissue-Tek II; Miles Laboratories Inc., Naperville, IL, U.S.A.
- Wallas 1260 Multigamma II Counter; LKB Producter AB, Bromma, Sweden.
- Liquid scintillation counter; Beckman LS system, Fullerton, CA, U.S.A.
- Automatic Cambridge Cell Harvester; Watertown, MA, U.S.A.
- Olympus inverted research microscope (model IMT); Olympus, Tokyo, Japan.
- Olympus BH_2 microscope; Tokyo, Japan.

2.2. Chemicals and Reagents

Products purchased from Flow Laboratories Inc., Sydney, N.S.W., Australia were Minimum Essential Medium (MEM; Modified Eagle's Medium with Earle's Salts, with glutamine, without sodium bicarbonate); RPMI 1640 medium (RPMI 1640); phosphate buffered saline (PBS); antibiotics (streptomycin/penicillin-G solution) and 0.05% trypan blue. Products bought from AJAX Chemicals, Sydney, Australia were sodium azide (NaN_3); sodium dihydrogen orthophosphate ($NaH_2PO_4.2H_2O$); di-sodium hydrogen orthophosphate dihydrate ($Na2HPO_4.2H_2O$) and acetone.

Products provided by Sigma Chemical Company, St. Louis, MO, USA were sodium chloride (NaCl), α-D[+]-glucose; sodium bicarbonate ($NaHCO_3$); calcium

chloride dihydrate ($CaCl_2.2H_2O$); penicillin–G sodium salt (PG); pyruvic acid sodium salt (Na Pyr); phenol red sodium salt (phenol red); DL–Lactic and sodium salt (Lactic acid); Hepes sodium salt (Hepes); Sigmacote; phytohemagglutein (PHA); phorbol myristic acetate (PMA), trypsin (type XII–5); diaminobenzidine (DAB); poly–l–lysine (PLL, MW $1.5–3x10^5$) and cycloheximade. Potassium chloride (KCl); magnesium sulphate ($MgSO_4.7H_2O$); potassium phosphate monobasic (KH_2PO_4) and magnesium chloride ($MaCl_2.6H_2O$) were purchased from Mallinckrodt, Paris, Kentucky, U.S.A.

Potassium phosphate, dibasic (K_2HPO_4), sodium hydroxide (NaOH), hydrogen peroxide (H_2O_2) and DePex were obtained from BDH, Poole, U.K. Medium 199 (M199) was purchased from GIBCO, Paisley, U.K. Methyl–^{3}H–thymidine (^{3}H–thymidine) with 5mCi in 5ml, TRA 120, specific activity 5Ci/mmol was provided by The Radiochemical Centre, Amersham, England.

Ficoll–paque was purchased from Pharmacia, Piscataway, NJ, U.S.A. Heparin (1,000 iu/ml) was obtained from Weddel Pharmaceuticals, NSW, Australia. Bovine serum albumin (BSA, fraction V, essentially fatty acid free) was provided by Boehringer–Mannheim, Mannheim, Germany. Percoll was purchased from Pharmacia LKB Biotechnology, Uppsala, Sweden. Human chorionic gonadotrophin (hCG), Profasi, (3,000 U/ml) was obtained from Serono Laboratories, Basel, Switzerland. O.C.T. Compound, Tissue–Tek II was bought from Miles Laboratories Inc., Elkhart, IN, U.S.A. Liquid scintillation cocktail was purchased from Beckman Instruments Inc., Fullerton, CA, U.S.A.

Radioimmunoassay (RIA) kits for IL–1β, IL–2, TNFα and cAMP were obtained from Amersham International P/L, Amersham, U.K. The RIA kits for progesterone (P_4) were purchased from Bioclone Australia Pty. Ltd., Sydney, NSW, Australia and oestradiol (E_2) from Farmos Diagnostica, Oulunsalo, Finland.

Recombinant human interleukin-1 (IL-1β), interleukin-2 (IL-2) and tumor necrosis factor (TNF) were purchased from Genzyme, Corp, Boston, MA, U.S.A.

Mayers haematoxylin, ammonia water (1ml concentrated NH_4OH per litre), ethanol and xylene were available from Clinical Histopathology, TQEH. MLA-144 cell line (gibbon T cell line) was provided by Dr. L.K. Ashman, Department of Immunology, University of Adelaide through Mrs. S. Robertson.

2.3 Antibodies and Sera

Primary antibodies (1^{o}Ab) are listed in Table 2.1. Fetal bovine serum (FBS) was purchased from Flow Laboratories Inc., Sydney, NSW, Australia. Normal horse serum (NHS), normal goat serum (NGS), biotinylated horse anti-mouse immunoglobulin (Ig) (2^{o}Ab), goat anti-mouse IgM (2^{o}Ab) and avidin-biotin-peroxidase complex (ABC reagents) were bought from Vector Laboratories, Burlingame, CA, USA. Str Avi Gen^{IM} Super Sensitive Universal Immunostaining Kit, which included biotinylated anti-rabbit Igs (BARI), biotinylated anti-mouse Igs (BAMI) and peroxidase-conjugated streptavidin (PCS) was purchased from BioGenex Laboratories, San Ramon, CA, USA.

2.4 Immunocytochemistry

Immunocytochemistry is the identification of a tissue constituent in situ by means of a specific antigen-antibody reaction trapped by a visible label. Two techniques in immunocytochemistry are applied in this thesis. One is the avidin-biotin-peroxidase complex (ABC) method which was developed by Hsu and his associates in 1981 and another is an improved biotin-streptavin amplified detection system (B-SADS) which was developed by Bio Genex Laboratories, San Ramon, CA, U.S.A.

Table 2.1 **List of Antibodies**

Name of antibody	Abbreviations	Source
Monoclonal antibody		
CD3	CD3	Dakopatts, Denmark
Leucocyte common antigen	LCA	"
L26	L26	"
UCHL–1	UCHL–1	"
Vimentin	Vimentin	"
Anti–Leu–4	Anti–Leu 4	Becton Dickinson, USA
Anti–Leu2a	Anti–Leu 2a	"
Anti–Leu–3a+3b	Anti–Leu 3ab	"
Anti–interleukin–2 Receptor	Anti–IL–2 receptor	"
Anti–Leu–12	Anti–Leu 12	"
Anti–Leu–14	Anti–Leu–14	"
Anit–Leu–7	Anti–Leu 7	"
Anti–Leu–M1	Anti–Leu M1	"
Anti–Leu–M5	Anti–Leu M5	"
MT1	MT1	Biotest Diagnostics, FRG
Anti–estradiol	Anti–E2	Sara–Lab Sussex U.K.
Polyclona antibody		
Anti–human placental 3β–hydroxysteroid dehydrogenase	Anti–3β–HSD	Provided by Dr. J.I. Mason, University of Texas, USA

The ABC method includes the following substances. The primary antibodies, Ig G or M, which are specific to the human tissue antigens, are derived from mouse or rabbit; secondary antibodies which are anti–mouse or –rabbit Ig G or Ig M, from horse or goat respectively, will bind to the corresponding primary antibodies; biotin, a small molecule vitamin, can be linked chemically to the secondary antibody and peroxidase. Biotinylated secondary antibody, when added to the tissue section, localizes to the sites of primary antibody which has bound to the antigen within the section. Avidin, a 68,000 molecular weight glycoprotein found in egg white, has an extraordinary affinity (dissociation constant 10^{-15}M) for the biotin. Therefore, avidin molecules are able to bind Igs or peroxidase molecules which have covalently coupled biotin. Peroxidase (horseradish peroxidase normally used) in conjunction with diaminobenzine (DAB, a chromogen) and hydrogen peroxidase as the substrate finally results in an intensive staining. Taken together, the ABC method involves an application of biotin–labelled secondary antibody followed by the addition of avidin–biotin peroxidase complex. During the formation of the complex, avidin acts as a bridge between biotin–labelled peroxidase molecules, and biotin–labelled peroxidase molecules, which contain several biotin moieties, serve as a link between the avidin molecules. Consequently, a "Lattice" complex containing several peroxidase molecules is likely formed. A high staining intensity therefore results from the binding of this complex to the biotin moieties associated with secondary antibody (Fig. 2.1).

The B–SADS method uses streptavidin instead of avidin. Streptavidin is a biotin–binding protein produced by Streptomyces (Chaiet and Wolf 1964). The binding affinity of streptavidin for the biotin molecules is over 1 million times higher than that of avidin. Unlike avidin, streptavidin contains no carbohydrate which can bind nonspecifically to lectin–like substances found in normal tissue (Naritoku *et al*, 1982). The secondary antibody in the B–SADS method attachs more biotin

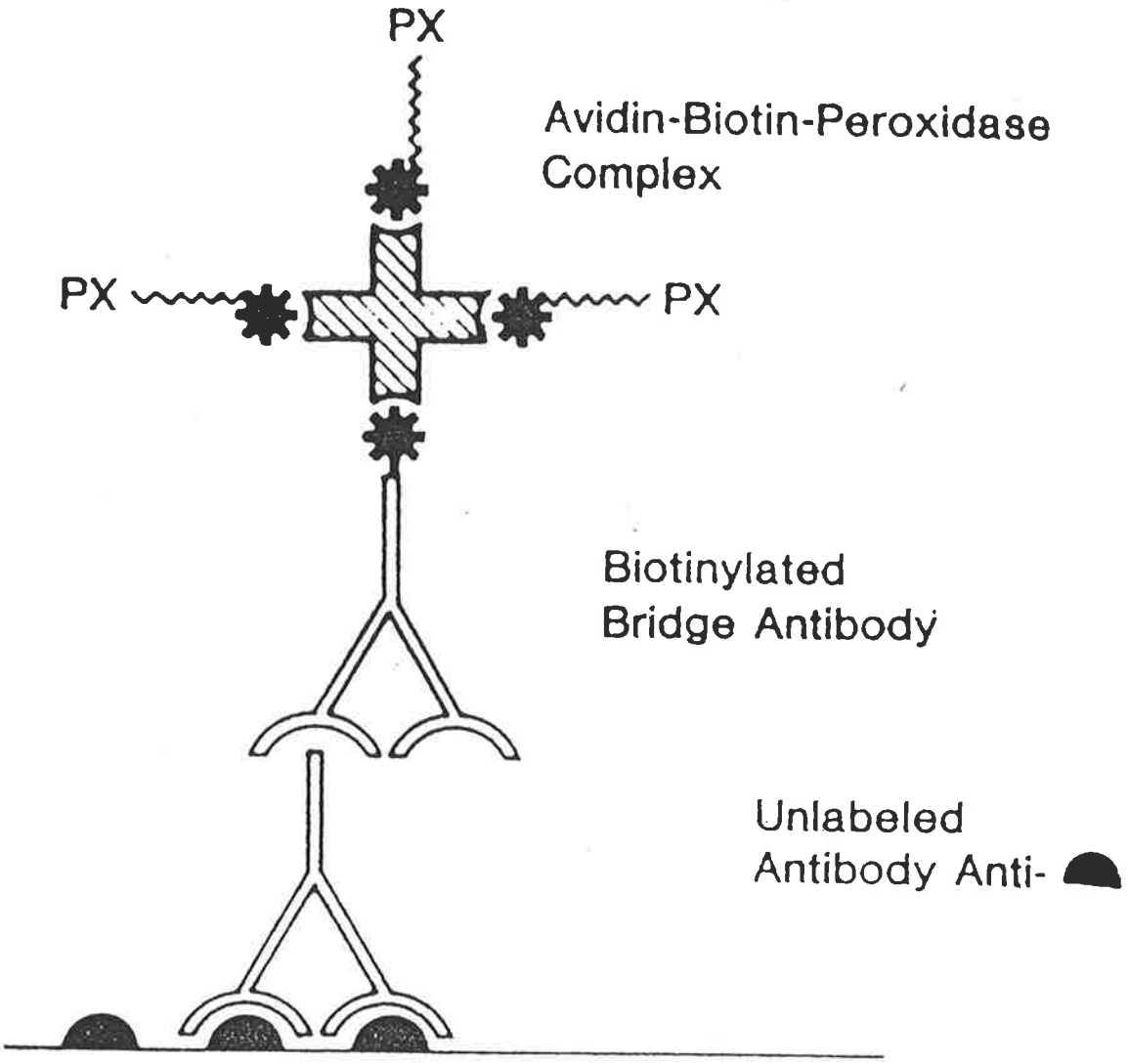


Fig 2.1 The pictorial illustration of the ABC method. Solid semicircle indicates antigen; PX, peroxidase, asterisk, biotin; and shaded open+, avidin (Reproduced from Falini and Taylor, 1983).

molecules without adversely affecting its binding affinity. In addition, the procedure for conjugation of the enzyme label to streptavidin has been optimized to permit maximum labelling of the streptavidin molecule with multiple enzyme labels. The combined effect of these improvements is a dramatic increase in the amount of signal generated per antigen–antibody binding event relative to any other procedures.

The dilution and predominant cellular reactivity of primary antibodies are summarized in Table 2.2. Immunocytochemistry solutions including peroxidase block; PBS pH 7.6; antibody diluent, stock solution of 0.5% DAB and poly–l–lysine (PLL) coated slides are mentioned in Appendix I. Pre–treatment of tissue section or cells on chamber slide and staining procedures are summarized in Appendix II. A positive reaction is demonstrated by a brown deposit surrounding the cell membrane. Tissue sections obtained from tonsil and granuloma are used as a positive control for all leukocyte markers. Corpus luteum tissue section is used as positive control for the antibodies against vimentin, E_2 and 3β–HSD. The selection of the negative antibody control is according to the standard procedure provided by Taylor (1986). The section is treated with PBS in lieu of primary antibody; the addition of secondary antibody and all other steps are followed unchanged. The evaluation of the results will be introduced in sections 3.2 and 7.2.

2.5 ^{3}H–thymidine uptake

The degree of cell proliferation can be determined in a number of different ways. The most common is measurement of ^{3}H–thymidine uptake. In this thesis, ^{3}H–thymidine incorporation into cultured human granulosa–lutein cells was performed to judge proliferation of these cells under the different culture conditions. Cell culture (see 2.6) was completed by removal of culture media at the required stage. Cell proliferation was assessed by adding freshly prepared ^{3}H–thymidine culture medium (see Appendix III) for a further 6h in an incubator at 37^{o}C with 5%

Table 2.2 Predominant Cellular Reactivity and Dilution of Antibodies

Name of antibody	Cluster Designation	Predominant Reactivity	Dilution	
			Frozen Section	Paraffin Section
Anti–Leu 4	CD3	T cell	1:5	
Anti–Leu 2a	CD8	T cytotoxic/ suppresor cell	1:40	
Anti–Leu 3ab	CD4	T helper/inducer cell	1:4	
Anti–IL2 receptor	CD25	Activated T cell	1:10	
Anti–Leu 12	CD19	B cell	1:50	
Anti–Leu 14	CD22	B cell	1:5	
Anti–Leu M5	$CD11_c$	Macrophage	1:25	
Anti–Leu 7		NK	1:20	1:50
Anti–Leu M1	CD15	Monocyte	1:50	1:20
LCA	CD45	Leukocyte common antigen positive cell	1:50	1:30
CD3		T cell		1:500
MT1	CD43	T cell		1:500
UCHL1	CD45RO	T cell		1:1000
L26	CD20	B cell		1:500
Vimentin		Cells of mesenchymal origin	1:200	
Anti–E_2		Cells containing E_2	1:100	
Anti–3β–HSD		Granulosa cell	1:300	

CO_2 and 95% air. After 6h incubation, ^3H-thymidine culture medium was aspirated. Cells were washed twice with MEM and digested in freshly prepared trypsin solution (see Appendix III) for 5–10 min. The cells in trypsin solution were collected with a multiple harvester onto a glass-fibre filter and after washing with water, the filters were dried and counted in a liquid scintillation counter. The results were expressed as mean counts per minute (c.p.m.) of ten culture wells.

2.6 Human Granulosa-Lutein Cell Culture

Since the IVF techniques were popularized, human granulosa-lutein cell culture has been widely used to study some of the properties of ovarian cells. These cells have been widely studied (Hsueh *et al*, 1984) due to their easy availability in relatively pure form through programmes of *in vitro* fertilization, and the ease of culturing them. Unlike most granulosa cell cultures obtained from rodents, human granulosa-lutein cells have been exposed to endogenous and exogenous gonadotrophins *in vivo*, while differentiating *in vitro*. Thus their function most likely resembles that of luteal cells *in vivo* (Hillier *et al*, 1987). Human granulosa-lutein cell culture remains a useful model which is applied as one of the study methods in this thesis.

2.6.1 Preparation of material and solutions

2.6.1.1 Preparation of silicon pipette

See Appendix IV

2.6.1.2 Preparation of cell culture media

See Appendix IV

2.6.1.3 Preparation of 50% percoll solution

50% percoll solution was made from HTF media and percoll solution which was produced by percoll stock solution and 10 x media. See Appendix V for details.

2.6.2 Collection of granulosa-lutein cells

Cells were recovered from follicular fluid obtained from women undergoing an *in vitro* fertilization (IVF) procedure at the Reproductive Medicine Unit, The Queen Elizabeth Hospital. Ovarian stimulation was used, with a combined therapy of 100mg clomiphene citrate/ day from days 5–9 and 75–150 IU human menopausal gonadotrophin every day from days 5–14, with slight dose variation depending on the individual response (Kerin *et al*, 1984). Follicular development was monitored by measuring daily levels of serum E_2 and by ultrasonographic measurements of follicle diameters. HCG (5000 IU) was injected intramuscularly when the size of at least two leading follicles reached 18 mm, with serum E_2 levels over 1–1.5 nmol/mature follicle. Laparoscopy or transvaginal ultrasound-guided oocyte retrieval was performed 36h later, and the contents of visible large follicles (>16mm) were aspirated. Oocytes were identified and separated. Follicular fluids from different large follicles of each woman were combined.

2.6.3 Cell culture procedures

- Combined follicular fluids from follicles of one case were transferred to a centrifuge tube using a sterile silicon coated pasteur pipette and spun at 200xg for 5 minutes (min);
- Supernatant was discarded, cells were washed with MEM and then spun at 200xg for 5 min;
- Supernatant was discarded and the required amount of MEM (cell:MEM about 1:1–2) was added to cells, with thorough mixing;
- A 50% percoll column (4ml) was placed in each new centrifuge tube, 2–2.5ml cell suspension was placed on the top of each percoll column and spun at

200xg for 20–30 min to pellet red blood cells;

- After centrifugation, the contents from the top to the bottom of the percoll column should be MEM, granulosa–lutein cells, 50% percoll solution and the red blood cells. A relatively purified granulosa–luteal cell population was aspirated from the interface using a new sterile siliconized pasteur pipette to a new tube with MEM (about 7 ml);
- The cell suspension was passed through a sterile nylon mesh into another new tube to make a single cell suspension, spun at 200xg for 5 min and supernatant was discarded;
- Cells were washed again in MEM (5ml), cell viability and number were determined by 0.4% Trypan Blue and a haemacytometer, spun at 200xg for 5 min and supernatant discarded.
- Cells were resuspended in 10ml of MEM–FBS, with mixing and the required amount of MEM–FBS was added.
- Cells were seeded to culture plates ($2.5–5x10^5$ cells with 1ml of MEM–FBS per well).

In most situations, more than 95% viable cells were obtained in every experiment by counting with Trypan Blue staining. Cultures were incubated in a humidified environment with 5% CO_2 and 95% air at 37°C. Culture media were changed every 48hrs with the same media or serum–free media, M199–FBS depending on the experiment. Each experiment such as, addition of hCG or cytokines consisted of four to six wells. These treatments were added at the required time. Culture periods were dependent on the requirement of observation. At the end of each culture period, the incubation media were collected and stored at –20°C until assayed

2.6.4 Evaluation of culture system

2.6.4.1 Identification and morphology

The majority of cells in the present culture system were steroid producing cells as demonstrated by immunocytochemistry (see 2.4) with 3β–hydroxysteroid dehydrogenase (HSD) polyclonal antibody (Fig. 2.2a,b). After 48hr of culture period, granulosa–lutein cells formed monolayers and assumed a fibroblastic appearance in the absence of any hormone. In the presence of hCG, cells were tightly packed with epithelial shape and tended to grow in multilayered aggregates. In the mean time, more granules were observed in the cytoplasm of cultured granulosa–lutein cells incubated with hCG compared to the culture without hCG. These observations may be in accord with the secretory activity of these cells under the different culture conditions.

2.6.4.2 Cell growth *in vitro*

Cell proliferation measured by [^{3}H]–thymidine incorporation into cultured cells (see 2.5) showed that cells gradually proliferated over a 10–day period starting with the original number ranging from $2x10^3$ to $1x10^6$ cells/well (Fig. 2.3). HCG (5iu/ml) did not alter cell proliferation with the original number of $5x10^5$ cells/well compared to the control. These observations indicate that the cell culture system applied in this study is suitable for the maintenance of human granulosa–lutein cell growth *in vitro*.

2.6.4.3 Daily and accumulative P4 secretion

In the absence of gonadotrophin stimulation in the culture system, granulosa–lutein cells retained their ability to produce P4. The values of P4 in every case peaked on the second or third day of culture (Fig. 2.4). The levels of P4 declined rapidly after the third day of culture, falling to much lower levels during the later stages of culture, although cell growth at this time was demonstrated to be unimpaired (Fig. 2.3). The cumulation of P4 in the culture medium also occurred at a decreasing

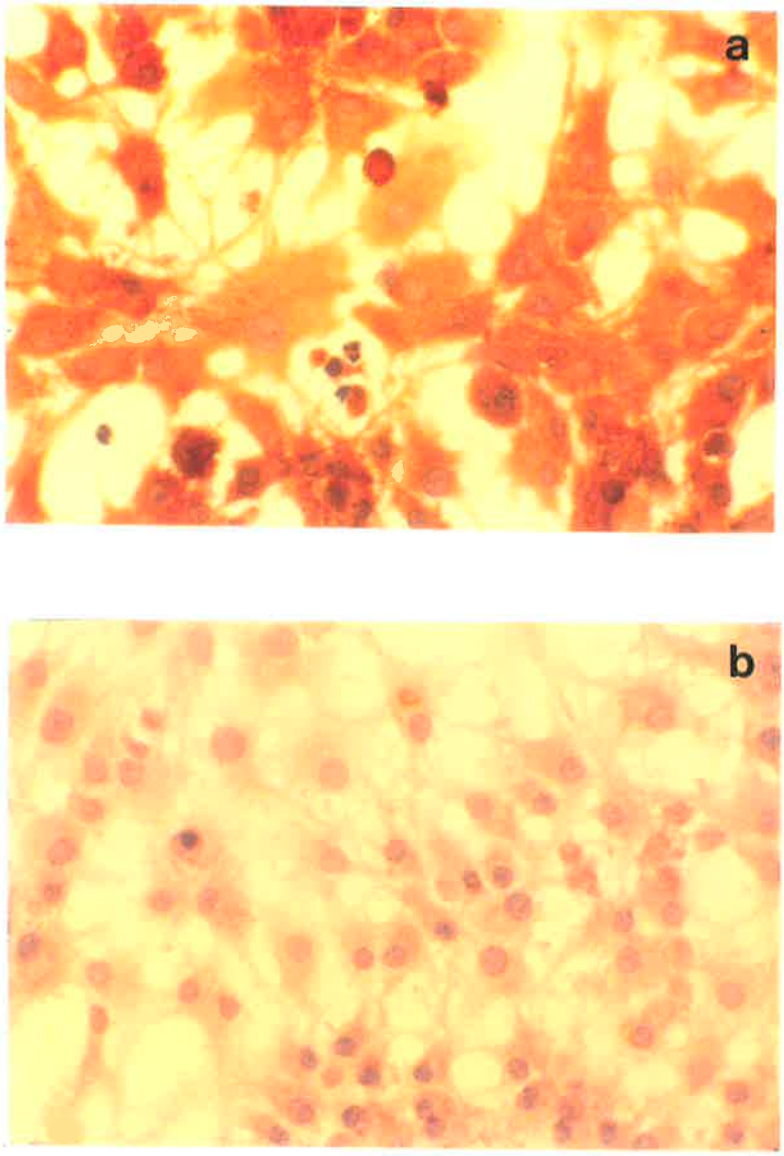


Fig. 2.2a 3β–HSD positive staining (brown) in cultured human granulosa–lutein cells (Magnification x555).

Fig. 2.2b Negative staining as a control in cultured human granulosa–lutein cells (Magnification x555).

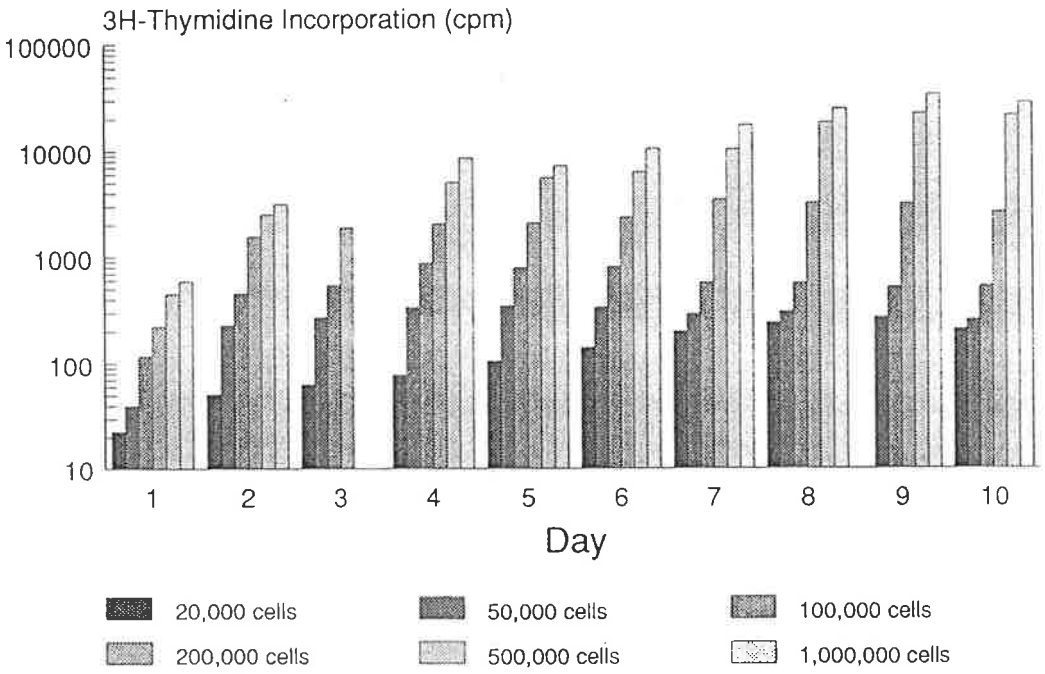


Fig 2.3 Cell proliferation was observated by (^{3}H)–thymidine incorporation into cultured human granulosa–lutein cells (starting cell numbers $2x10^4$ to $1x10^6$ per well, 1 case) grown for 10 days with media (MEM–FBS) changed every 48h.

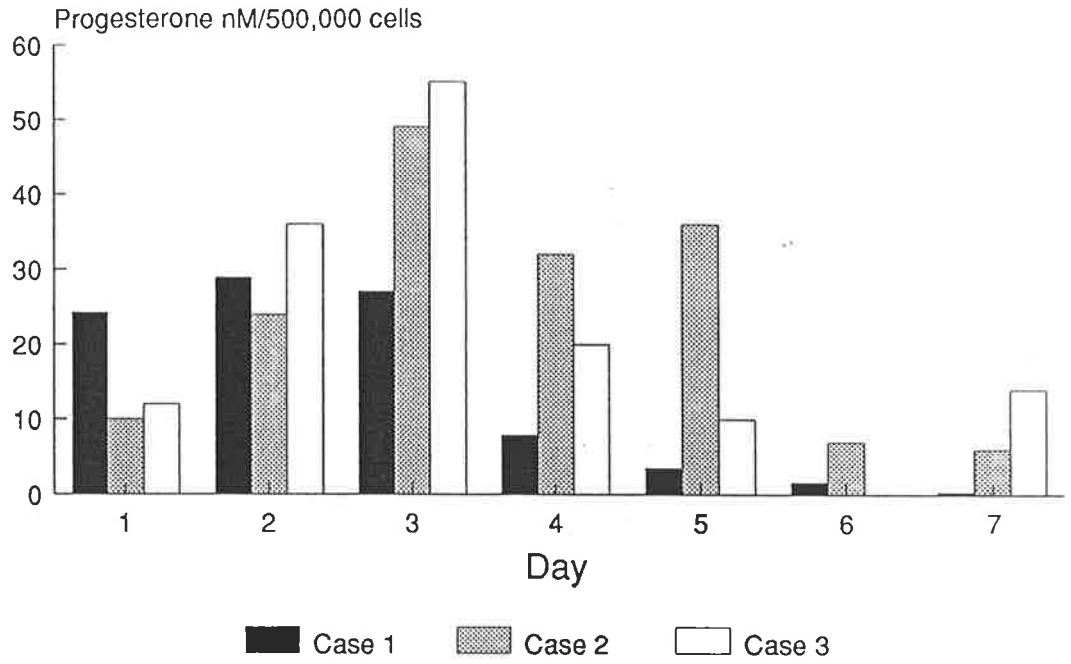


Fig. 2.4 Daily progesterone production from cultured human granulosa–lutein cells (3 cases).

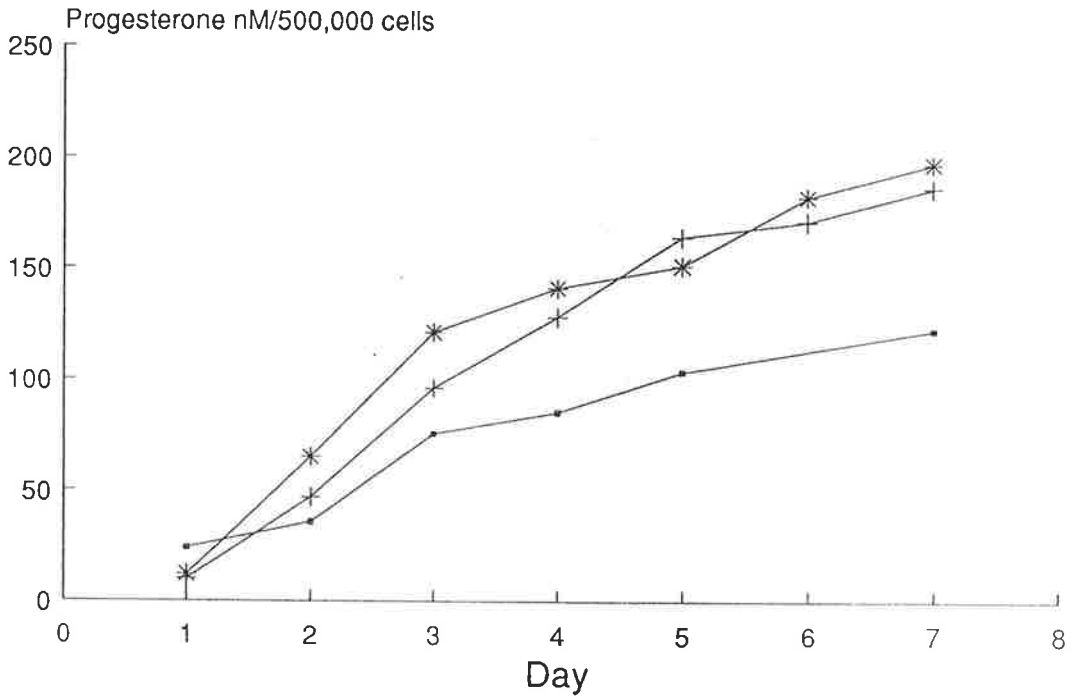


Fig. 2.5 Cumulative progesterone production from cultured human granulosa–lutein cells (3 cases).

rate after the fourth day of culture (Fig. 2.5). These results suggested that without gonadotrophin support, these cells can maintain their ability to proliferate but do not retain their P4 secretory function in parallel with their proliferative activity during long term culture.

2.6.4.4 Response to hCG on different days of culture

HCG was able to stimulate P4 production in granulosa–lutein cells, but the sensitivity of these cells to hCG stimulation differed at different stages of culture (Fig. 2.6). In the early stages of culture, cells demonstrated a high ability to produce P4 but a low sensitivity to hCG stimulation. Conversely, in the later stages of culture, cells secreted less P4 at a basal level but became more sensitive to stimulation with hCG (Fig. 2.6). Significant differences between P4 levels observed between control (basal levels) and hCG stimulated wells were found on day 2, 6 and 8 of culture ($p<0.001$). The results are similar to the findings of previous reports (Polan *et al*, 1984; Wickings *et al*, 1986; Hillier *et al*, 1987). These observations suggest that hCG hyperstimulation and the endogenous stimulation by preovulatory LH surge may therefore lead to a continued high secretory activity of these cells during the early days *in vitro*. The increased response to hCG of these cells *in vitro* on the later days may be related to the reduced effect of endogenous hCG and LH or the production of new cells which require gonadotrophin signals.

2.6.4.5 Thirty days of cell culture exposed to continual hCG stimulation

Although the granulosa–lutein cells retained their ability to produce P4 throughout the 8 days of culture, without hCG, a sharp decrease in their capacity to produce P4 occurred after day 3 or 4 onwards. If however the cells were incubated with hCG (5iu/5x10^5 cells), they retained their P4 biosynthesis for up to 30 days in culture (Fig. 2.7). This was demonstrated not only by the measurement of P4 levels, but also by the presence of substantially increased numbers of active secretory granules in the cytoplasm of hCG–stimulated cells, in comparison to those in control

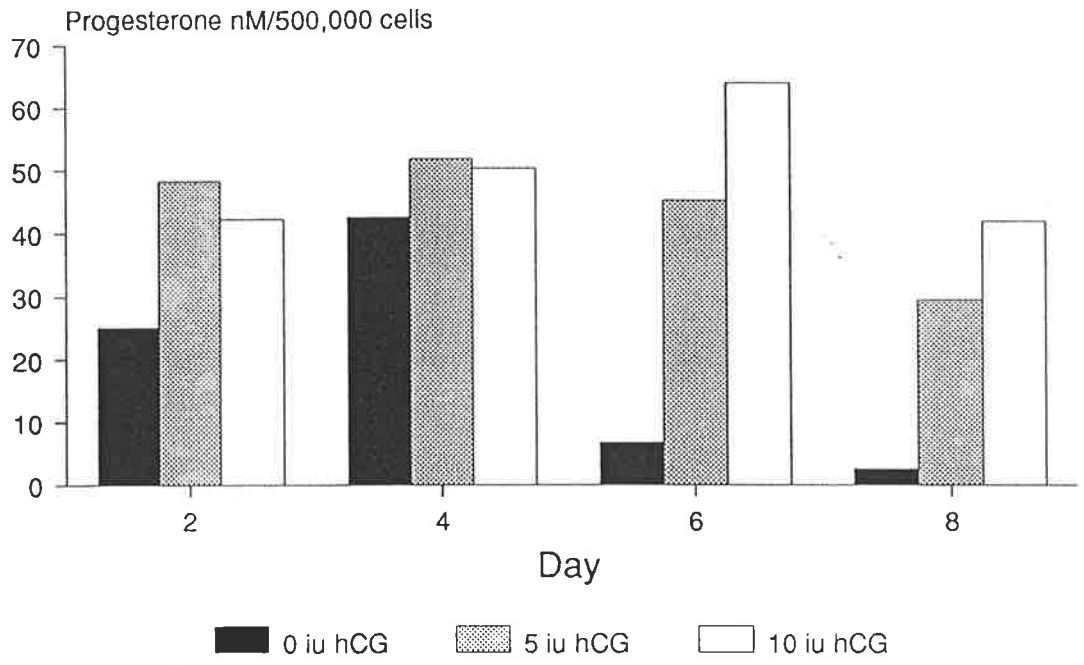


Fig 2.6 hCG (0, 5, 10 iu) stimulation of human granulosa–lutein cell culture on different culture days (three cases). Significant differences were found in progesterone secretion mean±SEM) between control and hCG–stimulated cells on days 2, 6 and 8 of culture ($P<0.001$).

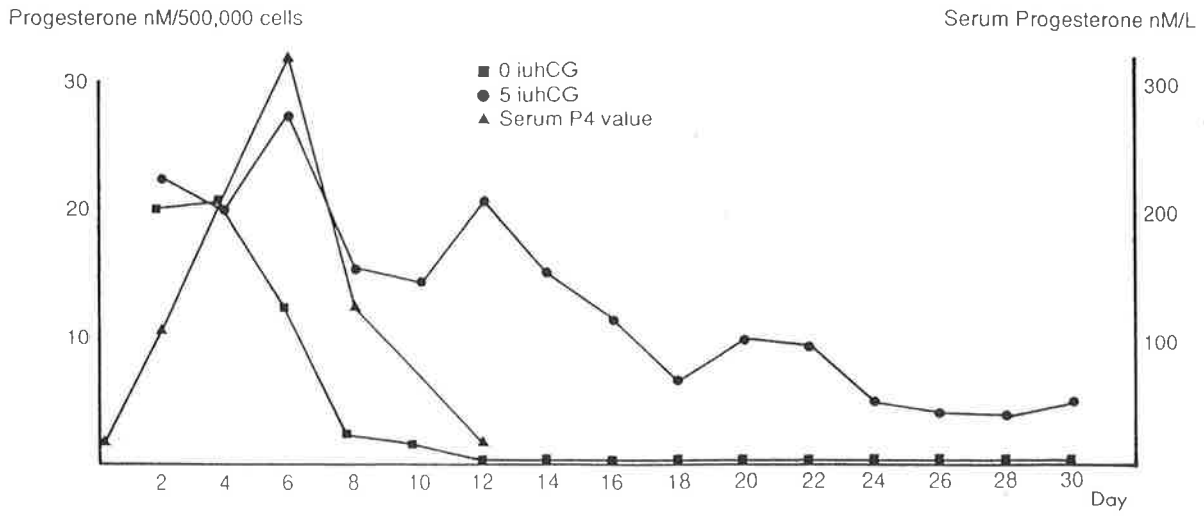


Fig. 2.7 Progesterone production from long term culture (30 days) of human granulosa–lutein cells (1 case) with or without hCG (5iu). Serum progesterone level (end on day 12) from the same case was almost parallel to that of cell secretion *in vitro*.

wells (Fig. 2.8a,b). During the first two weeks of culture, the *in vitro* P4 secretion was very similar to serum P4 measured in the same patient during the corresponding luteal phase. This *in vitro* model may be equivalent to the whole functional life-span of the corpus luteum formed in the natural ovulatory menstrual cycle, and also may imitate an early pregnant corpus luteum.

2.6.4.6 P4 secretion from cultured granulosa-lutein cells in serum-free media

In order to exclude the possibility that FBS contain endogenous cytokines which may influence the results of human granulosa-lutein cells with added cytokines, in some experiments, granulosa-lutein cells were precultured in MEM-FBS for 48h and followed in M199-BSA (serum free media) for another 48h. All experiments with cytokines showed similar responses in serum or serum free-medium. However concentrations of P4 in cultures with serum-free medium than serum containing medium were lower (results will be shown in chapter 5 and 7). The available assays were unable to detect immunoreactive and bioactive cytokines (IL-2 and TNFα) in serum-containing medium, which may be related to the inactivation of these peptides during the preheating procedure of FBS. Serum-containing medium was used therefore in the majority of cell culture in this thesis.

2.7 Leukocyte Culture

The purpose of *in vitro* culture of human leukocytes or MLA 144 cell line (gibbon T-cell line) in this thesis was to develop cytokine-enriched conditioned media which will be added to human granulosa-lutein cell culture to test the effect of cytokine on cell P4 production.

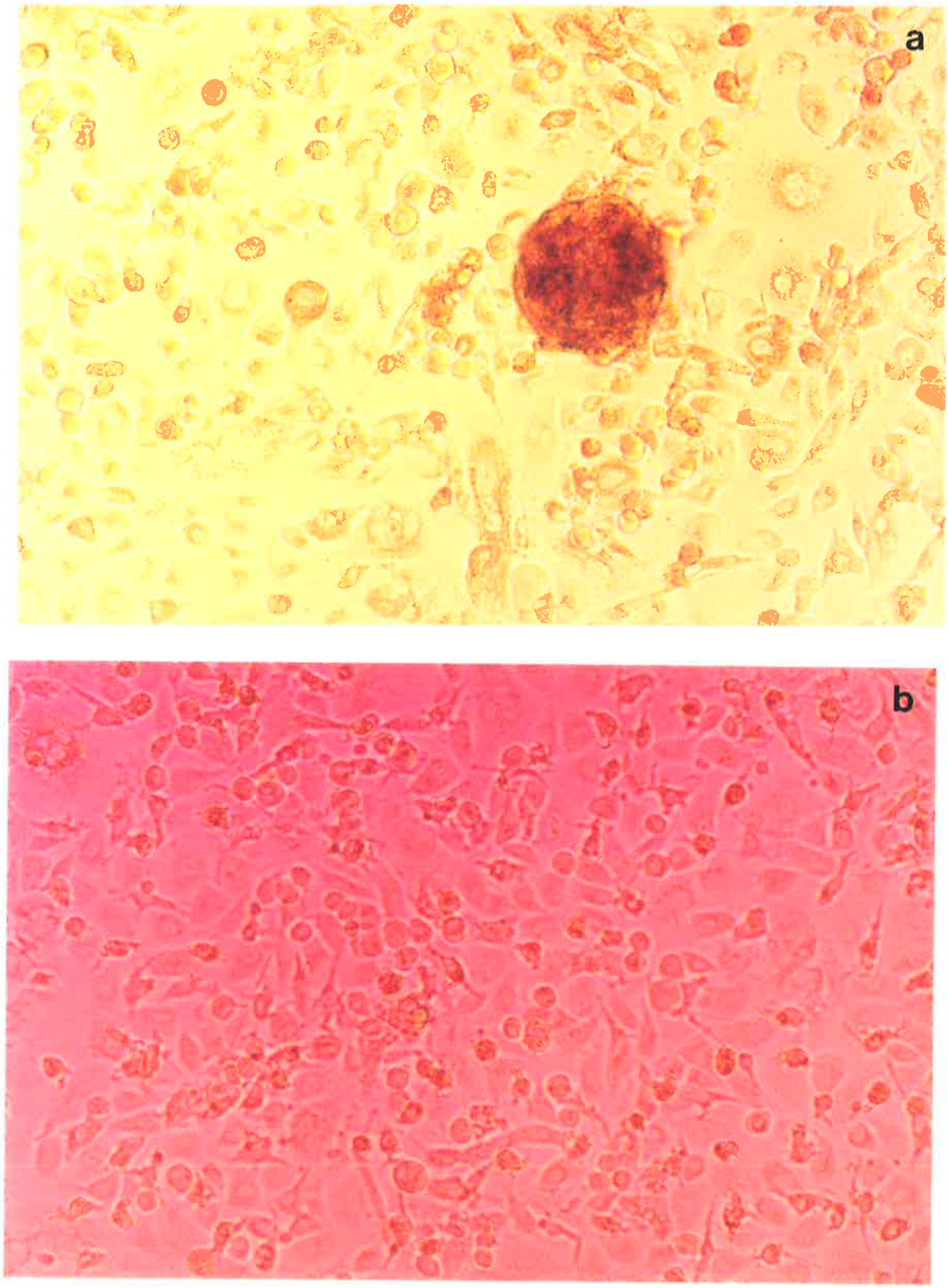


Fig. 2.8 Human granulosa–lutein cells were cultured *in vitro* with (a) or without (b) 5iu hCG for 24 days with media (MEM–FBS) change every 48h (1 case, Magnification x434).

2.7.1 Separation of peripheral blood mononuclear (PBM) cells

Blood (25ml) was drawn by sterile venepuncture into a syringe and immediately transferred into a sterile 50ml centrifuge tube with a concentration of approximately 5–10 iu/ml of heparin and mixed well. The blood was mixed with an equal volume of HBSS (see Appendix VI) and centrifuged at 1,500 xg, at 4°C, for 15 min. Approximately 2/3 of the supernatant was removed and HBSS added up to a total of 50ml and mixed well. Blood with HBSS (25ml) was added to one 50ml centrifuge tube; 15ml Ficoll–paque (15ml) was added to the bottom of the centrifuge tube by insertion of a mixing cannulae throughout the blood and the tube centrifuged at 300xg, 4°C for 30 min, then the PBM cells were recovered from the interface by a Pasteur pipette and washed twice with HBSS. The cell viability (normally more than 95% were viable cells) and cell number were assessed by staining with 0.5% trypan blue. Cells were diluted to the required number with MEM–FBS (see Appendix IV) or RPMI 1640–FBS (see Appendix VI).

2.7.2 Preparation of conditioned medium (CM)

1. Conditioned Medium 1 from cultured MLA–144 cell line (MLA–CM1)

MLA–144 cells ($5x10^6$) were added to each ml of MEM–FBS. Each 5ml of MEM–FBS containing the cells was transferred into a 25ml cell culture bottle. Cells were incubated in a humidified environment with 5% CO_2 and 95% air at 37°C for 48h. Conditioned medium was collected after centrifugation (500xg for 30 min) to remove the cells, it was then filtered, aliquoted and frozen at –70°C.

2. Conditioned Medium 2 from cultured MLA–144 cell line (MLA–CM2)

MLA–144 cells ($5x10^6$) were added to each ml of MEM–FBS. Each 5ml of MEM–FBS containing cells was transferred into 25ml cell culture bottle with 4μg/ml

PHA and 10ng/ml PMA. Cells were incubated in a humidified environment with 5% CO_2 and 95% air at 37°C for 48h. MLA-144 cells were removed from the harvested culture by centrifugation (500xg for 30 min). Culture medium (called MLA-CM_2) was dialysed twice against 2 litres of PBS and then once against 2 litres of MEM (MW cut-off, 3.5 KDa) at 4°C. MLA-CM_2 was filtered, aliquoted and frozen at -70°C.

3. Conditioned medium for cultured human peripheral blood cells (HPL-CM)

PBM cells ($5x10^6$) were added per ml of MEM-FBS. Each 5ml of MEM-FBS containing the cells was transferred into a 25ml culture bottle with 4μg/ml PHA and 10ng/ml PMA. Cells were incubated in a humidified environment with 5% CO_2 and 95% air at 37°C for 48h. PBM cells were removed from the harvested culture by centrifugation (500xg for 30 min). HPL-CM was dialysed twice against 2 litres PBS and then once against 2 litres of MEM (MW cut-off, 3.5KDa) at 4°C. HPL-CM was filtered, aliquoted and frozen at -70°C.

4. Control conditioned media (CM)

Control CM were prepared by equal amounts of MEM-FBS added with up to maximal concentrations of 40μg/ml PHA and 100ng/ml PMA using the same dialysis conditions.

2.8 Radioimmunoassays

Commercial radioimmunoassay (RIA) kits were used to assay IL-1β, IL-2, TNFα, cAMP, P_4 and E_2 in this study. The characteristics and procedures of each kit will be introduced as follows.

2.8.1 IL-1β RIA

IL-1β assay system utilized a high specific activity [125I]-IL-1β (human, recombinant) tracer, together with a highly specific and sensitive antiserum. Separation of the antibody bound from free fraction was achieved with a Amerlex-MN second antibody preparation, which was made from donkey anti-rabbit serum coated onto magnetizable polymer particles, thus allowing a simple magnetic separation. The assay was performed exactly as described by the manufacturer (Appendix VII) without prior extraction or dilution. The standards in this assay were calibrated in fmol/0.1ml (1fmol/0.1ml = 0.17μg/l) and the results were expressed in pmol/l. The cross-reactivity of the IL-1β antiserum for the interleukins was stated by the manufacturer to be 100% for human recombinant IL-1β and <0.01% for all other human recombinant interleukins tested, including IL-1α, IL-2, IL-2 [ala-125], IL-3, IL-4, IL-6, TNFα and IFNγ. The non-specific binding of IL-1β was 1.7%. The sensitivity, defined as the amount of IL-1β needed to reduce zero dose binding by two standard deviation was 0.22fmol/tube. All samples tested showed parallelism with standards.

2.8.2 IL-2 RIA

IL-2 immunoreactivity was evaluated by using an IL-2 RIA kit with the same principle as described in 2.8.1. The assay was performed exactly as described by the manufacturer (Appendix VIII) without prior extraction or dilution. IL-2 standards in this assay were calibrated in fmol/0.1ml (1fmol/0.1ml = 0.15μg/l) and the results were expressed in pmol/l. The cross-reactivity of the IL-2 antiserum for interleukins was stated by the manufacturer to be 100% for human recombinant IL-2 and <0.1% for all other human recombinant interleukins tested, including IL-1α, IL-1β, IL-3, IL-4, TNF-α, IFNγ and mouse recombinant IL-2. The non-specific binding of IL-2 was 2.5%. The sensitivity, defined as the amount of IL-2 needed to reduce zero binding to two standard deviations was 1.4fmol. All samples tested showed parallelism with standards.

2.8.3 TNFα RIA

Immunoreactive TNFα was analysed by using a TNFα RIA kit with the same principle as described in 2.8.1. The assay was performed exactly as described by the manufacturers (Appendix IX) without prior extraction or dilution. The standards in this assay were calibrated in fmol/0.1ml (1fmol/0.1ml = 0.17μg/ml) and the results were expressed in pmol/l. The cross-reactivity of the TNFα on antiserum for the interleukins was stated by the manufacturer to be 100% for human recombinant TNFα and less than 0.02% for all other cytokines tested including mouse recombinant TNFα, human recombinant TNFβ, IL-1α, IL-2, IL-3, IL-4, IL-6 and IFNγ. The non-specific binding of TNFα was 3.3%. The sensitivity, defined as the amount of TNFα needed to reduce zero dose binding by two standard deviation was 0.5fmol. All samples tested showed parallelism with standard.

2.8.4 cAMP RIA

The RIA for cAMP was performed using a kit with the same principle as described in 2.8.1. The assay followed the instruction from the manufacturer (Appendix X). cAMP standards were calibrated in fmol/0.1ml and the results were expressed in fmol/0.1ml. The antiserum cross-reactivity with related and other important compounds was determined by the 50% displacement technique stated by the manufacturer. Values for both acetylation and non-acetylation systems are 100% to cAMP and less than 0.4% for other compounds (including cIMP, cGMP, cCMP, AMP etc.). The non-specific binding was 2.3%. The sensitivity, defined as the amount of cAMP needed to reduce the zero dose binding by two standard deviations was 13.5fmol. Results were parallelism with the standard curve.

2.8.5 P4 RIA

The P4 kit was a double antibody RIA system. P4 in specimens (serum or cultured medium) competed with ^{125}I P4 for binding to a constant amount of anti–P4 antibody. The amount of ^{125}I P4 bound was inversely proportional to the amount of P4 present in specimens. A second antibody/polyethylene glycol (PEG) complex was used to separate antibody bound from free hormone. Assay procedures followed the instructions supplied with the kit (Appendix XI). The standards in this assay were calibrated in nmol/l and the results were also expressed in μmol/l or nmol/l (1nmo/l = 3.18ng/ml). All the specimens in the preliminary experiments were diluted $^1/_{50}$, $^1/_{100}$, $^1/_{500}$ or more in PBS–BSA (Appendix XI) in order to select the dilution closest to the medium point of the standard curve. When the correct dilution had been set up, all the samples from one batch of experiments were performed at the same time. The cross–reactivity of the antiserum for P4 was stated by the manufacturer to be 100% for P4 and less than than 2% for other steroids. Non–specific binding of P4 was 0.5%. The sensitivity, defined as the detectable mass equivalent to twice the standard deviation of the zero binding value was <0.04nmol/l. All samples showed parallism with standards. The intra–assay variation was less than 7% and inter–assay variation was less than 10% in serial consecutive assays.

2.8.6 E2 RIA

The Spectria direct [^{125}I] E2 coated tube RIA is designed for measurement of unconjugated 17β–E2 without chromatography or extraction of the sample and based on the double antibody RIA technique. The assay uses a rabbit polyclonal antiserum against E2 as first antibody and tubes coated with the second (anti–rabbit) antibody and was performed as advised by the manufacturer (Appendix XII). Assay procedures were followed by the instructions supplied with the kit (Appendix XII). Oestradiol values obtained were in μmol/l or nmol/l (1pg/ml = 272.4nmol/l). Sample dilution was the same as described in 2.8.5. When the correct dilution was established, all the samples from one batch of experiments were performed at the same time. The cross–

reactivity of the antiserum for E_2 was stated by the manufacturer to be 100% for E_2 and less than than 1% for other steroids. Non-specific binding of E_2 was 0.6% with standards. The sensitivity, defined as the detectable mass equivalent to twice the standard deviation of the zero binding value was less than 0.04 nmol/l. All samples showed parallism with standards. The intra-assay and inter-assay variabilities were less than 7% in serial consecutive assays.

2.9 Ethical Considerations

Applications for permission to obtain human granulosa-lutein cells and corpora lutea from patients were made to the Research and Ethics Committee of the University of Adelaide and the Queen Elizabeth Hospital, Woodville, S.A. The applications were approved. Patients, whose corpora lutea were removed, had read a consent form and signed their names to register before the operation.

2.10 Statistics

The details of statistics will be presented in the following individual section.

CHAPTER THREE

DISTRIBUTION OF LEUKOCYTE SUBPOPULATIONS IN THE HUMAN CORPUS LUTEUM

3.1 Introduction

While the distribution and function of leukocytes in the corpus luteum has been investigated in several species (Bulmer 1964; Paavola, 1979; Kirsch *et al*; 1983, Hume *et al*, 1984; Bagavandoss *et al*, 1988), there is relatively little data relating to the situation in the human female. Most of the previous investigations in man have been based entirely on morphological criteria for leukocyte and lymphocyte determination, and the only convincing studies have been those studying macrophages (Lei *et al*, 1991). With the increasing interest in cytokine mediated changes in steroidogenesis and proliferation in the corpus luteum and ovary of several species as discussed in 1.5.1 it is of importance to characterize leukocyte subpopulations in the human corpus luteum.

Mast cells are present in the rat ovary and are associated with ovulation, as shown by increasing numbers during the late follicular phase (Jones *et al*, 1980). Their product, histamine, is thought to be at least partially responsible for the initiation of the follicular hyperaemia (Krishna *et al*, 1989). Eosinophils also appear important in the sheep corpus luteum, where they infiltrate the ovary after ovulation and apparently degranulate either before functional or structural luteal regression (Murdoch, 1987). Macrophages have been identified in most species and are alleged to be important in the phagocytic degeneration of the corpora lutea cells. Several species such as the mouse (Kirsch *et al*, 1983; Hume *et al*, 1984), rat (Bulmer, 1964), guinea pig (Paavola, 1979), rabbit (Bagavandoss *et al*, 1988), and human (Katabuchi *et al*, 1989; Loukides *et al*, 1990), exhibit macrophages in the corpora lutea. There is

convincing evidence that the numbers of these macrophages increase as luteolysis proceeds. T-lymphocytes have also been reported in the rabbit corpus luteum (Bagavandoss *et al*, 1990), but have an unknown function in this organ. In addition, human pre-ovulatory follicular fluid has been shown to contain both T4 and T8 lymphocytes (Hill *et al*, 1987b; Droesch *et al*, 1988).

There are now several reports from animal species and humans suggesting that white cell products such as interleukins 1, 2, 3, and tumour necrosis factor α exhibit either stimulatory or inhibitory effects on steroid production (See 1.5.3.1 and 1.5.3.2). To demonstrate that cytokines play a physiological rather than pharmacological role in the ovary, it is important to demonstrate that white cells are present in the human corpus luteum and in sufficient numbers to produce concentrations of cytokines which could influence the corpus luteum function. The study reported here was undertaken to identify the leukocyte subpopulations which could potentially secrete cytokines in the human corpus luteum.

3.2 Materials and Methods

3.2.1 Preparation of tissue section

Ten corpora lutea were obtained prospectively from female patients undergoing hysterectomy for non-ovarian disease. The mean age of the patients was 37 years (range 23-55). Corpora lutea were fixed in 10% buffered formalin and routinely processed. In six of the corpora lutea, a piece was also snap frozen in Tissue-Tek O.C.T. Compound in liquid nitrogen and stored at -70°C until tested. Paraffin sections were stained with haematoxylin and eosin for light microscopic examination.

3.2.2 Immunocytochemistry

Immunocytochemistry was performed using the ABC method as described in 2.4 with the following monoclonal antibodies: LCA, UCHL-1, L26, CD3; Anti-Leu-4, Anti-Leu-2a, Anti-Leu-3a+3b, Anti-IL-2 receptor, Anti-Leu-7, Anti-Leu-12, Anti-Leu-14, Anit-Leu-M1 and Anti-Leu-M5. The source, predominant cellular reactivity and dilution of antibodies have been introduced in 2.2, Table 2.1 and 2.2. For method details see Appendix II. A positive reaction was demonstrated by a brown deposit surrounding the cell membrane. The results were analysed by counting the number of positive cells in a total of 500 cells within a randomly chosen field of a counting grid on an Olympus BH_2 microscope and expressed by the mean and the mean of standard error.

3.3 Results

Cells positive for monoclonal antibody were observed mainly in three regions (Fig. 3.1): theca-lutein, granulosa-lutein and loose connective tissue. The latter region originates from the theca externa and penetrates into the lutein mass, covering the inner surface of the lutein cells and forming a main component in the centre of the corpus luteum (Copenhaver, 1964).

Leukocyte subpopulations detected in human corpora lutea included: macrophages, cells positive for leukocyte common antigen (LCA), T lymphocytes and subsets, T helper/inducer (T4) and T cytotoxic/suppressor (T8) cells, activated T (Ta) cells (IL-2 receptor positive cells), monocytes and natural killer (NK) cells. B lymphocytes were not found in any human corpus luteum tissue. The density distribution of leukocytes in the different parts of the corpus luteum was found to be in the order: thecal-luteal area > loose connective tissue area > granulosa-luteal area. The number (mean ± SEM) of the monoclonal antibody positive cells per total 500

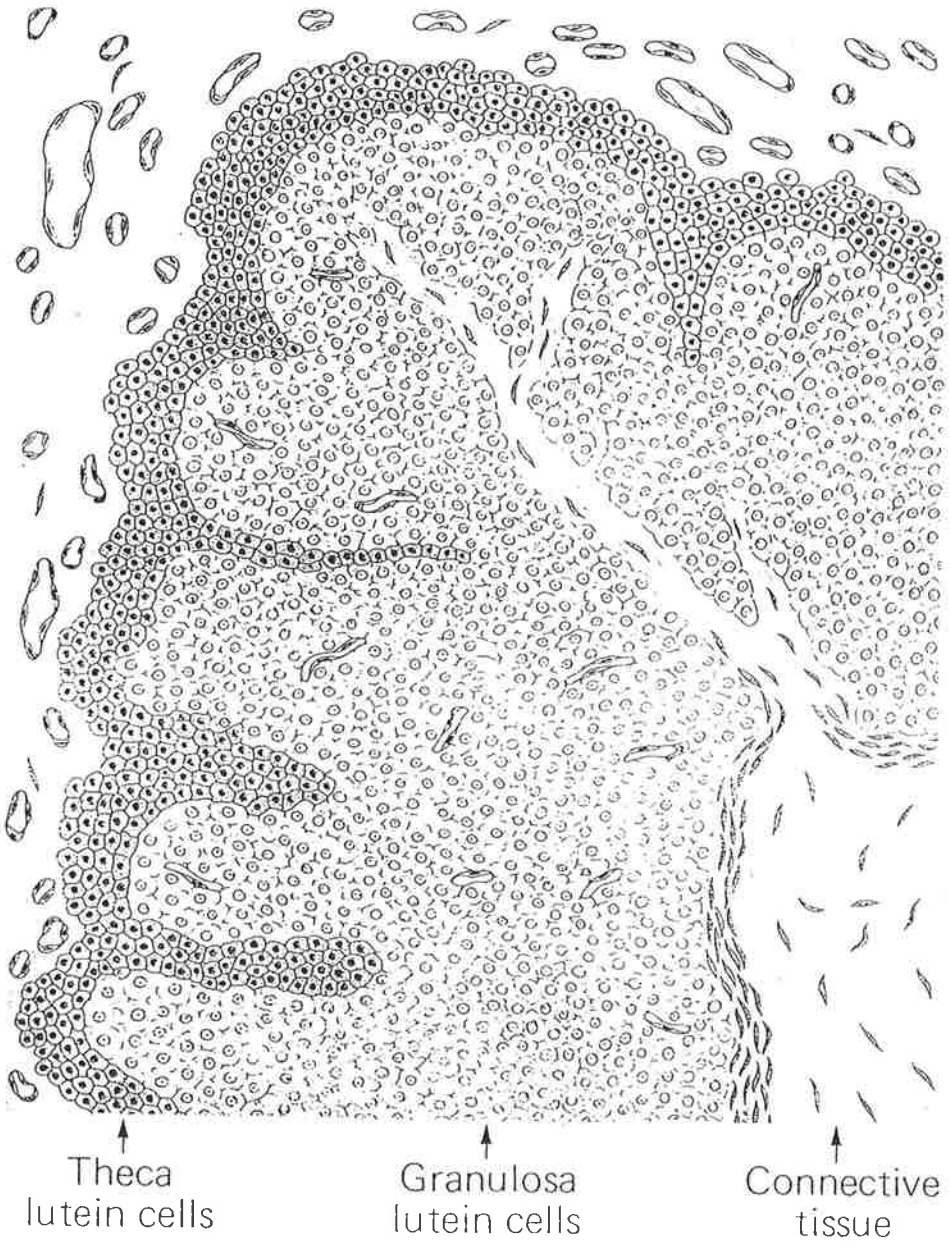


Fig. 3.1 The pictorial illustration of a small portion from a human corpus luteu. Granulosa–lutein cells derived from the granulosa layer are large and less darkly stained than the theca lutein cells, which derive from the theca intera. Loose connective tissue is in a remnant of the follicular cavity (Reproduced from Jungueira et al, 1986).

cells in the theca–luteal and granulosa–luteal areas respectively were as follow: 48±14 and 50±10 for Anti–LeuM5 cells (macrophages);28±5 and 15±6 for LCA cells; 14±6 and 5±3 for CD3 cells (T cells); 15±6 and 2±0.6 for Anti–Leu3ab cells (T4 cells); 11±2 and 2±1 for UCHL1 cells (T cells); 10±1.4 and 4±1 for MT1 cells (T cells); 7±1.2 and 2±1 for Leu2a (T8) cells; 3±1 and 3±1.7 for Anti–LeuM1 cells (monocytes) and 1±0.3 and 0.6±0.2 for Anti–Leu7 cells (NK cells). The quantitative relationship of these cells is shown in Fig. 3.2 from which it is clear that the majority of leukocytes were localized in theca–luteal area . There were relatively few leukocytes in the granulosa–luteal area. A cell border composed mainly of macrophages, LCA cells and T4 cells was usually seen in the theca–luteal and loose connective tissue surrounding the granulosa luteal area. (Fig. 3.3a, b and c). Macrophages were the most common cell type of the leukocyte subpopulations in the corpus luteum. They represented almost 10% of the total cells and were equally distributed in both the theca and granulosa luteal area. Morphologically, the cytoplasm of macrophages spread widely and was in contact with neighbouring cells (Fig. 3.3a).

Leukocyte common antigen positive cells were present in this study representing different types of leukocytes (Fig. 3.3b), excluding macrophages. The distribution of positive cells was greater in the theca–luteal area than in the granulosa–luteum area.

There were fewer T lymphocytes (Fig. 3.3c) than LCA cells in the human corpus luteum. There were more T4 than T8 cells and there were also a larger number in the theca–luteal and in loose connective tissue than in granulosa–luteal area. The ratio of T4/T8 was approximately 2:1 and 1:1 in the theca–luteal and granulosa–luteal areas respectively and they were found predominantly in proximity to blood vessels (Fig. 3.3d and 3.3e). In five out of six frozen sections, Ta cells were found only in loose connective tissue (Fig. 3.3f) where they co–existed with T4 but not T8 cells.

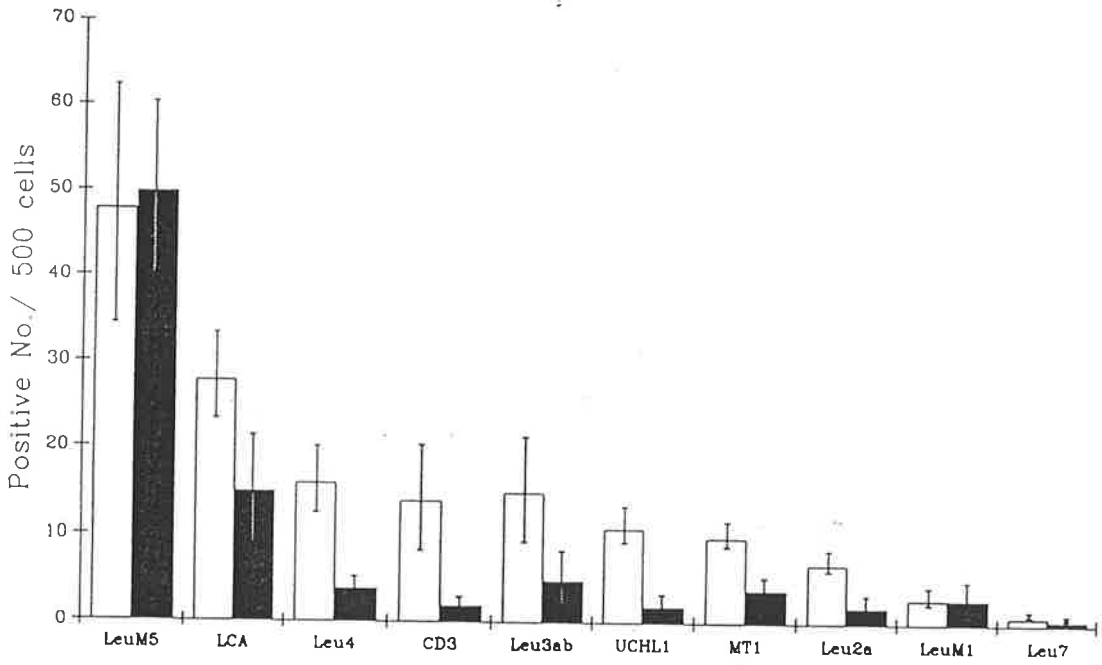


Fig. 3.2 Numbers of cells positive for monoclonal antibody (mean ± SEM) in the thecal–luteal (open bar) and granulosa–luteal (solid bar) areas of the human corpus luteum. Designations of antibodies are as follows: LeuM5 for macrophages; LCA for cells positive for leukocyte common antigen; Leu 4, CD3, UCHL1, MT1, for T cells; Leu 3ab for T cells; Leu 2a for T8 cells; Leu M1 for monocytes and Leu 7 for NK cells.

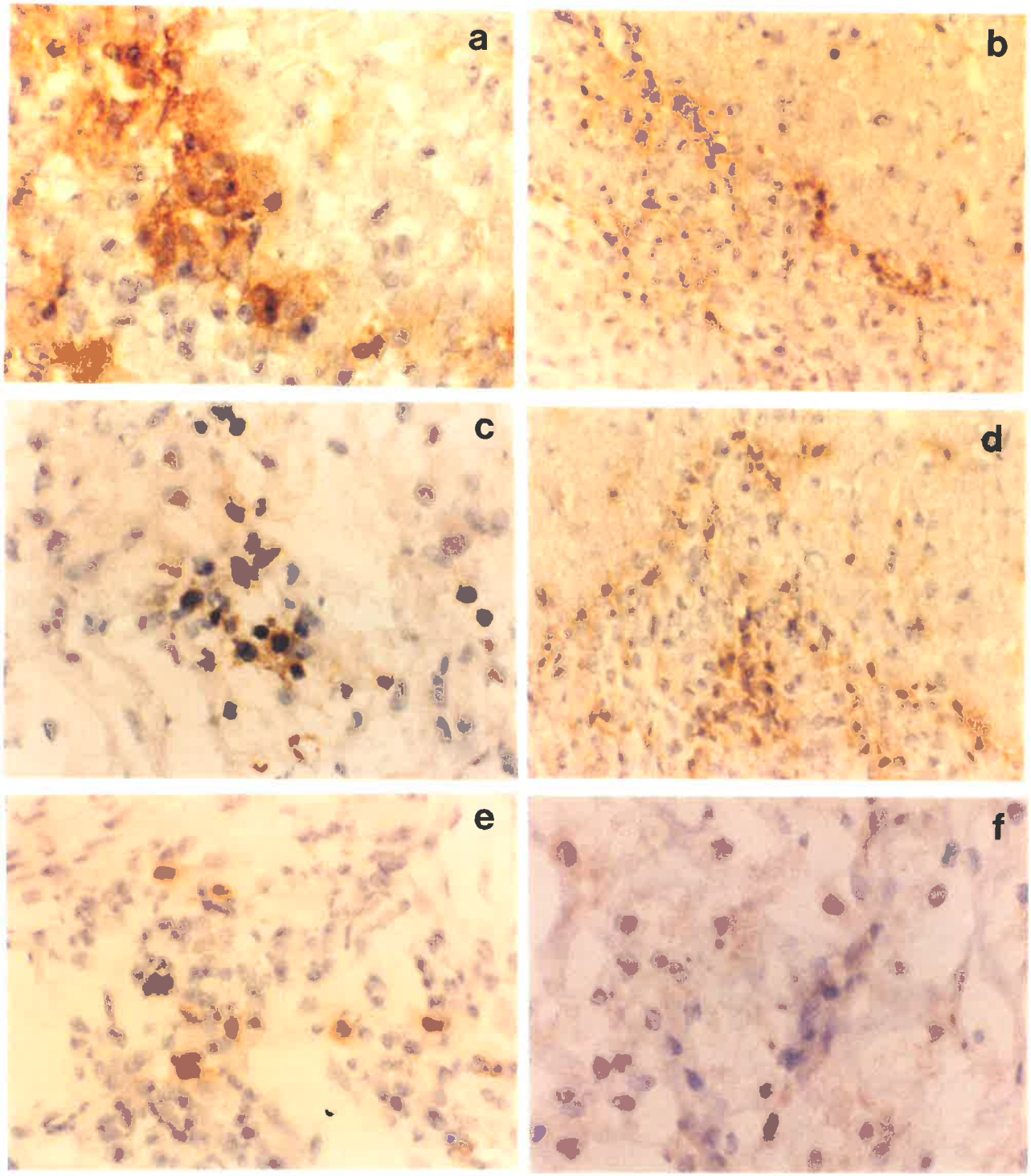


Fig. 3.3 **a.** Macrophages, stained brown, detected by Anti–LeuM5 antibody in the human corpus luteum. (Magnification x 400); **b.** Cells positive for leukocyte common antigen (staining brown) detected by LCA antibody in the human corpus luteum. Note the concentration along the border between granulosa–lutein (GC) and theca–lutein (TC) cells. (Magnification x 250). **c.** T lymphocytes, stained brown, detected by Anti–Leu 4 antibody in the human corpus luteum. (Magnification x 400). **d.** Helper/inducer T lymphocytes (T4 cells; staining brown) detected by Anti–Leu 3ab antibody in human corpus luteum. (Magnification x 250). **e.** Cytotoxic/suppressor T lymphocytes (T8 cells; staining brown) detected by Anti–Leu 2a antibody in the human corpus luteum. (Magnification x 400). **f.** Activated T lymphocytes (Ta cells; staining brown) detected by Anti–IL–2 receptor antibody in the loose connective tissue of the human corpus luteum. (Magnification x 400).

Monocytes detected were present mostly in the lumen of blood vessels of the corpus luteum. Very few NK cells could be observed in the theca– and granulosa–lutea areas.

3.4 Discussion

This is the first study to demonstrate conclusively the presence of a wide range of white cell types in the human corpus luteum. Macrophages, T–lymphocytes and monocytes were all detected in significant numbers with very few NK cells in the tissue. There were no B lymphocytes found at any stage. While the current study does not comprehensively cover the changes in white cells that occur throughout the life span of the corpus luteum it does give an indication of a large white cell population which could potentially produce substances regulating steroidogenesis, proliferation of cells, and other factors related to the life span of the corpus luteum. Lei *et al*, (1991) have recently confirmed that there are significant numbers of white cells, particularly macrophages in both human and bovine corpora lutea.

The human corpus luteum is composed of various regions. The majority of steroid secreting cells (granulosa–luteal cells) are the main substance of the corpus luteum. However, in the centre there is a region of loose connective tissue that increases rapidly as luteolysis proceeds. Around the perimeter of the steroid secreting cells, is the thecal zone, in which there are several cell layers which also appear to secrete steroids. The predominant sites of white cell distribution were in the junction between the theca– and granulosa–luteal area and in the loose connective tissue. However, a significant number of white cells were also found in the main body of steroid secreting granulosa–luteal cells. The predominant type of leukocyte in the human corpus luteum was the macrophage subpopulation. Macrophages are found in many organs in the body and can occupy up to 75% of all cells in organs such as the liver and the spleen (Unanue, 1989). Macrophages have been identified in other

endocrine organs particularly the adrenal gland, pituitary, thyroid, parathyroid gland, pineal gland, and testes (Hume *et al*, 1984). Katabuchi *et al*, (1989) have suggested that in the resting stages of the human ovary there are relatively few macrophages but numbers increase dramatically in the developing follicle and immediately prior to ovulation. Human follicular fluid contains a significant number of macrophages (Loukides *et al*, 1990). The current study indicated that macrophages comprised at least 10% of the total cells in the corpus luteum, with distribution equally between the thecal–luteal and granulosa–luteal area. Significant number of macrophages could also be detected in the central loose connective tissue area of the corpus luteum. Of particular interest was a concentration of macrophages in the boundary area between the granulosa–luteal and thecal–luteal cells, indicating that there may be a production of a chemoattractant by cell types in that area. Herriot *et al* (1986), have suggested that there is a leukocyte chemoattractant in human follicular fluid and this, or a similar substance may be produced by granulosa–luteal cells. Macrophages extend their cytoplasm to encourage cell to cell contact with neighbouring tissues. It is therefore reasonable to suggest that products of macrophages could have a paracrine effect on cells in the human corpus luteum. Substances known to be produced by macrophages include IL–1, TNFα, IL–3 and IL–6 (Unanue, 1989). Some of these substances have been suggested to have effects on ovarian function in other species. The cells of the macrophage system circulate in the blood as monocytes and subsequently migrate into various tissues to become macrophages. In the present study a few monocytes were found mostly localized inside the blood vessels of the human corpus luteum.

The second most common positive staining was with the antibody to leukocyte common antigen which detects cells of normal lymphoreticular origin, including those of bone marrow origin (Forbes and Leong, 1987). Cells staining with LCA antibody were particularly common in the theca luteal area suggesting that in the early corpus luteum the white cells may first emerge in this area.

Several reports have suggested that T lymphocytes can be detected in human follicular fluid, but given the possibility of blood contamination in this fluid at the time of recovery of the oocyte for *in vitro* fertilization there has been uncertainty about the significance of these lymphocytes. The present results are the first definitively to describe T lymphocytes in the human corpus luteum. Using different antibodies, T4 and T8 cells (the subsets of T lymphocytes) were found in ratio of 2:1 in the thecal–luteal area and 1:1 in the granulosa–luteal area. In peripheral blood this ratio is approximately 2:1 (Hill *et al*, 1987b). There were also a significant number of T cells in the spongy connective tissue surrounding the steroid secreting cells. It was in this area that activated T cells were detected by an anti–IL–2 receptor antibody and these cells corresponded with T4 cells in that area. This indicated that there are functional T cells in the human corpus luteum with IL–2 receptors permitting the possible effect of IL–2 on cells of the corpus luteum. Small groups of T cells were frequently observed near the capillaries of the thecal–luteal area, indicating that these cells may migrate from the blood vessels into the substance of the corpus luteum. One of the most important functions of the T4 cells is to help the B cell respond to protein antigen. However, B cells were not found in the human corpus luteum, suggesting that the presence of the T4 cell is probably unrelated to its normal co–operation with B cells. Activated T lymphocytes expressing class II histocompatibility antigen have previously been found in the rabbit corpus luteum (Bagavandoss *et al*, 1990). Although IL–2, one of the T–cell–derived lymphokines, has shown an inhibitory effect on steroidogenesis *in vitro* (Guo *et al*, 1990), the significance of the activated T cells and T cell–derived lymphokines in the corpus luteum remains uncertain. NK cells which have the ability to lyse their targets including a range of tumour cells were also detected in this study. The significance of these cells is also unknown in the human corpus luteum.

In this study, freshly frozen corpora lutea were used as well as paraffin tissue blocks which had been fixed with formalin. It has been known from a variety of other

studies, the antibodies which recognize antigens in frozen tissue are often not effective in formalin fixed tissue. Using a variety of antibodies which detect antigens of white cells in formalin fixed tissues, the presence of white cells was confirmed in this study but their numbers were considerably less than that of freshly fixed tissue. Accordingly all immuno–histological studies of this organ would require tissue which has been snap frozen prior to analysis, which has therefore restricted the accessibility of tissue. This study has not described the range of white cell changes throughout the life–span of the corpus luteum, although this is the subject of an ongoing larger study to describe the time span of infiltration of white cells.

The purpose of leukocytes in the human corpus luteum must remain speculative. Most attention has been focussed on the ability of white cell derived cytokines to influence the steroidogenic potential of the ovary during the life–span of the corpus luteum. There is also considerable evidence that macrophage derived cytokines may influence the steroidogenic potential of the developing corpus luteum as well as many other physiological functions (prostaglandin production, blood flow, protein production, etc.) which occur in the corpus luteum and may also be regulated by white cell function. The presence of white cells in the ovary, led to the decision to investigate whether cytokines, the products of white cells, are present in the human ovary.

CHAPTER FOUR

CYTOKINE DETERMINATION IN HUMAN FOLLICULAR FLUID

4.1 Introduction

As described in chapter 3, immune cells have been identified in the human corpus luteum. The aim of the next study was to see whether cytokines, products of the immune cells, are present in the human ovary. Investigations could be directed to determine of cytokines in whole ovarian tissue or in the ovarian functional units, i.e. the follicle and corpus lutuem. In this chapter, study was performed to examine the presence of cytokines, IL–1, IL–2 and TNFα in human preovulatory follicular fluid, which was more accessible than tissue. The follicular fluid was available from patients on the *in vitro* fertilization–embryo transfer (IVF–ET) programme. Cytokines, IL–1, IL–2 and TNFα have been shown to have an action on ovarian function (see 1.5.3). The determination of ovarian cytokines is therefore important to understand their physiological roles in the ovary by their paracrine or autocrine mechanisms.

4.2 Materials and Methods

4.2.1 Collection of follicular fluid and blood

Blood–free samples of follicular fluid were obtained from preovulatory ovarian follicles of 29 patients participating in the IVF programme at the Reproductive Medicine Unit, The Queen Elizabeth Hospital (excluding patients with polycystic ovarian disease). For measurement of immunoreactive IL–1 and IL–2, a total of 46 fluid samples were divided into two groups. Group I contained 20 specimens from 20 individual patients and group II had 26 fluids from 7 patients (2 to 7 in each patient).

For the measurement of immunoreactive and bioactive TNFα-levels, a total of 32 fluids were selected from 9 patients (2 to 7 from each patient). Contamination by red blood cells in these samples was confirmed microscopically to be $<4x10^5$ cells/ml, which is probably less than 1% of normal circulating values (Tietz and Finley, 1983). All follicles in group I were > 15mm in size and every sample was matched with a plasma sample taken at the same time from the same patient. The collected samples were frozen at -20^oC until assay. The stimulation protocols used was a gonadotrophin releasing hormone agonist (leuprolide acetate) with HMG, as described previously (Kerin *et al*, 1984; Norman *et al*, 1991).

4.2.2 Immunoassays

Immunoassays used in this study included RIA's for IL-1β, IL-2, TNFα, P_4 and E_2. For details of these assays see 2.8.

4.2.3 TNFα Bioassay

TNFα was assayed in a cytotoxicity assay employing the TNFα sensitive cell line L929 as described by Matthews and Neale (1987). Duplicate serial dilutions were incubated with $2x10^4$ L929 cells in 200μl of RPMI-FBS and 4mg/L cycloheximide overnight in flat-bottomed 96-well microtitre trays. Cell lysis was measured by methyl violet uptake (0.5% in 20% methanol for 10 min at room temperature). Incorporated dye was dissolved in 50% acetic acid and quantitated by measuring absorbence at 570nm using a multiwell ELISA reader. The assay was standardized against recombinant cytokine and the minimum detectable amount was 117.6pmol/l (equal to 2μg TNFα/L, 5μg/L gave 50% cytolytic activity).

4.2.4 Specificity of the assays for interleukins

The cross-reactivity of the antisera for the interleukins has been reported previously (see 2.8.1, 2.8.2 and 2.8.3). All samples tested showed parallelism with the standards, suggesting identity between the substance being measured and IL-1 or

IL–2 or TNFα. Several samples of follicular fluid and plasma from the same patients were lyophilized and applied to a Sephacryl S200 column (dimensions 1.3 x 35.4 cm) in 0.05 M ammonium acetate buffer. The column had previously been calibrated with molecular weight markers and the elution volumes of recombinant [^{125}I]IL–1 and [^{125}I]IL–2 in carrier protein determined. Both recombinant [^{125}I]IL–1 and immunoreactive material from follicular fluid and plasma eluted at M_r 23 000 (Fig. 4.1a). A single peak of IL–2 immunoreactivity eluted at M_r 23 000, while the radioactive recombinant material eluted at M_r 16 000 (Fig. 4.1b). The differences between radioactive and immunoreactive IL–2 were attributed to the glycosylation of the natural (follicular fluid) material and the subsequent effect on mobility in a gel column. It was concluded that immunoreactive IL–1 and IL–2 corresponded to authentic material in follicular fluid and blood. Chromatography was not performed for TNFα because of it's scarce supply.

4.2.5 Analysis of Data

The data were normally distributed and expressed as the mean ± SEM. Statistical analysis was performed using Students t'–test and Pearson's linear correlation analysis. Statistical significance was accepted at $P<0.05$ for two tailed analysis.

4.3 Results

4.3.1 IL–1 and IL–2

Group I

This group contained twenty specimens from 20 individual patients. The volume of follicular fluid collected in this study ranged from 1.5 to 13.5ml per follicle (Table 4.1).

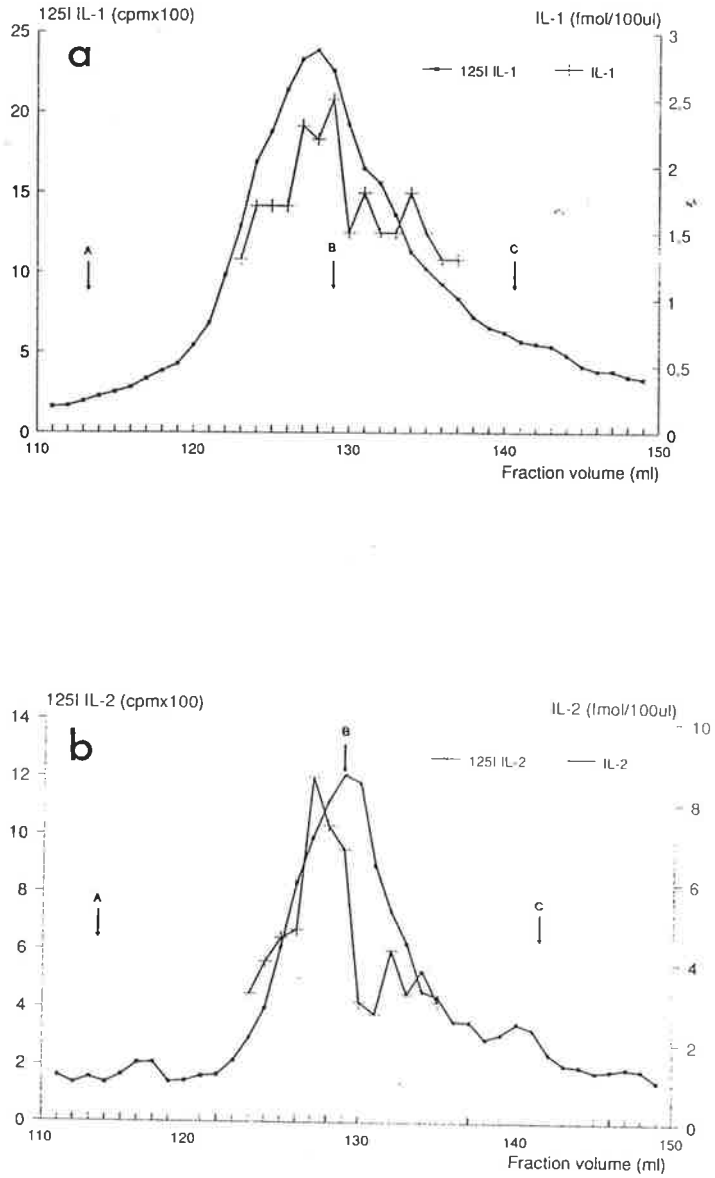


Fig 4.1 Chromatography of recombinant ^{125}I–labelled interleukin–1 (IL–1), ^{125}I–labelled interleukin–2 (IL–2) and immunoreactive IL–1, IL–2 from follicular fluid on a Sephacryl S200 column (2.6 x 35.4 cm). Arrows indicate the elution position of reference molecular weight markers on the same column: **A**, ovalbumin (M_r 43 kDa; K_{av} 0.294); **B**, chymotrypsinogen (M_r 25 kDa; K_{av} 0.437) and **C**, ribonuclease (M_r 13.7 kDa; K_{av} 0.544). **a**. Pattern of recombinant [^{125}I]IL–1 and immunoreactive IL–1 from follicular fluid. **b**. Pattern of recombinant [^{125}I]IL–2 and immunoreactive IL–1 from follicular fluid.

Table 4.1 Concentrations of IL–1, IL–2, P_4 and E_2 in Follicular Fluid and Plasma (Group I)

Case No.	Vol. of Follicle (ml) (A)	IL–1 F.F. (B)	(pmol/l) plasma (C)	IL–2 F.F. (D)	(pmol/l) plasma (E)	P_4 F.F. (F)	(μmol/l) plasma (G)	E_2 F.F. (H)	(μmol/l) plasma (I)
1	1.5	6	20	30	52	40.7	0.021	6.3	0.004
2	1.8	9	18	32	52	74.5	0.03	10.3	0.004
3	3.0	15	18	25	40	89.8	0.04	6.0	0.070
4	3.2	9	18	43	56	40.9	0.02	9.0	0.011
5	3.9	3	13	23	50	51.8	0.02	9.5	0.005
6	4.0	8	18	25	43	87.6	0.003	10.3	0.011
7	4.5	9	18	30	58	67.7	0.013	4.3	0.004
8	4.5	11	16	30	50	91.5	0.022	9.3	0.015
9	5.0	9	17	38	62	3.6	0.021	0.8	0.008
10	5.6	6	16	36	65	25.9	0.028	0.8	0.008
11	6.5	9	19	32	62	0.36	0.016	8.8	0.009
12	7.2	11	20	38	68	63.5	0.01	8.3	0.006
13	7.5	9	20	30	62	66.6	0.01	10.0	0.003
14	9.0	9	20	36	58	78.6	0.02	7.5	0.070
15	8.0	12	25	50	88	75.7	0.02	4.0	0.011
16	8.7	11	20	36	68	77.3	0.04	8.3	0.006
17	9.0	6	17	38	86	44.8	0.01	11.5	0.002
18	9.7	17	22	43	65	65.6	0.02	5.3	0.005
19	9.7	9	17	52	68	55.3	0.05	9.5	0.013
20	13.5	8	18	50	62	73.7	0.01	2.5	0.004
Mean	**6.3**	**9**	**19**	**35**	**61**	**58.7**	**0.02**	**6.7**	**0.007**
SEM	0.68	0.1	0.4	2	3	5.8	0.002	0.8	0.0007

F.F. Follicular Fluid

Significant differences ($P<0.001$) were found between B and C, D and E, F and G, H and I.

Significant positive correlations ($P<0.001$) were found between A and D ($r=0.66$), A and E ($r=0.59$), B and C ($r=0.56$), D and E ($r=0.65$).

IL–1 concentrations in follicular fluid ranged from 3–17pmol/l (mean 9pmol/l) in follicular fluid and from 13–25pmol/l (mean 19pmol/l) in plasma (Table 4.1). IL–1 concentrations were significantly higher in plasma compared to follicular fluid ($P<0.001$). There was a positive correlation between the follicular IL–1 and plasma IL–1 concentrations ($r=0.56$, $p=0.01$). The mean IL–2 concentration was 35pmol/l (2.3–52pmol/l) in follicular fluids, and 61pmol/l (40–88pmol/l) in plasma (Table 4.1). There was a significant difference between follicular fluid and plasma IL–2 ($P<0.001$) and a positive correlation between follicular and plasma concentrations ($r=0.65$, $P<0.01$).

A positive correlation was found between the volume of follicular fluid and IL–2 levels in follicular fluid ($r=0.66$) or plasma ($r=0.59$) ($P<0.001$). No similar significant correlation was found between the volume of follicular fluid and IL–1 levels in fluid or plasma.

In contrast to IL–1 and IL–2, follicular fluid P_4 and E_2 concentrations were much higher than those in plasma (3000 fold difference in P_4 and 1000 fold difference in E_2, $P<0.001$, Table 4.1). Follicular fluid P_4 values ranged from 0.36 to 91.5μmol/l with the mean value of 58.7μmol/l and plasma P values ranged from 0.003 to 0.05μmol/l with the mean value of 0.02μmol/l. Follicular E_2 levels were from 0.8 to 11.5μmol/l with the mean of 6.7μmol/l and plasma levels were from 0.002 to 0.070μmol/l with the mean of 0.007μmol/l (Table 4.1). There was no significant relationships between IL–1, IL–2 and the follicular fluid steroids. To further investigate whether follicular fluid volume related to cytokine concentrations, IL–1, IL–2 and the steroids were measured in 7 patients when a size range of follicles were aspirated.

Group II

This group had 26 fluids from 7 patients (2 to 7 in each patient). The follicular volume, IL–1, IL–2, P_4 and E_2 concentrations are shown in Table 4.2. There was no significant correlation between cytokines and steroid levels, or between follicular volume and cytokines or steroid concentrations.

4.3.2 TNFα

In a total of 32 samples of follicular fluid from 9 patients the mean concentration of TNFα in follicular fluid was from 12–41pmol/l with the mean of 21.2 ± 1.2pmol/l (Fig 4.2). Bioactive TNFα was not detected in any sample of FF previously stored at –70°C. The experiment was repeated using fresh FF; 5/5 samples showed bioactivity close to the lowest standard equivalent to which was 117.6pmol/l. The mean follicular fluid volume was 6.8ml (range 2–11.4) and the mean concentrations of P_4 and E_2 in these fluids were 13.2±1.1μmol/l (from 1.9 to 23.8μmol/l) and 2.3±0.3μmol/l (from 0.1 to 4.9μmol/l) respectively (Fig. 4.2). No statistically significant correlations were found between concentrations of TNFα and follicular volume (r=0.10, p=0.59), P_4 (r=0.11, p=0.56) or E_2 (r=0.1 p=0.59) concentrations.

4.4 Discussion

The presence of immunoreactive IL–1, IL–2 and TNFα in human follicular fluid has been confirmed in this study; concentrations of IL–1 and IL–2 in follicular fluid were approximately one–half of those in plasma. The possibility that the IL–1, IL–2 and TNFα are derived from direct blood contamination during follicle aspiration can be excluded because the red blood cell counts in these fluids were absent or very low. Evidence is presented that immunoreactive cytokines corresponded with authentic natural cytokines based on antibody specificity, parallelism with authentic standards and chromatographic mobility (except TNFα).

Table 4.2 Concentrations of IL–1, IL–2, P_4 and E_2 in Follicular Fluid (Group II)

Case No.	Vol. of Follicle (ml)	IL–1 (pmol/l)	IL–2 (pmol/l)	P_4 (μmol/l)	E_2 (μmol/l)
1	2	8.8	34	5.7	2.6
	3	10.0	61	7.0	2.1
	4	9.6	31	8.2	1.9
	6	7.6	31	13.2	2.8
	6	8.8	28	10.6	3.2
	7	7.6	58	12.5	1.9
	9	9.4	26	10.8	2.6
2	2	8.0	31	11.9	1.3
	3	7.8	34	14.1	0.9
	3	2.5	62	22.4	0.5
	4	6.6	34	22.1	1.0
	5	7.8	51	20.2	1.1
	7	9.4	34	11.9	0.9
3	2	8.0	68	16.3	2.3
	6	7.6	62	7.7	3.2
	8	8.0	68	14.8	2.3
	10	6.4	31	4.2	1.5
4	5	8.4	75	9.9	2.2
	6	7.8	52	8.2	3.4
	11	9.6	64	8.3	2.8
5	3	7.8	22	1.9	1.3
	8	5.6	34	5.8	0.1
6	3	8.0	38	13.8	0.6
	11	8.0	38	14.2	1.1
7	8	10.0	34	23.8	4.9
	11	9.4	22	13.2	4.9

There was no positive correlation found among these results.

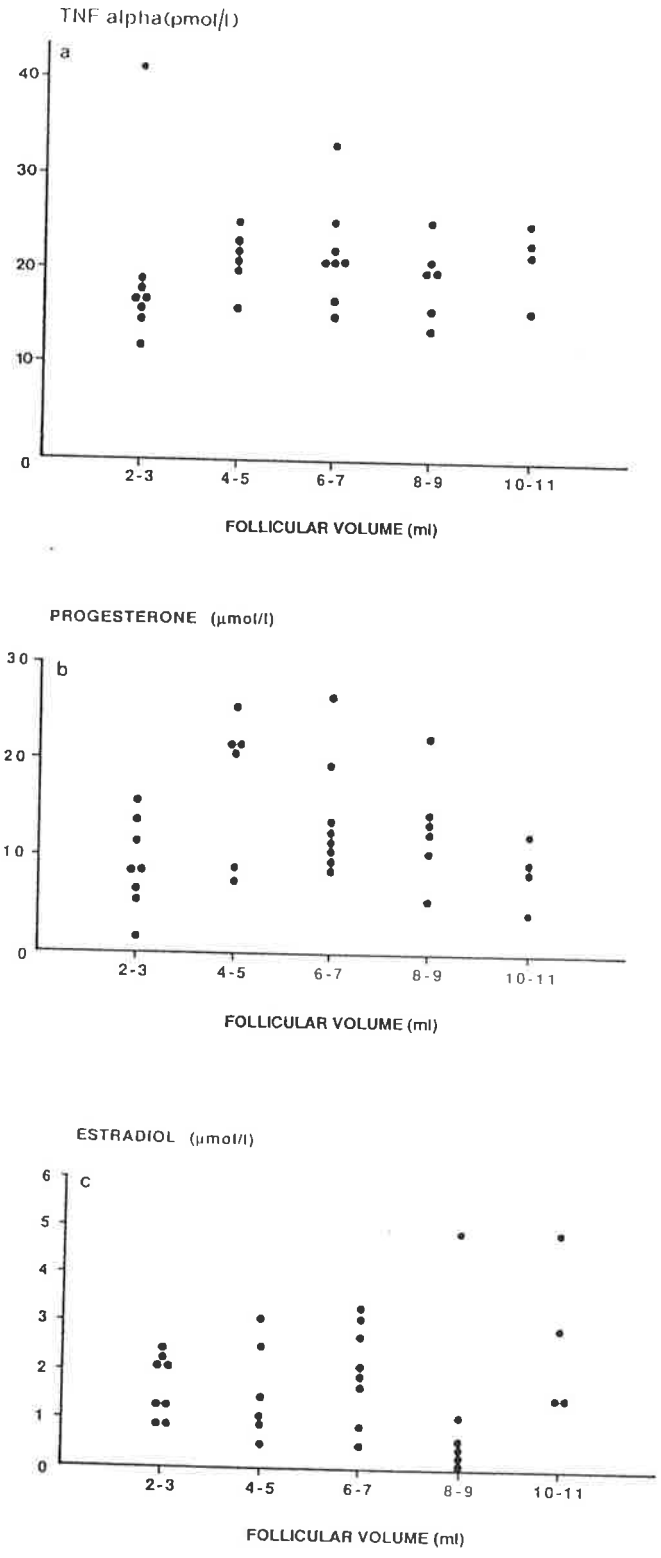


Fig. 4.2 Immunoreactive concentrations of TNFα (a), P_4 (b) and E_2 (c) in individual FF in relation to follicular volume.

The origin of the follicular fluid cytokines remains undetermined from this study. Macrophages, the IL-1 and TNFα producing cells, have been identified in human follicular fluid (Loukides *et al*, 1990) and the corpus luteum (Lei *et al*, 1991; chapter 3), while T lymphocytes, including activated T lymphocytes which secrete IL-2, have been recognized as one of the cellular components of human follicular fluid and corpus luteum (Hill *et al*, 1987b; Droesch *et al*, 1988; and chapter 3). A recent study has confirmed that ovarian theca-interstitial cell is a source of IL-1 in the rat as IL-1β gene expression has been detected from these cells (Hurwitz *et al*, 1991). If these cytokines were produced by the developing follicle, it might be expected that increasing concentrations would be found in the larger follicles. In group I, the 20 samples of fluid came from different sizes of follicles from different patients. A significant positive correlation existed between the volume of follicular fluid and IL-2 levels, indicating that a possible mutual relationship may exist between the development and maturity of the follicle and IL-2 concentrations (i.e. an increasing size of follicle may lead to increasing IL-2 concentration, or IL-2 may be one of the factors related to promoting follicle growth). However, this finding was not confirmed in group II, nor for IL-1 and TNFα. This does not disprove an ovarian origin for IL-1, IL-2 and TNFα and their possible physiological role as there was no indication of a progressive increase in oestradiol as the volume of follicular fluid increased, nor was there a positive correlation between the volume of follicle and oestradiol and progesterone levels in follicular fluid. This is consistent with previous observations that the largest follicle is not necessarily the most steroidogenic (Bomsel-Helmreich *et al*, 1979; Carson *et al*, 1982). In addition, if the cytokines are produced predominantly in the stroma or thecal area, follicular fluid may not adequately reflect the production of these products. The low bioactivity of TNFα may relate to the limited working range of the TNFα bioassay because mean level of immunoreactive TNFα were 20.0pmol/l but the lowest standards of the TNFα bioassay was close to 117.6pmol/l therefore limiting the value of this assay to detect bioactive TNFα in the follicular fluid.

There was also no obvious relationship between the cytokines and both oestradiol and progesterone in follicular fluid or plasma, as assessed by simple correlation statistics. This questions any role that IL–1, IL–2 and TNFα may have in the regulation of steroidogenesis in the stimulated preovulatory follicle. Other growth factors, such as epidermal growth factor and insulin–like growth factor–I, have also been found in human follicular fluid but also do not show a correlation with steroid concentrations (Westergaard and Andersen 1989; Rabinovici *et al*, 1990). However IL–1, IL–2 and TNFα in cultured rat or porcine granulosa cells, at the lowest concentrations (IL–1 2.9pmol/l equal to 0.05μg/l, Fukuoka *et al*, 1989; Yasuda *et al*, 1990; IL–2, 1.86nmol/l equal to 28μg/l, Kasson and Gorospe 1989; TNFα 5.9pmol/l equal to 0.1μg/l, Adashi *et al*, 1989b) show an effect on steroid producing function including LH/hCG receptor formation, cAMP accumulation and production of oestrogens and progestins. These effective concentrations are, at least, very close to the detectable levels of IL–1 and TNFα in the current study. Therefore, the *in–vitro* effects of IL–1, IL–2 and TNFα on steroidogenesis (see 1.5.3) and the presence of these cytokines in human preovulatory follicular fluid suggests the possibility that those cytokines may play regulatory roles in ovarian physiology *in vivo*.

In summary, the current study has shown that IL–1, IL–2 and TNFα are found in follicular fluid in the concentrations range known to have effects upon ovarian steroidogenesis *in vitro*. Their role in ovarian physiology requires investigation.

CHAPTER FIVE

INTERLEUKIN–2 EFFECTS ON GONADOTROPHIN–STIMULATED PROGESTERONE PRODUCTION AND CELL PROLIFERATION BY HUMAN GRANULOSA–LUTEIN CELLS

5.1 Introduction

The reported studies in chapter 3 and 4 showed the presence of immune cells and cytokines in the human ovary. It becomes important therefore to investigate whether cytokines have any function in the ovary. Previous studies on IL–1 and IL–2 on gonadotrophin dependant ovarian steroidogenesis were in animal cell culture systems. In these species IL–1 has both inhibitory or stimulatory effects on steroidogenesis at different stages of granulosa cell differentiation (see 1.6.3.1) while IL–2 significantly enhances FSH–stimulated production of progesterone (P4) or the 20–hydroxy–progesterone, but it does not affect FSH–stimulated oestrogen production or LH/hCG receptor induction in rat granulosa cell culture (Kasson and Gorospe, 1989). However, Gottschall et al (1988b) and Fukuoka et al (1988) described no effect of IL–2 on FSH–stimulated and LH–stimulated P4 production or LH receptor development from both rat and porcine granulosa cells. The reason for studying these compounds is the presence of significant quantities of macrophages and T lymphocytes in the corpus luteum. In addition, the effects of lymphocyte conditioned medium was examined as this contains a range of cytokines rather than a single factor and may be more "physiological" than using recombinant hormones.

It is not known if human granulosa cells are similarly influenced by cytokines. The purpose of the present study is therefore to examine the effect of human IL–1 and IL–2 on human granulosa–luteal cell differentiation with or without hCG stimulation. These results *in vitro* could contribute to the understanding of both normal regulation

of ovarian steroidogenesis and provide insight into the possible causes of abnormal corpus luteum function during pregnancy and in luteal phase defects.

5.2 Materials and Methods

5.2.1 Granulosa–Lutein Cell Collection and Culture Method

The details of collection and culture procedure of granulosa–lutein cells have been described in 2.6.2 – 2.6.3.

In order to observe the effect of cytokines on granulosa–lutein cells in the present study, 2.5–5 X 10^5 cells were seeded in each well with 1ml MEM–FBS or M199–BSA (Appendix IV). Media was changed every 48 hours. On the morning of the fourth day of culture, 0.1ml of hCG, IL–1, IL–2, conditioned media with MLA–144, or forskolin were added to four to six wells per treatment. At 24 or 48h hours later, the culture media was collected from each well and stored at –20°C until assay for P_4 and cAMP.

5.2.2 [^{3}H] Thymidine Incorporation into Granulosa–lutein cells

In some experiments, cell proliferation effected by cytokines was observed by the assay of ^{3}H–thymidine incorporation (see 2.5). In brief, at the end of the co–culture period with hCG and IL–2, or at various other days of cultures, 0.5uCi of ^{3}H–thymidine was added to each well. Cells were harvested on an automatic Cambridge Cell Harvester after 6 hours incubation and ^{3}H–thymidine incorporation was determined by liquid scintillation counting.

5.2.3 The Preparation of Conditioned Media from MLA–144 Cell Line and Human Peripheral Blood Leukocytes

Two forms of conditioned media (CM) called MLA–CM_1 and MLA–CM_2 were prepared from MLA 144 cell line (gibbon T–cell line, which produces high

concentration of IL-2 *in vivo*). Briefly, MLA-CM_1 was obtained from 5 X 10^6 MLA-144 cells incubated with RPMI-FBS (see 2.7 and Appendix VI). CM_2 was similar to MLA-CM_1 but the cells were mitogen stimulated using 4μg/ml of purified phytohemagglutinin (PHA) and 10ng/ml of phorbol myristic acetate (PMA). A further medium (HPL-CM) was obtained from mitogen stimulated human peripheral blood leukocyte culture which contains most leukocyte subpopulation. Control conditioned media (control CM) were made up by addition of a range of concentrations of PHA (up to 40μg/ml) and PMA (up to 100ng/ml). All mitogen incubated media (MLA-CM_2) were extensively dialysed before use (see 2.7 for details).

Immunoactive IL-2 concentrations as determined by specific human IL-2 RIA (see 2.8.2) were 986pmol/l in MLA-CM_1, 366pmol/l in MLA-CM_2 and 653pmol/l in HPL-CM. (The immunoactivity of gibbon IL2 in the RIA was not determined and the significance of these results uncertain). Background concentrations of IL-2 in MEM-FBS and in media from control granulosa-lutein cell culture were less than 25pmol/l which is the lowest point of the IL-2 standard curve.

5.2.4 RIA

RIAs for IL-2, cAMP and P_4 have been described in 2.8.2, 2.8.4 and 2.8.5 respectively.

5.2.5 Data Analysis

Each experiment was repeated 2 to 7 times, using different patient samples. Progesterone concentrations (nmol/per $5x10^5$ cells) were expressed as the mean and SEM. Student's t and Wilcoxon test were used, depending on parametric or non parametric distribution of data. Significance was assigned at 5% levels for two-tailed statistics.

5.3 Results

5.3.1 The effect of CM with or without hCG on progesterone production from granulosa-lutein cells

Conditioned media (0.1ml) added to 5 X 10^5 granulosa-lutein cells in 1ml culture media significantly increased P_4 production in MLA-CM_1 cultures compared to controls ($P<0.001$, Fig. 5.1). Cells cultured in MLA-CM_2 demonstrated reduced P_4 production compared to MLA-CM_1 ($P<0.001$) but not compared to the control wells (Fig. 5.1). HPL-CM significantly inhibited P_4 production compared to control wells and MLA-CM_2 (Fig. 5.1, $P<0.05$). Progesterone production stimulated by 5iu hCG was significantly inhibited by MLA-CM_2 and HPL-CM ($P<0.001$) but was not influenced by MLA-CM_1 (Fig. 5.2). Control CM did not show any effect on P_4 production.

5.3.2 The effect of IL-2 with or without hCG or FK on progesterone production

The possibility that the regulation of P_4 secretion in the cytokine conditioned media was attributable to IL-2 was investigated using recombinant material. Preliminary experiments indicated granulosa cells were most sensitive to stimulation of hCG after day 4 of culture (see 2.6.4). Although addition of IL-2 (2.6 to 2600pmol/l) for 24 hours at this time did not show any change in P_4 levels compared to control values, all concentrations of IL-2 significantly inhibited P_4 secretion from those cells stimulated by 5iu of hCG (7 cases, $P<0.01$) (Fig. 5.3). A dose-dependent inhibitory relationship was not obvious in those experiments. To show that this effect was not due to constituents of the FBS, the experiment was repeated with serum-free medium (M199-BSA Fig. 5.4) where a similar finding was shown although values were lower.

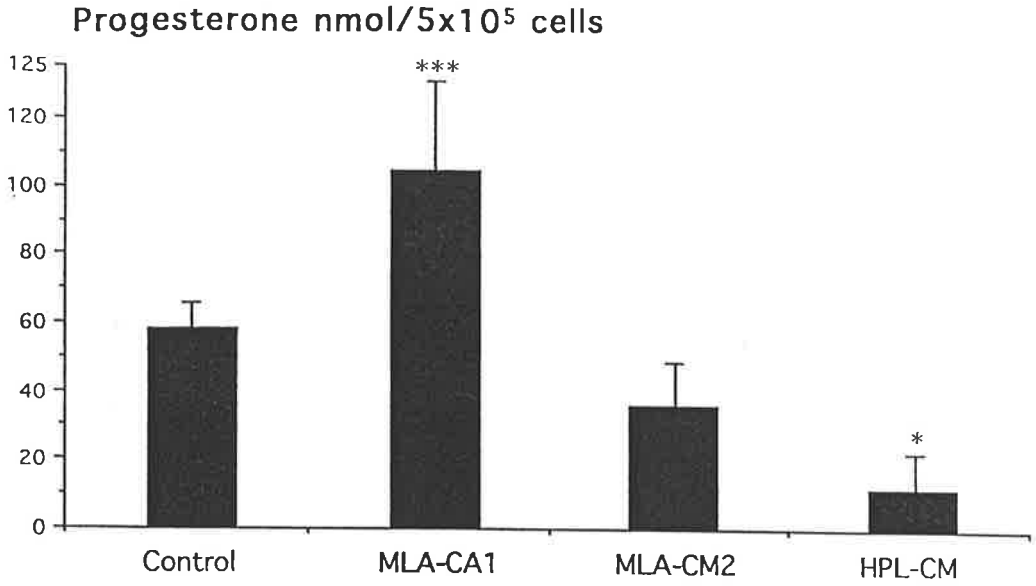


Fig. 5.1 The effects of CM* (0.1ml) on progesterone production (mean ± SEM; 3 cases). The comparisons between CMs and control showed that significant differences were present between MLA–CM_1 and control (***P<0.001) and between HPL–CM and control (*P<0.05). Cells were co–cultured with CMs for 24h from the fourth day of culture.

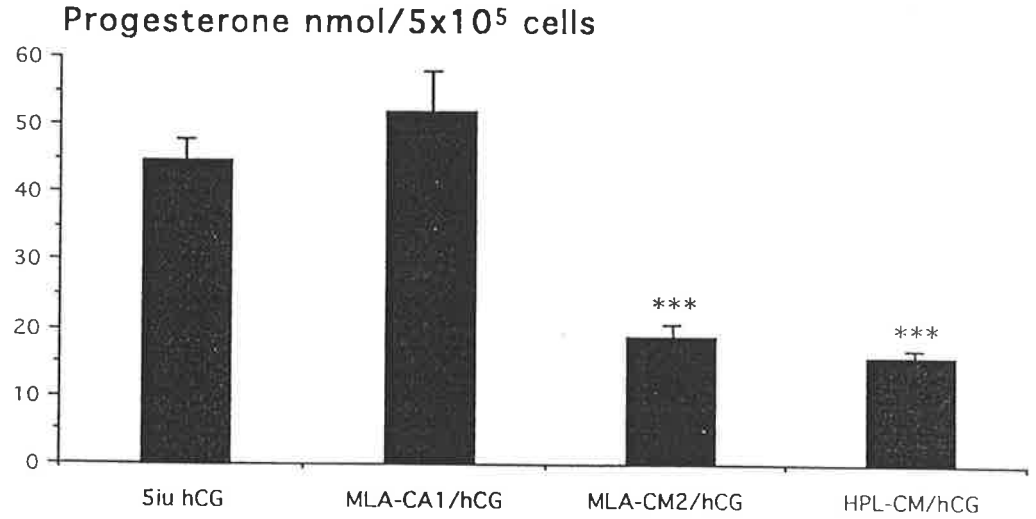


Fig. 5.2 The effects of CMs on progesterone production (mean ± SEM) stimulated by hCG (5iu; 3 cases). Significant differences were found between hCG and MLA–CM_2/hCG wells (***P<0.001) and between hCG and HPL–CM/hCG (***P<0.001). Cells were co–cultured with hCG or CM with hCG for 24h from the fourth day of culture. This was a different set of experiments from that shown in Fig. 5.1; hence, the different values of progesterone.

*
MLA–CM1:	conditioned media obtained from cultured MLA–144 cell line.
MLA–CM2:	conditioned media obtained from cultured MLA–144 cell line stimulated by mitogens (PHA and PMA)
HPL–CM	conditioned media obtained from cultured human peripheral blood leukocytes stimulated by mitogens (PHA and PMA)

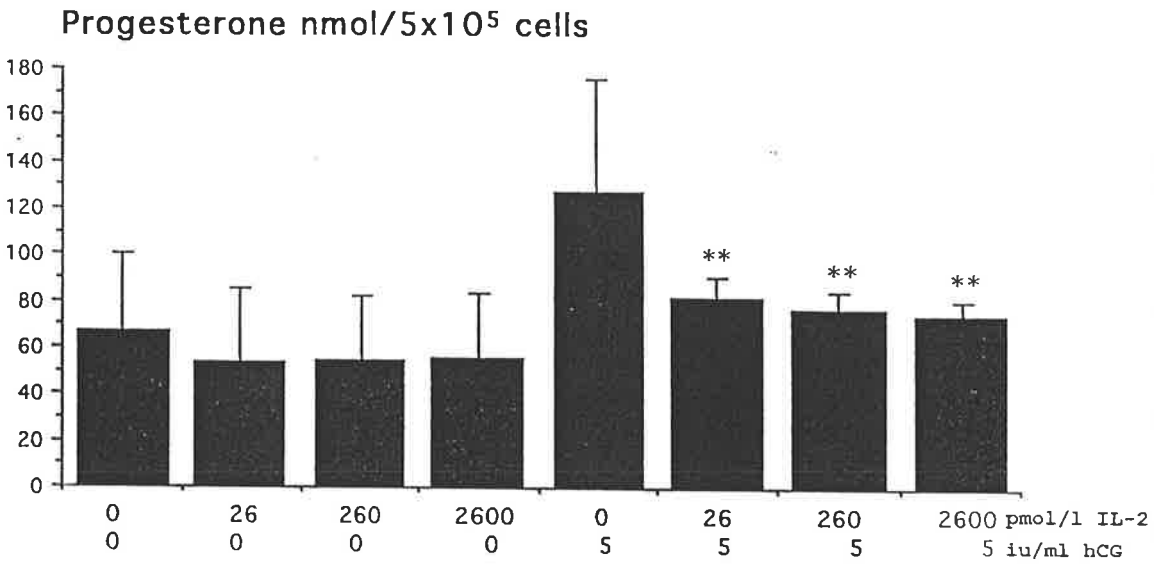


Fig. 5.3 The effect of human recombinant IL–2 on progesterone production (mean ± SEM; 7 cases) with serum containing media. Significant differences were found between hCG alone and 26–2600pmol/l IL–2 plus 5iu/ml hCG (**P<0.01).

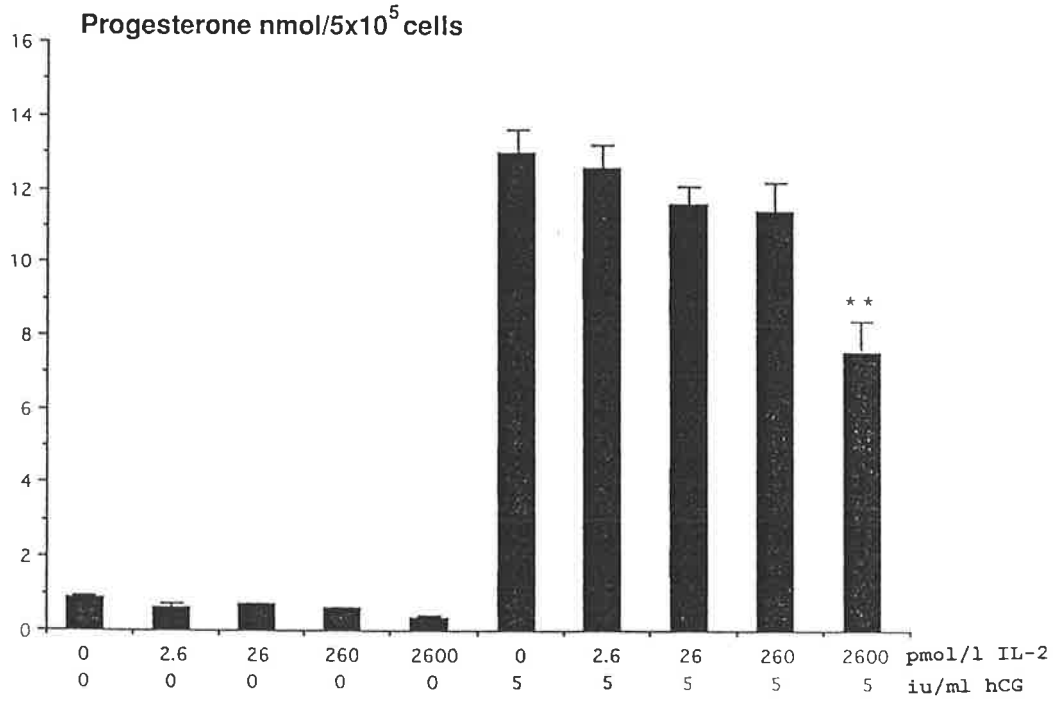


Fig. 5.4 The effect of human recombinant IL–2 on progesterone production (mean ±SEM; 1 case) with serum–free medium. A significant difference was found between 5iu/ml hCG and 2600pmol/l IL–2 IL with 5iu/ml hCG (** P<0.01).

The mechanism of IL–2 effect on hCG–stimulated P4 production was further investigated by addition of the adenylate cyclase stimulator, forskolin (FK) which should stimulate accumulation of the hormone second messenger, cAMP. Concentrations of cAMP in medium from cultured cells were significantly increased by FK (100μmol/l) ($P<0.05$, Fig. 5.5). Forskolin increased P4 production but the enhanced P4 level stimulated by FK was also significantly inhibited by 260pmol/l IL–2 ($P=0.01$) (Fig. 5.6). This effect however was not mediated through decreased cAMP since the FK enhanced cAMP level ($p<0.05$, compared to control) was not influenced by IL–2 ($p>0.05$, Fig 5.5).

5.3.3 IL–2 effects with or without hCG on granulosa–lutein cell proliferation

To determine whether altered P4 secretion resulted from an effect of IL–2 on cell proliferation, this parameter was assessed using ^{3}H–thymidine incorporation. In these experiments, granulosa–lutein cells were cultured for eight days and, hCG alone, IL–2 alone, and IL–2 with 5iu hCG were added to cultures for 24 hours on days 2, 4, 6 or 8. At the end of co–culture, ^{3}H–thymidine incorporation was performed as described previously (see 2.5). Cells stimulated by hCG alone did not show any increased proliferation on any day of culture compared to the results of control samples. Interleukin 2 alone, at 26pmol/l increased cell proliferation on days 2, 4 and 8 of culture compared to the control ($P<0.001$, Table 5.1) and with 5iu of hCG also showed significantly stimulatory effects on cell proliferation on days 2, 4 and 8 compared to 5iu hCG alone ($P<0.001$, Table 5.1).

5.3.4 The effect of IL–1 with or without hCG on P4 production

The possibility that the inhibitory activity observed in HPL–CM was attributable to IL–1 (which would be present in HPL–CM, but probably not in MLA

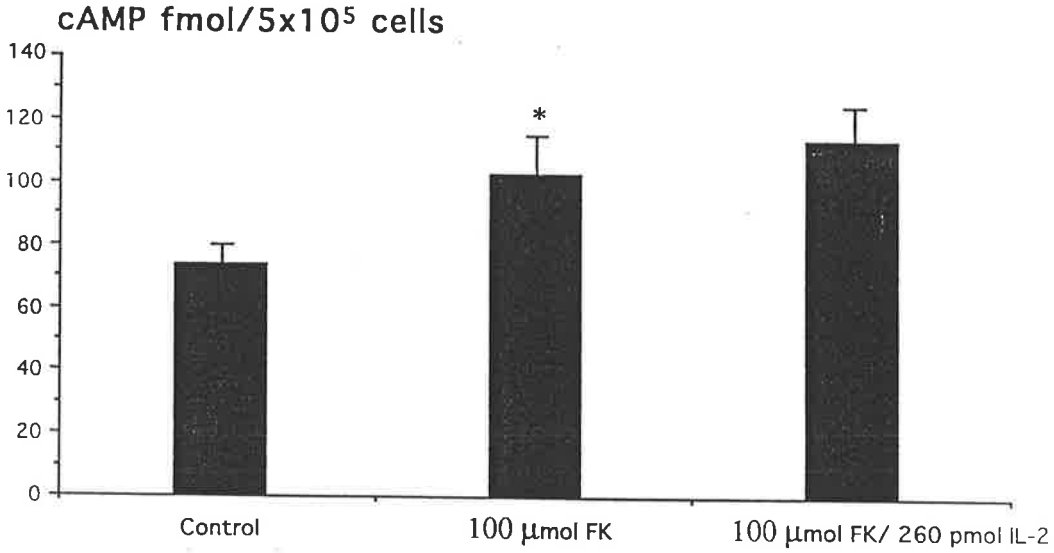


Fig. 5.5 cAMP concentrations (mean ± SEM) in granulosa–lutein cell culture (3 cases). FK significantly increased cAMP secretion (*P<0.05). Recombinant human IL–2 (260pmol/l) did not influence the stimulatory effect of FK (100μmol/l) (P>0.05).

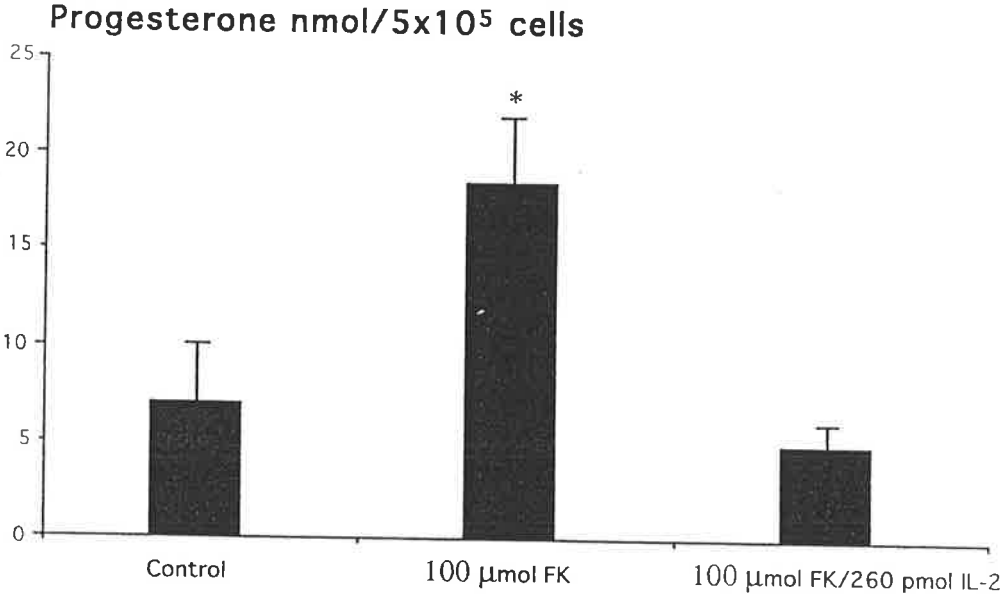


Fig. 5.6 Recombinant human IL–2 inhibits the stimulatory effect of FK on progesterone production (mean ± SEM; 3 cases). Significant differences were found between control and 100μmol/l FK–treated wells (*P<0.05) and between 100μmol/l FK–treated wells and 260pmol/l IL–2 with 100μmol/l FK–treated wells (P<0.01).

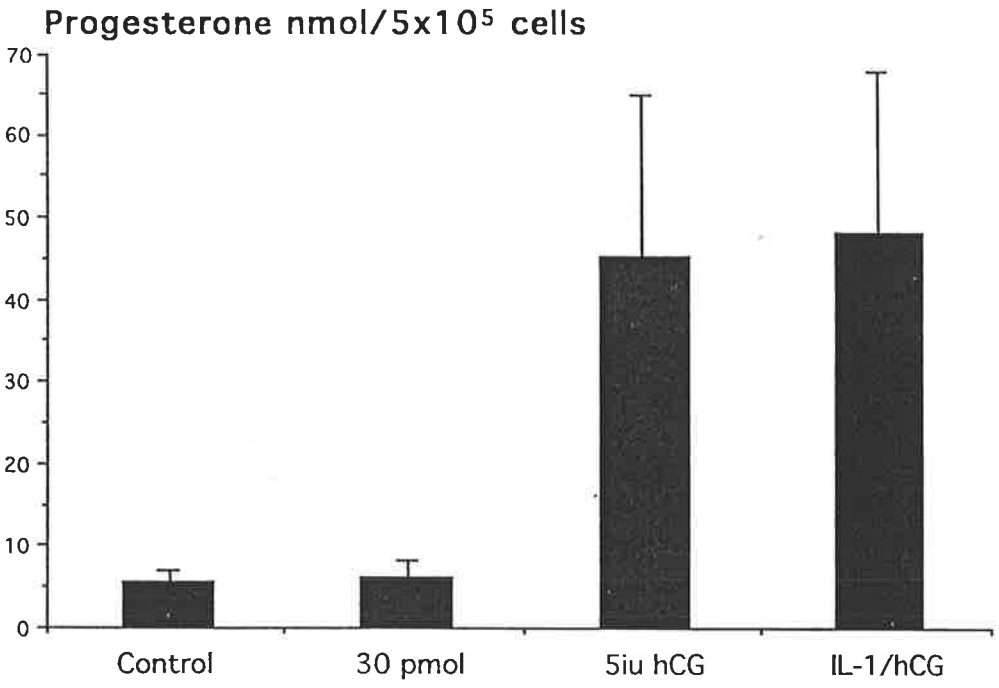


Fig. 5.7 The effect of recombinant human IL–1, with or without hCG, on progesterone production (mean ± SEM, 3 cases) for 48h of culture in serum containing medium. There was no significant difference between control and 30pmol/l IL–1 treated wells or between 5iu hCG–treated wells and 5iu/ml iuhCG/30pmol/l IL–1 treated wells

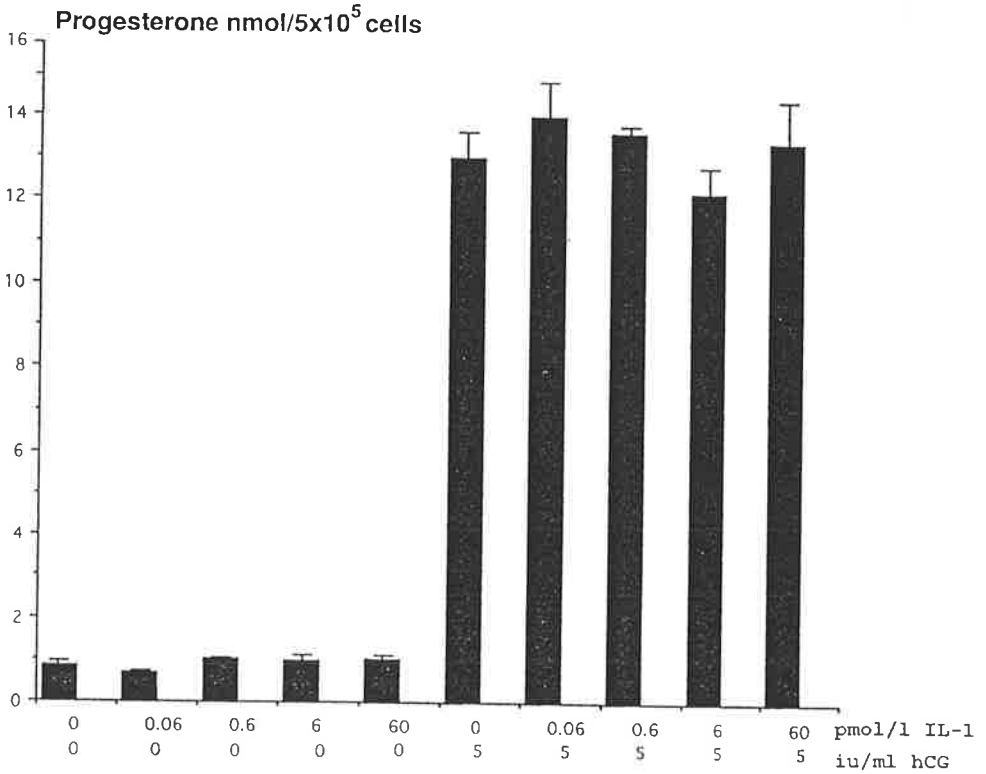


Fig. 5.8 The incubation human recombinant IL–1 with human granulosa–lutein cell culture with serum–free medium. IL–1 did not show any significant effect on progesterone secretion (mean ± SEM, 1 case).

−CM_1 or MLA−CM_2 since T cells are not a source of IL−1) was investigated using recombinant IL−1. However, IL−1 concentrations (from 0.06 to 60pmol/l) with or without hCG (5iu/ml) cultured with cells for 24 hours did not show any significant effect on P_4 secretion. Further experiments were performed with 3pmol/l IL−1 and 5iu/ml hCG incubation with cells for 48 hours. There was no significant effect of IL−1 on progesterone production in either serum containing medium (Fig. 5.7) and serum free medium (Fig. 5.8).

5.4 Discussion

The objective of this study was to examine the putative contribution of cytokines secreted by macrophages and T cells which have been shown to be present in human ovarian tissue and follicular fluid. Preliminary studies of cytokine action on granulosa cell function in other species has indicated that they may play an important role in ovarian steroidogenesis along with other paracrine and autocrine regulators in the ovary (see 1.3.4.3e and 1.5.3).

The experiments described utilised cells obtained from follicular fluid in women undergoing IVF. The luteinized cells were grown in culture with or without added hCG and the effects of CM and recombinant cytokines on P_4 production were studied. Fisch *et al*, (1989) have shown that the results of this type of cell culture correlates well with events occurring *in vivo* in the corpus luteum. In the present study, [^{3}H] thymidine incorporation into cells and progesterone secretion into media indicated ongoing cell proliferation and normal steroidogenic function. In the previous study, addition of hCG to the cultures on different days (see 2.6.4.4) reflected the capacity of cells to respond to hCG stimulation over the time period studied and was in accord with previous reports (Polan *et al*, 1984; Fisch *et al*, 1989).

Table 5.1 [3H] Thymidine incorporation (counts per min) in granulosa–luteal cells

	DAY			
	2	4	6	8
Control	2119 (174)	3259 (166)	9094 (1091)	14,429 (1589)
5 iu/ml hCG	1717 (120)	3541 (323)	8806 (1399)	16,540 (745)
26pmol/l IL–2	3842 (285)	5141 (212)	8715 (796)	16,608 (1610)
26pmol/l IL–2 with 5 iu/ml hCG	2493 (169)	5283 (234)	6734 (623)	21,229 (1729)

Values are the mean (SEM). The experiments were carried out for 8 days (two cases). Cells were cultured with hCG, IL–2, or hCG plus IL–2 for 24 h on days 2, 4, 6, and 8. At the end of the culture period [^{3}H] thymidine incorporation was performed. Significant differences were found between controls and 26pmol/l IL–2 on days 2, 4, and 8 ($P<0.001$), between 5 iu/ml hCG and 26pmo/l IL–2 with hCG on days 2, 4, and 8 ($P<0.001$).

Conditioned media showed different effects upon P_4 production. Initial experiments using media conditioned by MLA–CM (obtained from the cultures of gibbon T cells) and mitogen–activated human peripheral blood monocytes demonstrated that leukocyte products are able to modulate P_4 production by granulosa–luteal cells. In the case of MLA–CM_1, at least, its products are non–dialysable and hence likely to be proteinaceous in nature. All three CM used contained high concentrations of immunoactive IL–2, but also should have a variety of other cytokines (see 1.2.2.2). Different effects on P_4 synthesis were observed with the different CM's. Stimulation with MLA–CM_1 as compared with inhibition caused by MLA–CM_2 and HPL–CM may reflect an interaction of synergic cytokine activities. Other non–dialysable products of the activated leukocytes may be responsible for the inhibitory effects observed in the experiments described here. Toxicity from residual mitogens was ruled out as a possible cause since control CM previously treated with PMA or PHA at a variety of doses had no effect on steroidogenesis and the media was extensively dialysed before use. It therefore became important to examine the effects of individual cytokines separately using recombinant products.

Recombinant IL–2 by itself in concentrations similar to those in CM did not influence P_4 secretion by cells in the absence of hCG. However, IL–2 had a significant influence on gonadotrophin induced P_4 secretion even at small doses which was closed to the physical concentration in the human preovulatory follicular fluid and, may account for at least some of the inhibitory activity observed in the experiments with MLA–CM_2 and HPL–CM. This effect was studied further with FK, a stimulator of adenylate cyclase and, hence, cAMP. Interleukin 2 also had a marked inhibitory effect on FK–induced P_4 release but did not influence the increased cAMP level stimulated by FK. This suggested that the influence of IL–2 on P_4 synthesis may be downstream in the signal transduction pathway from cAMP activation. The inhibitory effect of IL–2 on hCG–stimulated P_4 production was not an indirect result of cytotoxicity, since [^{3}H] thymidine incorporation demonstrated that, on the contrary,

IL–2 has a mitogenic effect on cells. A dose–dependent relationship between IL–2 and inhibition was not observed over the concentration range studied (26–2600pmol/l), but may have become apparent at lower and higher IL–2 concentrations. Clearly many other cytokines could interact with IL–1 and IL–2, and are of the main products of macrophages, TNFα, is another important candidate requiring investigation. The effects observed with conditioned media are different in character from those obtained with recombinant IL–2, suggesting that other leukocyte products may moderate or over ride the influence of IL–2; these need to be investigated. These results are evidence that the actions of human recombinant IL–2 are not limited to target cells of the immune system and indicate a potential role in the regulation of ovarian cell proliferation and function.

IL–1 apparently did not influence P4 production under the conditions studied whether added with hCG or not. Fukuoka *et al*, (1988, 1989) previously reported that IL–1 inhibited P4 secretion in porcine granulosa cells but the species difference and the unluteinized nature of their cells may be important in explaining the apparent lack of effect of IL–1 on human luteal cells.

When human granulosa–lutein cells were cultured in serum free media, their ability to provide progesterone was obviously reduced which may suggest the essential role played by serum precursors such as androgens and LDL cholesterol in this culture. However, similar findings of effects of hCG, IL–1 and IL–2 on these cells in serum free medium were further confirmed suggesting the observed effects of IL–1 and IL–2 were not a result of serum but of the added cytokines themselves.

While the physiological significance *in vivo* of these findings remains uncertain, the following conclusions can be made. 1) Human lymphocytes produce substances that alter granulosa–luteal cell proliferation and steroidogenesis *in vitro* and by extension may do so in the ovary especially the corpus luteum. 2) IL–2

exhibits a gonadotrophin–dependent effect on stimulating mitogenesis, while inhibiting steroidogenesis in the ovary. 3) Other white cell–derived products may also modify the proliferation and function of progesterone–secreting cells. These conclusions result from *in vitro* experiments, and it is important to evaluate their significance *in vivo*.

These results extend previous reports on the effects of cytokines, suggesting a physiological influence on the human granulosa–luteal cell. Concentrations of IL–1 and IL–2 in human follicular fluid are of the same magnitude as those used *in vitro* in this study (chapter 4), and the influx of lymphocytes and macrophages into the ovary before and after ovulation would provide a source of production of cytokines to affect steroid–secreting cells. A recent study has shown markedly higher circulating serum concentrations of IL–2 in pregnancy compared with those in nonpregnant subjects (Favier *et al*, 1990), and while the source of this remains uncertain, elevated concentrations of lymphokines could also have a significant influence on the ovary, possibly by modulating the effect of hCG.

CHAPTER SIX

TUMOR NECROSIS FACTOR α: EFFECTS ON GONADOTROPHIN-STIMULATED PROGESTERONE PRODUCTION AND CELL PROLIFERATION BY HUMAN GRANULOSA-LUTEIN CELLS

6.1 Introduction

Tumour necrosis factor α (TNFα) is a cytokine with pleiotropic actions on several cell types. It is produced by leukocytes, particularly macrophages, and by several cells not classically associated with the immune system such as smooth muscle cells (Beutle, 1988). Local communication between immune cells and endocrine glands has been inferred in several animal species and in the human (Adashi, 1990). In particular, the roles of macrophage-derived cytokines such as TNFα and interleukin-1 have been investigated following the finding of a large population of macrophages in the stroma surrounding the preovulatory follicle (Hume *et al*, 1984) and inside the corpus luteum (Bagavandoss *et al*, 1990 and chapter 3). As discussed in 1.5.2.2, TNFα has been reported to be secreted from the corpus luteum during *in vitro* incubation of rabbit corpora lutea, and by cultured human granulosa-lutein cells (Roby *et al*, 1990). Furthermore, specific binding sites for TNFα on swine granulosa cells were recently demonstrated by Veldhuis *et al* (1991). The effects of TNFα alone or in combination with gonadotrophic hormones on ovarian cells seem to vary between species and cell preparations used (see 1.5.3.2). TNFα decreases FSH-stimulated aromatase activity (Emoto and Baird, 1988), and LH-stimulated progesterone (P4) production (Adashi *et al*, 1990) in cultured granulosa cells from diethylstilbestrol-treated rats. TNFα alone stimulates steroidogenesis in incubated preovulatory follicles from the rat (Roby and Terranova, 1990) by action on theca cells but inhibits hCG-stimulated androgen accumulation in cultures of

theca/interstitial cells from immature rats (Andreani *et al*, 1991). In the present chapter we extend these observations to human granulosa–lutein cells in culture.

6.2 Materials and Methods

6.2.1 Collection and culture of human granulosa–lutein Cells

The details of collection and culture of granulosa–lutein cell have been introduced in 2.6.2 and 2.6.3. In the present study, cells ($3x10^5$) were seeded in each well in 1ml MEM with 10% Fetal Bovine Serum (Appendix IV) or under serum–free conditions in medium M199 with 0.1% Bovine serum albumin (Appendix IV). There were three to five replicate wells in each experiment. Each experiment was repeated at least three times using individual samples.

In order to determine if recombinant human TNFα on its own, affected production of prostaglandin E2 (PGE_2) and prostaglandin F2α ($PGF_{2\alpha}$), the cells were cultured for periods of up to 48h in either serum–containing or serum–free medium. To investigate the possible role of TNFα on basal and hCG–stimulated P_4 accumulation the cells were cultured in serum– containing medium for 24, 48 and 72h with or without TNFα (6–6000pmol/l). The effects on P_4 by TNFα with or without hCG were also tested during the subsequent 24h periods starting from 73 and 97h, because of the better response of human granulosa–lutein cell to hCG after 2 to 3 days in culture (see 2.6.4.4) . In other experiments, cells were incubated with M199–0.1% BSA for 48h, followed by incubation in TNFα with or without hCG in M199–0.1% BSA for 48h. To measure TNFα production, the cells were cultured for periods of up to 48h, with or without hCG (5IU). The media were stored at –70°C until further analysed.

6.2.2 [^{3}H] Thymidine Incorporation into Granulosa-lutein Cells

To assess the effect of TNFα on cell proliferation, granulosa-lutein cells were incubated in various concentrations of TNFα for 48h before the addition of 0.5μCi [^{3}H] thymidine for the [^{3}H] thymidine incorporation test (see 2.5).

6.2.3 RIA

RIAs for immunoreactive TNFα and P_4 have been described in 2.8 and 2.8.5. PGE_2 and $PGF_{2\alpha}$ were measured by RIA as described previously (Kelly *et al*, 1986; Reddi *et al*, 1987). In brief, PG assays utilized the competition between unlabelled PGs and the fixed quantities of ^{3}H-labelled PGs for the specific antibodies raised against specified PG (PGE_2 or $PGF_{2\alpha}$). The antigen-antibody complex formed after incubation are separated from unbound antigen by the addition of cold Dextran-Charcoal. The standards in PGE_2 assay ranged from 0.4 to 21 nmol/l and in the $PGF_{2\alpha}$ assay they were 0.2 to 21 nmol/l. The sensitivity by 95% B/Bo method was 3.3pg/100μl for PGE_2 and 34.0pg/100μl for $PGF_{2\alpha}$. All samples showed parallism with standards. Intra-assay variations in all assays were less than 5% and inter-assay variations were less than 10%. All samples in the same experiment were measured in the same assay to reduce variations.

6.2.4 Data Analysis

The values of P_4, PGs and immunoreactive TNFα were expressed as the mean and SEM. Student's t test was used and significance was assigned at $P<0.05$ for two tailed analysis.

6.3 Results

6.3.1 TNFα Concentrations in Medium from Cultured Human Granulosa-lutein Cells

Media from granulosa-lutein cells cultured with or without hCG (5IU) for 24 or 48h was collected and TNFα concentrations were measured using RIAs and bioassays (see 4.2.3). There were no detectable levels of immunoactive TNFα in a total of 40 samples measured by RIA (<10pmol/l) and in 7 samples measured by bioassay (<117.6pmol/l)

6.3.2 The Effect of TNFα on Cell Proliferation

A dose-dependent increase of ^{3}H-thymidine incorporation into cultured cells was observed in the presence of TNFα (Fig. 6.1). Significant differences were found between control and 60 pmol/l or 600 pmol/l of TNFα ($p<0.05$).

6.3.3 The Effect of TNFα on PG

TNFα stimulated accumulation of both PGE_2 and $PGF_{2\alpha}$ in a dose-dependent manner during a 48h culture period both in serum-containing medium (Fig. 6.2ab) and serum-free medium (Fig 6.3ab). Time course studies revealed that maximum levels of PGE_2 and $PGF_{2\alpha}$ in the presence of TNFα (600 pmol/l) were reached within 12h in serum-containing medium (Fig. 6.4ab).

6.3.4 The Effect of TNFα on P4 Production in Granulosa-lutein Cell Culture

TNFα did not alter basal P4 release in serum-free granulosa-lutein cell culture during a 48h culture period (Table 6.1). In serum-containing medium the basal and hCG-stimulated P4 levels were 6 and 2-fold higher, respectively, than in serum-free medium, but no effect of TNFα (6-6000 pmol/l) was seen during incubation periods up to 72h or during 24h periods starting at 73h or 93h of culture (Table 6.1).

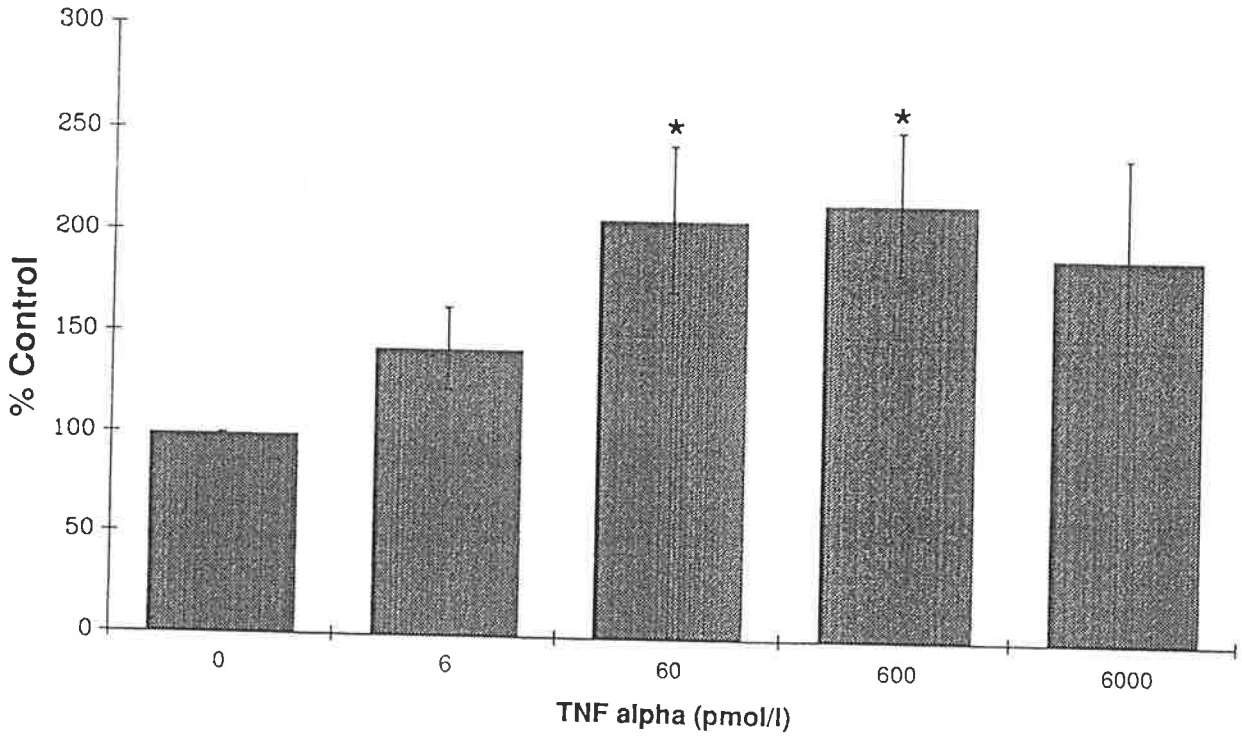


Fig. 6.1 Incorporation of ^{3}H-thymidine into cultured human granulosa-lutein cells during a 6 hour period (48 to 54h) in the presence of TNFα. Vlaues are expressed as % of control incoporation. Significantly (*P<0.05) higher than control at 60 and 600pmol/l.

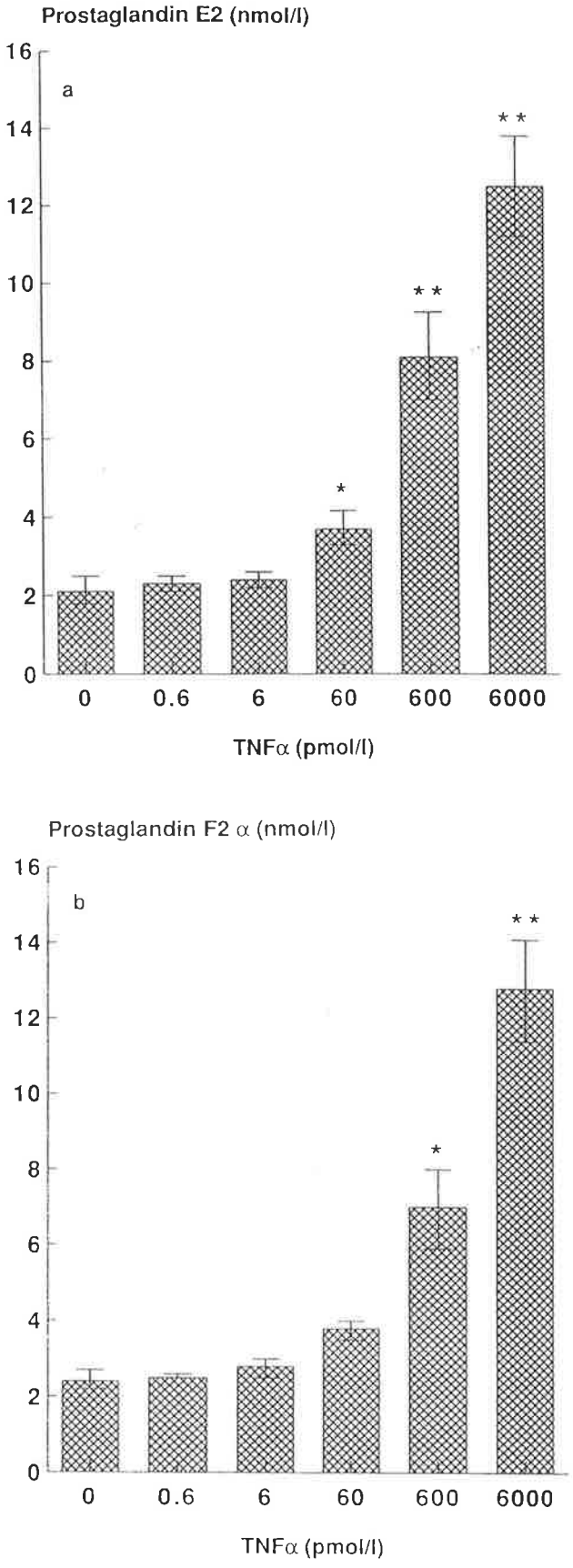


Fig. 6.2 Dose response curve of PGE2 (a) and PGF2α (b) production in the presence of TNFα (6 to 6000pmol/l) during a 48h culture period in serum-containing medium. Values are mean $\pm$ SEM (n=3–5). *P<0.05 **P<0.01.

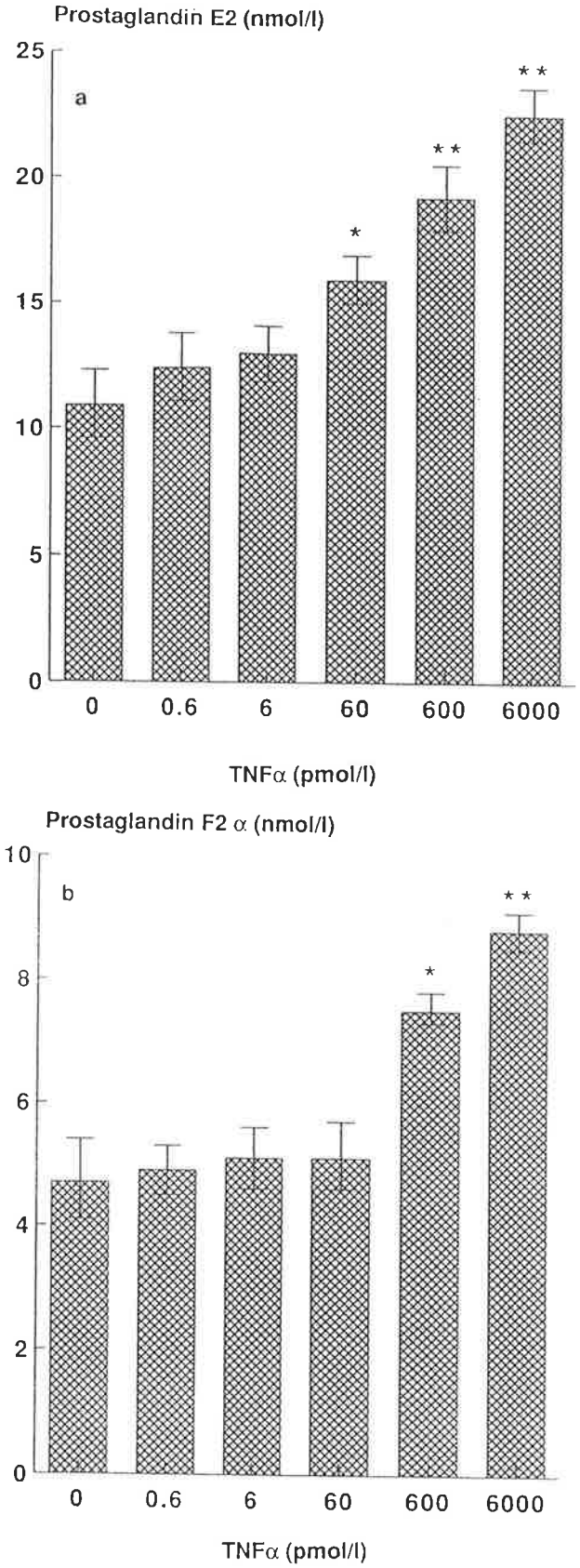


Fig 6.3 Dose response curve of PGE2 (a) and PGF2α (b) production in the presence of TNFα (6 to 6000pmol/l during a 48h culture period in serum-free medium. Values are mean ± SEM (n=3-5). *P<0.05 ** P<0.01.

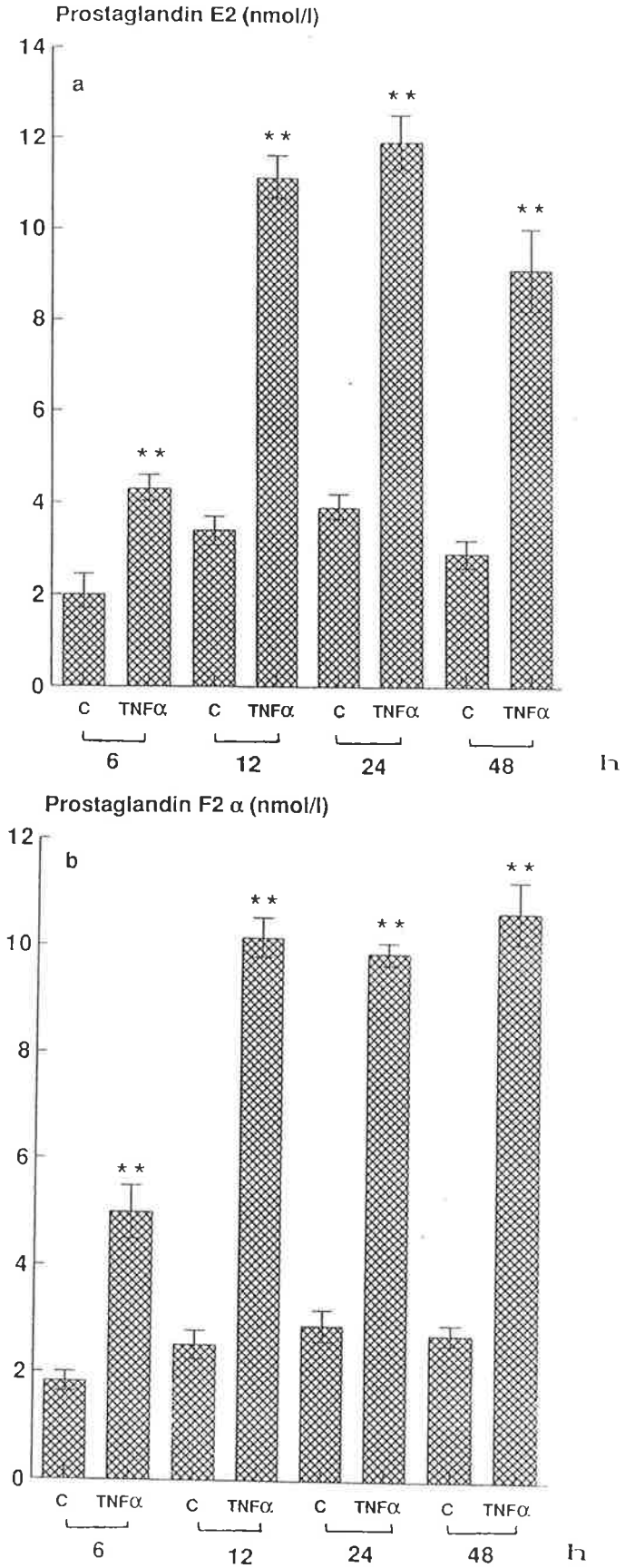


Fig. 6.4 Time course of PGE2 (a) and PGF2α (b) production in the presence of 600 pmol/l of TNFα in serum containing medium. Values are means ± SEM (n=4–5). ** P<0.01 compared to control.

Table 6.1. Progesterone Concentrations (nmol/5x10^5 cells) Detected in Cultured Human Granulosa-Lutein Cells

TNFα Incubated period (Hours)	TNFα (pmol/l)					TNFα (pmol/l) with hCG (5iu/ml)				
	0	6	60	600	6000	0	6	60	600	6000
0-24[a]	12 (±1)	11 (±1.4)	10 (±0.6)	8 (±0.2)	9 (±0.1)	12 (±2)	10 (±0.5)	9.6 (+0.5)	9 (+0.9)	9 (+0.3)
0-72[a]	30 (±0.5)	33 (±2.8)	26 (±1.7)	25 (±1.9)						
49-96[b]	1.1 (±0.2)	1.1 (±0.1)	1.3 (±0.4)	2.7 (±1.0)	1.4 (±0.5)	8.2 (±1.9)	9 (±2.5)	8.2 (±3.4)	9.3 (±2.1)	9.4 (±2.6)
49-120[a]	15 (±0.7)		16 (±0.03)	17 (±0.03)	17 (±0.1)	16 (±0.1)		18 (±0.3)	16 (±0.6)	16 (±0.5)
97-120[a]	7.5 (±2.4)	6.3 (±1.5)	6.2 (±1.4)	6.4 (±1.2)		10.9 (±0.8)	10.4 (±1.5)	10.5 (±0.8)	10.3 (±0.7)	

p=N.S. a. Culture was carried out in Serum-containing medium b. Culture was carried out in serum-free medium.

6.4 Discussion

Although TNFα has been localized to the theca luteal region of the human ovary by immunocytochemistry (Roby and Terranova, 1989), its specific cellular origins remain to be identified. TNFα is characteristically a product of activated macrophages although other tissues can also produce this cytokine. Macrophages are a major component of the human preovulatory ovarian–leukocyte population (Loukides *et al*, 1990) and in the corpus luteum co–localized with the Leu M5 (a marker for macrophages) –positive (see 5.3), TNFα–positive (Roby and Terranova, 1989), 3β–hydroxysteroid dehydrogenase–negative (Sasano *et al*, 1990) theca–luteal area suggesting strongly that these cells may contribute to ovarian TNFα production. Alternatively TNFα may originate in tissue from circulating concentrations of this cytokine and preliminary observations from our group have shown detectable concentrations of TNFα with peak concentrations in the luteal phase of the cycle. This explanation would appear to be less likely than local production but awaits further data relating to the distribution of cytokine and its mRNA.

Surprisingly, TNFα was not detected in the supernatants of cultured human granulosa–lutein cells by either immuno– or bioassay, in contrast to the finding of others (Roby *et al*, 1990; Zolti *et al*, 1990). The reason for this discrepancy is not clear, but it is possible that different cell harvesting protocols may yield different proportions of cytokine producing cells, particularly if the culture–derived cytokine originates from macrophages (which are notoriously 'sticky' *in vitro*), or another minor tissue– or blood–derived cell population. This data suggests that granulosa cells at the preovulatory follicle stage are not the major source of follicular fluid TNFα and may support the possibility of thecal or white cell origins of TNFα. Recent data has also failed to confirm immunohistochemical evidence for TNFα in the human ovary (Chen *et al*, 1992).

TNFα was found to stimulate uptake of ^{3}H-thymidine by granulosa-lutein cells in a dose-dependent manner, although others have failed to demonstrate an effect of TNFα on rat (Adashi *et al*, 1989b) or pig (Veldhuis *et al*, 1991) granulosa cell proliferation. This difference in responsiveness may be accounted for either by inter-species variations, the nature of the cell preparations, or be a consequence of the state of differentiation of the cell populations studied. In this study, hyperstimulation of patients with high doses of hCG *in vivo* gives rise to fully luteinized granulosa cells, whereas rat and swine granulosa cells employed by others are unluteinized. TNFα-mediated stimulation and inhibition of cell proliferation have both been described in a variety of normal and transformed cells (Sugarman *et al*, 1985). Human granulosa-lutein cell populations have a significant number of white cells in the culture (Beckman *et al*, 1991) and an effect on non-steroid secreting cells can not be excluded as will be shown in the next chapter.

TNFα, either alone or in combination with hCG, had no effect on P4 production by granulosa lutein cells *in vitro*. This contrasts with earlier reports of TNFα-mediated stimulation of P4 production (Zolti *et al*, 1990; Clinton *et al*, 1991), although those investigators assayed steroid synthesis after either longer (Clinton *et al*, 1991) or shorter (Zolti *et al*, 1990) culture periods than in the present study. Interestingly, in rat and swine granulosa cells, FSH-stimulated P4 production was inhibited by TNFα (Adashi *et al*, 1990; Veldhuis *et al*, 1991), as was hCG-stimulated androgen synthesis by rat thecal cells (Andreani *et al*, 1991). The degree of luteinization between human and animal cells may account at least in part for the dissimilarities in these results.

TNFα induced a marked stimulation of PG production in cultured granulosa-lutein cells. As PGs are important mediators both in the human ovulatory process (Killick *et al*, 1987) and in facilitating P4 production in the corpus luteum in the human menstrual cycle and early pregnancy (Hahlin *et al*, 1988), TNFα may act both

as a local stimulatory agent in ovulation and a local regulator of corpus luteum function. It is well known that PGE_2 acts to stimulate and $PGF_{2\alpha}$ to inhibit P_4 production in the human corpus luteum, and it has been proposed that the ratio between the tissue concentration of PGE_2 and $PGF_{2\alpha}$ may be important in regulation of the human menstrual corpus luteum (Hahlin *et al*, 1988).

In summary, TNFα is present in follicular fluid of the human (chapter 4) and it stimulates proliferation and production of PG by cultured human granulosa–lutein cells. Follicular TNFα, which may be produced by local macrophages might be of importance as a facilitator of PG production during ovulation and of cell proliferation during the formation of the early corpus luteum. Since TNFα is chemotactic for monocytes and granulocytes (Ming *et al*, 1987), it may therefore account at least in part for the chemotactic activity of human follicular fluid (Herriot *et al*, 1986) and have a role in leukocyte recruitment into the ovulating follicle and corpus luteum (see 5.3).

CHAPTER SEVEN

CELLULAR COMPOSITION OF PRIMARY CULTURES OF HUMAN GRANULOSA-LUTEIN CELLS AND THE EFFECT OF CYTOKINES ON CELL PROLIFERATION

7.1 Introduction

Studies in chapters 5 and 6 have shown that conditioned lymphocyte medium and recombinant cytokines presumed to be produced by resident white cells influence proliferation, steroid secretion and progesterone production by human granulosa-lutein cells in culture. This culture system has been a common source of human ovarian cells to study functional and morphological events (Polan *et al*, 1984; Hillier *et al*, 1987; Amsterdam and Rotmensch 1987). The previous studies have shown that the cytokines, IL-2 and TNFα, stimulate thymidine uptake in cultures of human granulosa-lutein cells (see Chapter 5 and 6) . In these investigations IL-2 and TNFα did not affect basal progesterone (P4) secretion while IL-2 inhibited hCG-stimulated P4 production. Thus the stimulation of cell proliferation and the contradictory effect on P4 release by IL-2 and TNFα could indicate that the stimulatory and proliferative effects were not necessarily due to direct actions on the granulosa-lutein cells. Pure granulosa-lutein cells are difficult to obtain due to blood contamination from ruptured blood vessels during the aspiration procedure and the concomitant presence of blood cells in the follicular antrum at this stage. Density cell separation techniques or lysis of red blood cells (Beverley 1986) are widely used for removal of red blood cells from other constituents in ovarian follicular cells. However, these techniques may not eliminate leukocyte contamination in human granulosa-lutein cell culture. A certain proportion of the leukocytes among the granulosa cells is expected knowing the cell composition resulting from follicular rupture when there is bleeding into the follicular cavity before formation of the corpus luteum. This is illustrated by the subsequent

high proportion of leukocytes demonstrated the human corpus luteum (see Chapter 3). Moreover several studies have shown the effects of growth factors (Olsson *et al*, 1990, Gospodarowicz and Biatechi 1979) on the proliferation of cells in the primary granulosa–lutein cell culture without elucidating if the effect was due to a direct response of the steroidogenic cells or via cells of white cell lineage. A recent report (Beckmann *et al*, 1991) has suggested that leukocytes are a major contaminant of human granulosa–lutein cell culture and this could have significant implication for physiological studies. In the present study the aim was to see whether some of the changes described were due to differences induced in granulosa–lutein cells or accompanying white cells.

7.2 Materials and Methods

7.2.1 Granulosa–lutein cell collection and culture method

This study was designed to examine the nature of the cell population affected by IL–2 or TNFα since these two cytokines increased cell proliferation as shown in the previous study (chapter 5 and 6). Cultures were grown in chamber slides to permit subsequent staining of the cells. Details of the collection and culture method are described in 2.6.2 and 2.6.3. In the present experiment the cell number in 1 ml MEM with 10% FBS (Appendix IV) was adjusted to 2–3x10^5 and the cell cultures were performed with or without human recombinant IL–2 (26–2600 pmol/l) or human recombinant TNFα (60–6000 pmol/l). Each chamber well contained 0.45 ml of cell–suspensions with duplicates of each treatment. The cell culture was maintained for 48h or media changed every 48h until the end of 144h. Previous sections have characterized the steroid secretion and gonadotrophin sensitivity of the cells from this method (see 2.6.4.3 and 2.6.4.4).

7.2.2 Immunocytochemistry

Immunocytochemistry was performed using an improved biotin–streptavin amplified detection system (B–SADS) as described in section 2.4. Primary antibodies used in the present study were monoclonal antibodies, anti–E_2, vimentin, anti–Leu 4, anti–Leu 2a, anti–Leu 3ab, anti–IL–2 receptor, anti–Leu 14, anti–Leu M1, anti–Leu M5, anti–Leu 7, LCA and polyclonal antibody to 3β–HSD. The source, predominant cellular reactivity and dilution of these antibodies are described in chapter 2 (Table 2.1 and 2.2). A positive reaction was demonstrated by a brown deposit. The results were analysed by counting the number of positive cells in a total of 500 cells within a randomly chosen field of each slide.

7.2.3 Data Analysis

Each culture condition was repeated two to four times, using samples from different patients. Antibody positive cells were expressed as the number per 500 counted cells. Significance was assigned at 5% levels for two–tailed statistics (students' t test).

7.3 Results

7.3.1 Cellular composition of unstimulated human granulosa–lutein cells

About 35% of all cells were of bone–marrow lineage as judged by positive staining with LCA (20%, range 17–25%, Fig. 7.1a) and antibodies specific for macrophages/monocytes (15%, range 8–29%, Fig. 7.1bc). The LCA–positive cells included B lymphocytes (39%, range 33–47%, Fig. 7.1d), T lymphocytes (31% range 28–34%, Fig 1e) and NK cells (18%, range 15–21%, Fig. 7.1f). In the group of T lymphocytes, 75%(range 54–95%) were helper T and 25% (range 14.5–35%) were cytotoxic T cells. All T lymphocytes stained positive with antibodies against the IL–2 receptor(Fig. 7.1g). The non–lymphohematopoeitic cells were in general bigger in

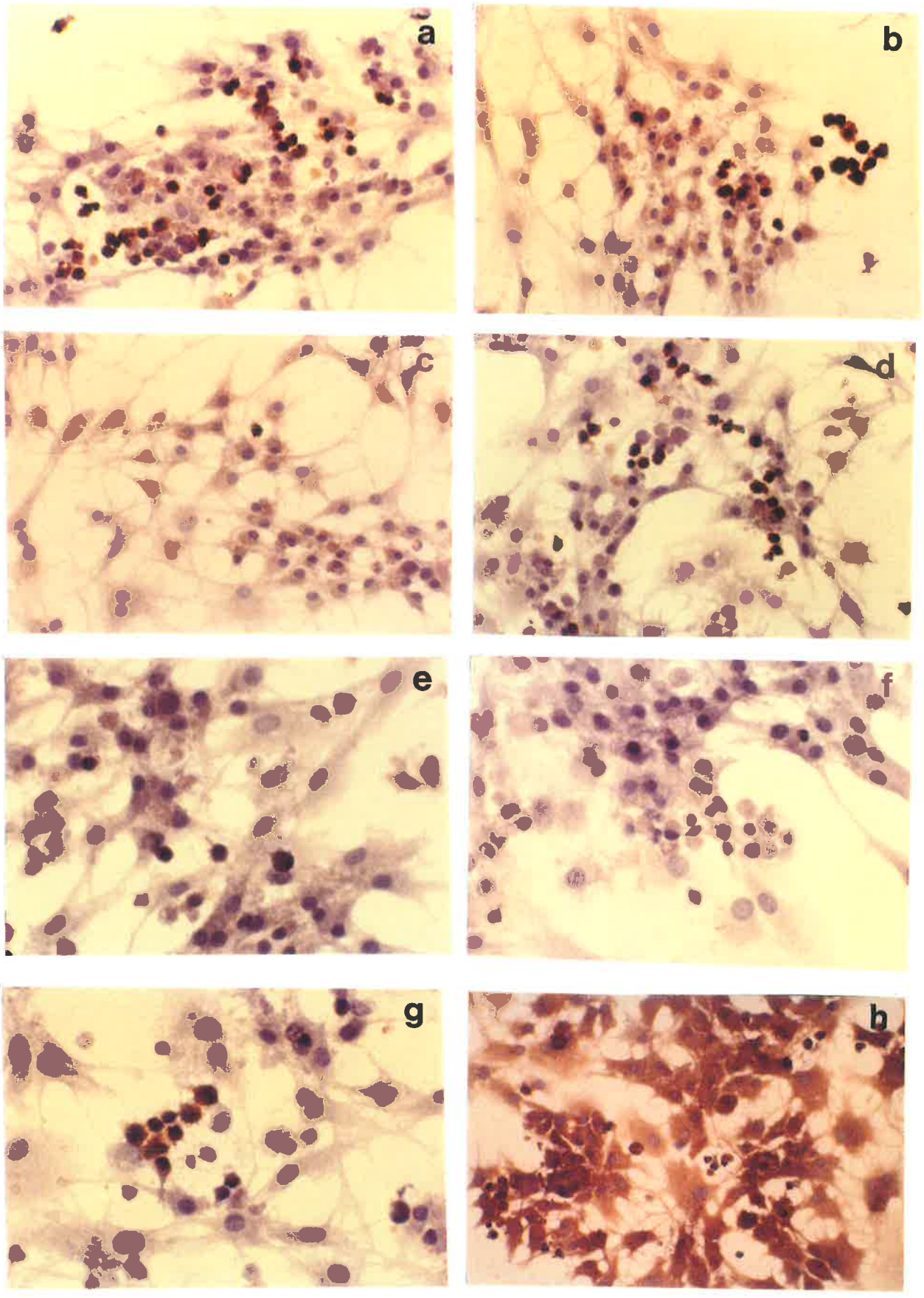
size and most of them spread in a fibroblastic like manner. They stained positive with antibodies against vimentin, E_2 and 3β–HSD. The percentages of visible positively stained cells with antibodies against vimentin, E_2 and 3β–HSD(Fig. 7.1h) were around 98%, 95% and 82% respectively. Many of the lymphohaematopoeitic cells in the culture were covered by steroid producing cells and were only revealed by staining with specific antibodies to white cells.

7.3.2 Changes in cellular composition in response to IL–2 and TNFα during a 48h culture

Cells were cultured in the presence or absence of IL–2 (26–2600 pmol/l) and TNFα (60–6000 pmol/l) for 48h. Both IL–2 and TNFα at concentrations of 2600 pmol/l and 6000 pmol/l respectively decreased the proportion of 3β–HSD positive cells ($p<0.05$ Fig. 7.2). The proportion of monocytes and LCA positive cells were increased dose–dependently by IL–2 and TNFα ($p<0.05$ Fig. 7.3 and 7.4).

7.3.3 Changes in cellular composition over a 6 days culture period

Cytokine–influenced changes of cellular composition were found within 48h of culture but not on the following days. In the absence of cytokines, the proportion of granulosa–lutein cells remained constant while the percentages of LCA positive cells and monocytes tended to decrease with time (Tables 7.1, 7.2 and 7.3).


a
b
c
d
e
f
g
h

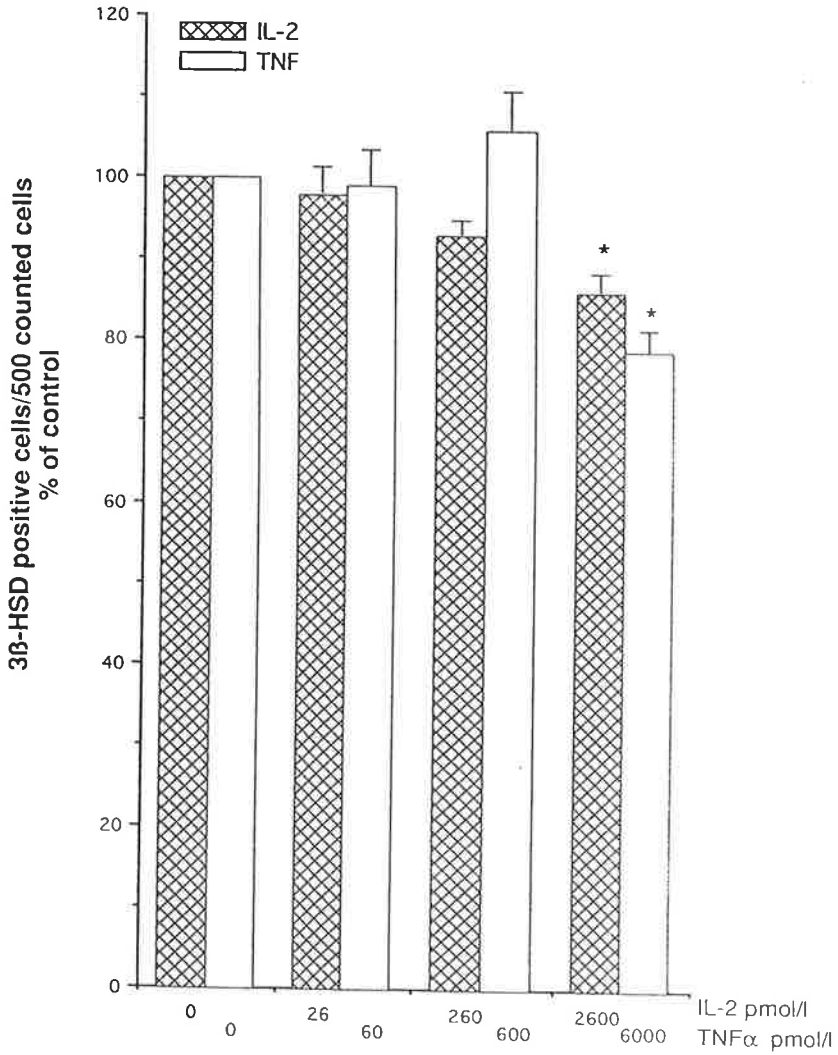


Fig 7.2 Proportion of 3β–HSD positive cells in primary culture of human granulosa–lutein cells with or without IL–2 (26–2600pmol/l) and TNFα (60–6000pmol/l) for 48h. Significant differences were found between control and cytokine treatment at the highest concentration (IL–2 2600pmol/l and TNFα 6000pmol/l, *P<0.05).

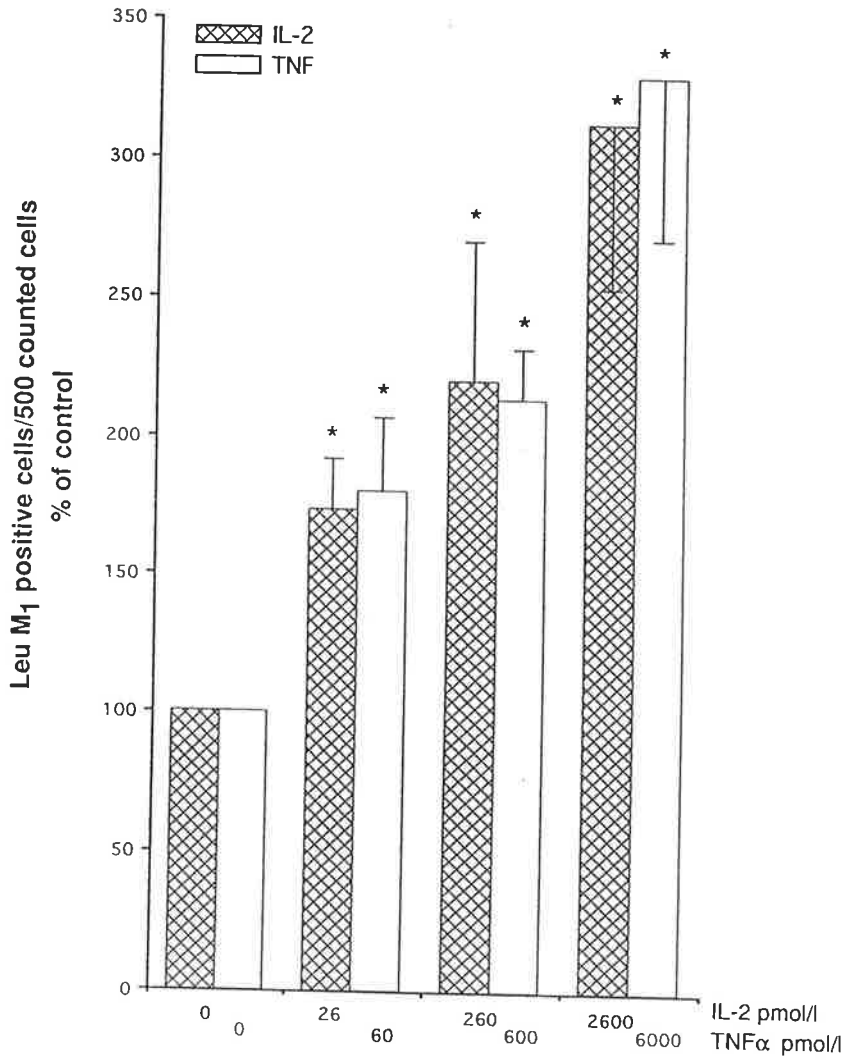


Fig. 7.3 Proportion of monocytes in primary culture of human granulosa–lutein cells with or without IL–2 (26–2600pmol/l) and TNFα (60–6000pmol/l) for 48h. Significant differences were found between control and all cytokine treatments (*P<0.05).

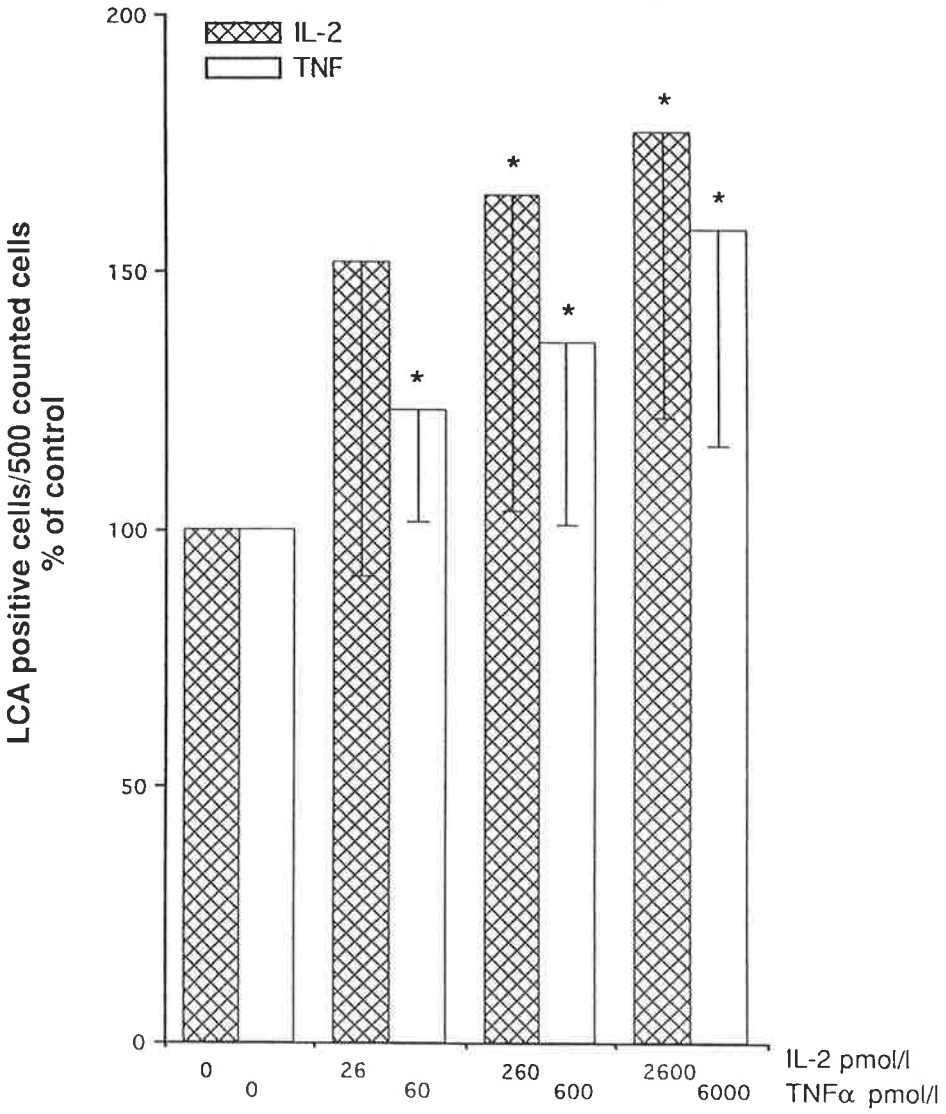


Fig. 7.4 Proportion of LCA positive cells in primary culture of human granulosa–lutein cells with or without IL–2 (26–2600pmol/l) and TNFα (60–6000pmol/l) for 48h. Significant differences were found between control and IL–2 treatment from 260–2600pmol/l and between control and all TNFα treatments (* $P<0.05$).

Table 7.1 3β–HSD positive cells (Mean±SEM) in the human granulosa–lutein cell culture on different days with or without IL–2 or TNFα[a]

	Control	IL–2 (260pmol/l)	TNFα (600pmol/l)
Day 2	418±11	306±54	392±10
Day 4	386±38	394±30	364±8
Day 6	400±25	384±13	379±7

Table 7.2 LeuM1 positive cells (Mean±SEM) in the human granulosa–lutein cell culture on different day with or without IL–2 or TNFα[a]

	Control	IL–2 (260pmol/l)	TNFα (600pmol/l)
Day 2	30±8	67±17[b]	64±5[b]
Day 4	18+3	40±9	42±11
Day 6	3±1.7	20±8	10±3.8

Table 7.3 LCA positive cells (Mean±SEM) in the human granulosa–lutein cell culture on different day with or without IL–2 or TNFα[a]

	Control	IL–2 (260pmol/l)	TNFα (600pmol/l)
Day 2	98±35	162±60[b]	133±35[b]
Day 4	57±32	70±34	73±29
Day 6	69±47	83±44	63±37

a Five hundred cells were counted in each well (x2) from each case (n=2). The significantly reduced number of 3β–HSD cells in Fig. 7.1 were at the highest concentrations of IL–2 (2600pmol/l) and TNFα (6000pmol/l).

b $P<0.05$ compared to control

7.4 Discussion

Several publications have used the secretory profile of human–granulosa lutein cells in culture to examine the influence of hormones and non–hormone substances on ovarian function (Hsueh *et al*, 1984) as described in previous sections. Basal and gonadotrophin–stimulated steroid production and cell differentiation have been described in short and long term culture (chapter 5 and 6) and these have been shown to correlate well with the previously described results from human granulosa–lutein cell culture system (Polan *et al*, 1984, Hiller *et al*, 1987). Growth factors such as IGF–1, EGF and FGF (Gospodarowicz and Biatechi 1979, Olsson *et al*, 1990) appear to induce proliferation of these cells *in vitro* and it has been assumed that steroid–secretory cells respond directly to these and other trophic agents. However there are several lines of evidence to suggest that other non granulosa–lutein cells may be present and could influence the results of cell proliferation and hormone secretory experiments. Human preovulatory follicular fluid contains a considerable number of white cells including macrophages, monocytes, B lymphocytes and T lymphocytes (Castilla *et al*, 1990; Loukides *et al*, 1990). Recent data also suggests that white blood cells are an important constituent of the human corpus luteum constituting up to 15% of all the cells (Lei *et al*, 1991, and Chapter 3). Lymphocytes and other bone marrow–derived cells have also been shown to have receptors for many sex steroids, ovarian trophic hormones and growth factors (Cohen *et al*, 1983; Rouabhia *et al*, 1987; Marchetti *et al*, 1988; Johnson *et al*, 1992). This suggests that the contribution of cells normally regarded as unwelcome contaminants of cell culture can not be ignored. Accordingly this study attempted to characterize the cellular composition and proportion of individual cell types in the total cell population and their response to IL–2 and TNFα.

The antibodies used to detect human granulosa–lutein cells were directed against vimentin, E_2 and 3β–HSD. Vimentin positive cells represent all the cells of mesenchymal origin besides granulosa–lutein cells including fibroblasts and endothelial cells. Oestradiol positive cells represent those containing E_2 which may be synthesized by cells themselves or bound to the E_2 receptor. 3β–HSD is a key steroidogenic enzyme and 3β–HSD positive cells would therefore represent the steroidogenically–active granulosa lutein cells. The present study confirms that the granulosa–lutein cell is indeed the major component of this primary culture system but that significant numbers of macrophages/monocytes, and lymphocytes including T and B cells and NK cells are also resident in the culture. The proportion of monocytes/macrophages in this study is similar to the proportion found in human follicular fluid (Loukides *et al*, 1990) which indicates that standard separation methods using Percoll gradients do not eliminate these cells from the culture. There was a broad variation in the percentage of cell types present probably reflecting variations in the degree of blood contamination of follicular fluid between cases. All methods commonly used for preparation of granulosa–lutein cells will probably have the same proportions of non–steroid secreting cells.

In the present study of IL2 and TNFα, both cytokines reduced the proportion of 3β–HSD positive cells and dose–dependently increased leukocytes and monocytes during the first 48h of cell culture. This indicates that the main target cells for the proliferative effects of IL–2 and TNFα are leukocytes rather than granulosa–lutein cells in this culture system during this stage. Other substances elaborated by the resident leukocytes and stimulated by IL–2 and TNFα could also cause the proliferation and altered physiology of granulosa cells.

Cellular proportions in the culture periods beyond 48h did not show any statistical significant changes depending on the presence or absence of cytokines. This could be attributed to the observation that a considerable proportion of cytokine-responsive leukocytes were removed at the media change at 48h and 96h when there are leukocytes floating in the media. The proportion of 3β-HSD cells remained unchanged at the end of 96 or 144h of culture and therefore suggests that the reduced number of 3β-HSD cells during 48h culture may be a secondary effect from resident and floating leukocytes which have been stimulated by IL-2 and TNFα rather then due to a direct influence on the cells. The disappearance of this secondary effect later in culture may relate to the removal of floating leukocytes during media change.

IL-2 has been shown to enhance both the *in vitro* proliferation of cytolytic T cells (Smith 1980) and the *in vitro* induction of NK cell activity through induction of interferon-γ gene transcription and translation (Kuribayashi *et al*, 1981). TNFα also has multiple stimulatory activities including stimulation of cell proliferation on activated T cells, B cells and macrophages (Chang and Lee 1986; Scheurich *et al*, 1987; Kehrl *et al*, 1987). Both interferon γ and IL-1 production have been confirmed to be induced by TNFα (Pober *et al*, 1986, Scheurich *et al*, 1987). We have also detected bioactive interleukin-1 from media of human granulosa-lutein cells cultured with TNFα, (preliminary study). White blood cells are also the target cells of hormones that are secreted from the granulosa cells as human lymphocytes and eosinophils have E_2 receptors (Cohen *et al*, 1983; Danel *et al*, 1983; Gleich and Adolphson 1986). Since physiological concentrations of IL-1, IL-2 and TNFα have been detected in the human preovulatory follicular fluid from IVF cycles (chapter 4) and concentrations of these cytokines are effective in regulation of proliferation of granulosa/lutein cells and leukocytes in vitro, they may be active *in vivo* in regulation of activity in granulosa cells.

Contamination of cultures by white cells may not be unrepresentative of the

physiological situation *in vivo* given the documented presence of these cells in the follicle and corpus luteum but attribution of direct effects of growth factors and cytokines to granulosa–lutein cells should be treated with caution. Cytokine signals to steroid–secreting cells may be an essential component of ovarian function; the specific signal however may not be easy to prove given the effects of cytokines on white blood cells to promote secretion of other cytokine messengers.

CHAPTER EIGHT

CONCLUSIONS

Ovarian function includes the production of female germ cells and secretory activities. The regulation of ovarian function is controlled by the hypothalamic–pituitary–ovarian axis either through classic endocrine pathways or by intraovarian regulators through paracrine or autocrine mechanisms (see 1.5). An additional regulation by immune cell–derived cytokines, as part of immune regulation has been investigated in the last five years. Studies reported in this thesis provided some evidence of this third type of regulation in the human ovary.

The identification of immune cells in the human ovary is an important element in establishing this regulation. Macrophages and T lymphocytes are the major cellular components of immune system in human as well as in animals. These two types of immune cells also constitute a major source of cytokines (see 1.2). In this study, macrophages, T lymphocytes and other immune cells such as NK cells and monocytes have been systematically identified in the human corpus luteum by modern methods of immunocytochemistry. Immune derived cells comprised up to 15% of the total cell population in this endocrine organ. Macrophages predominated with an almost equal percentage in both granulosa–luteal and theca–luteal areas. The central loose connective tissue of the corpus luteum also contained a significant number of macrophages. T lymphocytes were detected in the human corpus luteum with greater numbers in the thecal luteal than granulosal–luteal area. The ratio of T4 to T8 cells was about 2:1 in the theca–luteal area and 1:1 in the granulosa–luteal area. Activated T cells were observed in the central loose connective tissue of the corpus luteum. It is obviously important in the future to know the distribution of these immune cells throughout the menstrual cycle and this was limited because of the scarcity of available specimens and will be part of an ongoing study. It has been observed that

the infiltration of these immune cells into human ovary at the preovulatory stage was most likely related to the chemoattractive activity locally produced from the follicle (Herriot *et al*, 1986; Murdoch and McCormick, 1989). The further invasion of large amounts of these immune cells into human corpus luteum may result from ovulation when the blood vessels of the follicular wall were ruptured and the haemorrhagicum was subsequently formed. The maintenance of these resident immune cells in the human corpus luteum may still require locally produced chemoattractants (Herriot *et al*, 1986; Murdoch and McCormick, 1989) including immune cell–derived cytokines themself (Wang *et al*, 1987) as well as ovarian steroids (see 1.5.4.3). Little attention has been given to the properties and roles of these cells. Apart from heterophagy of damaged luteal cells by ovarian macrophages (Paavola, 1979) the evidence provided by this study strongly suggests a possible relationship between presence and function. It is therefore possible that local paracrine secretion of cytokines may be implicated.

Demonstration of immune cells in the human corpus luteum led to the further investigation of cytokine production in the human ovary. Interleukin 1 and TNFα are products of macrophages while IL–2 is a product of T cells. Immunoreactive IL–1, TNFα and IL–2 were demonstrated in this study by radioimmunoassay in human preovulatory follicular fluid. Concentrations of IL–1 and IL–2 in follicular fluid were approximately half those of plasma. In one study (group I), a significant positive correlation existed between the volume of follicular fluid and IL–2 levels indicating that a possible mutual relationship may exist between the development and maturity of the follicle and IL–2 concentrations (see 4.3.1). However, further observations in group II (see 4.3.1) could not confirm this correlation measuring follicles with a range of sizes in the same patient. No significant correlation was observed between concentrations of cytokines and steroids (P_4, E_2) in follicular fluid or plasma. The follicular fluid IL–1, IL–2 and TNFα may be produced by the peripheral immune cells and diffuse into the follicle when blood circulated through ovary or oocytes were aspirated. It was confirmed in this study by the significantly positive correlation was

existed in the concentrations of IL–1 and IL–2 between the plasma and follicular fluid levels. However, ovarian origin of these cytokines should not be excluded as preovulatory follicles contains immune cells, macrophages and T cells (Hill *et al*, 1987b; Droesch *et al*, 1988; Loukides *et al*, 1990; Castilla *et al*, 1990). The detection of IL–1 gene expression in rat ovarian theca–interstitial cells (Hurwitz *et al*, 1991) suggested its origin in ovarian endocrine cells. The existence of cytokines at the preovulatory stage may be important in ovulatory mechanisms, because cytokines have been found to be stimulators of prostaglandin (PG) (Winter *et al*, 1990) which are important mediators in the human ovulatory process (Killick and Elstein, 1987). Furthermore, the physiological concentration of cytokines in human ovary will provide a reference to concentration of cytokines for *in vitro* studies of human granulosa–lutein cells.

Human granulosa–lutein cells are the most easy available *in vitro* model to investigate ovarian function, and were used to study the effect of cytokines on cultured human granulosa–lutein cells. The initial experiment was designed by addition of cytokine–conditioned media to human granulosa–lutein cell culture. HPL–CM and MLA–CM were respectively prepared from mitogen–stimulated human peripheral leukocytes and a gibbon T cell line with or without mitogen stimulation (see 2.7.2). HPL–CM showed significantly inhibitory effects on P_4 production, both with or without hCG stimulation, but MLA–CM_1 stimulated basal P_4 secretion and MLA–CM_2 reduced hCG stimulated P_4 secretion. HPL–CM contained a lower level of IL–2 than MLA–CM. Thus, the various results from three kinds of CMs could not contribute to the only effect from IL–2. Human recombinant IL–1 was therefore added into the same culture system and was not found to have any effect on P_4 production. However, human recombinant IL–2 showed a significant inhibitory effect on hCG–induced P_4 secretion and cell proliferation but no effect on the basal P_4 level. The mechanism of IL–2 action in hCG–stimulated P_4 production was partly studied. IL–2 also had a marked inhibitory effect on forskolin (FK), a stimulator of adenylate

cyclase, induced P4 release, but did not change the increased cAMP level stimulated by FK. This suggests that the influence of IL–2 on P4 synthesis may be down–stream in the signed transduction pathway from cAMP activation. Human recombinant TNFα did not alter basal or hCG stimulated P4 production but significantly increased PGE_2 and $F_{2\alpha}$ release and cell proliferation. Taken together, cytokine effects resulting from conditioned media showed either inhibitory or stimulatory influence on P4 production and cell proliferation. These results may be explained by synergic cytokine influences. With recombinant materials, IL–2 inhibited hCG–stimulated P4 production and enhanced cell proliferation, while TNFα stimulated both PG release and cell proliferation.

Based on the findings that both IL–2 and TNFα increasing cell proliferation without changing basal progesterone levels in the human granulosa–lutein cell culture, the final experiment was designed to examine the cellular components and the type of cell influenced by cytokines with the combined methods of cell culture and immunocytochemistry. This study showed that primary culture of human granulosa–lutein cells contained a large number of immune cells which were macrophages/monocytes, T and B lymphocytes, NK cells and other cells of bone–marrow lineage. Cytokines, IL–2 and TNFα significantly increased the numbers of monocytes and LCA (Leukocyte common antigen) positive cells and reduced the number of 3β–HSD positive cells in the first 48h of culture. Results suggested that any observed effects by added cytokines in the human granulosa–lutein cell culture may be due to an indirect effect from cytokine–activated resident leukocytes on granulosa–lutein cells. It may also infer an *in vivo* situation in which the presence of immune cells and cytokines may have both direct or indirect action on immune cells themselves or on steroidogenic cells.

A hypothesis is proposed based on all studies including in this thesis. Immune cells and their secretory products, the cytokines, play a role in the regulation of human

ovarian function, influencing the well established control of ovarian cells by pituitary gonadotrophins and locally produced peptides regulators (1.3.4.3e). It is postulated that the changing population of immune cells is recruited by cytokines produced in the human ovary and is part of an orchestrated epithelial cell organized network. Evidence to support this hypothesis firstly include that cytokines have been detected in human ovary (chapter 4 and 1.4.2). These cytokines have been shown to affect decisively the behaviour and functions of immune cells themselves (1.2.2.1 , 1.2.2.2). The second is that several of these cytokines are chemotactic for and activate white blood cells (Wang *et al*, 1987; Brown *et al* 1991). Thirdly, the cytokines known to be produced by immune cells attracted to the ovary have been shown in *in vitro* studies to alter the turnover and endocrine function of ovarian cells (1.5.3 and chapter 5.6.7). Thus the potential exists for resident ovarian cells under the influence of gonadotrophins to increase expression of adhesion molecules on the vascular endothelium and to establish a chemotaxic gradient in ovarian tissues which would result in the trapping of white blood cells within the ovarian microvasculature, their passage through blood vessel walls and movement to specific sites within ovarian tissues, where they could be activated in situ to participate in the processes of ovulation, corpus luteum development and luteolysis (Fig. 8.1). This hypothesis, if shown to be correct, would radically revise the current understanding of the relationship between reproductive endocrine organs and the cells of the immune system.

In conclusion, studies reported in this thesis have described the distribution of immune cells in the human corpus luteum, concentrations of immunoreactive cytokines, IL–1, IL–2, TNFα, in human preovulatory follicular fluid and cytokine effects on human granulosa–lutein cell culture with observation, on the production of P4 and PGs, cell proliferation and the alteration of cellular composition. The existence of immune cells and their derived cytokines in human ovary and the influence of cytokines on human granulosa–lutein cells *in vitro*, suggest that cytokines

may have roles in human ovarian physiology.

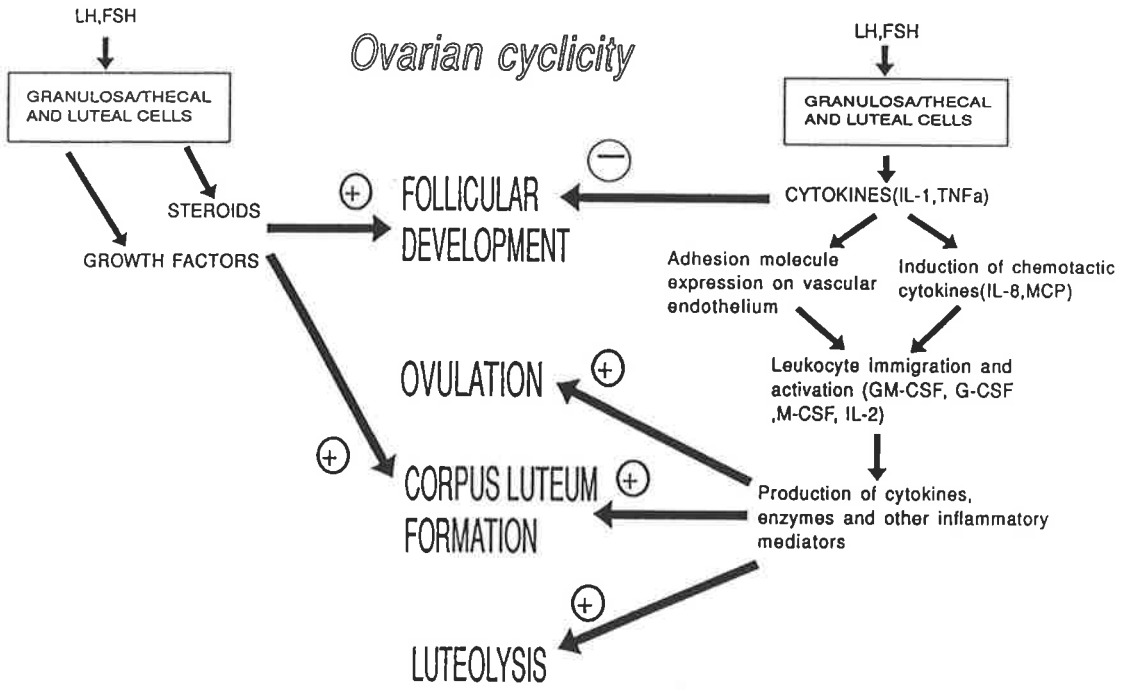


Fig. 8.1 Illustration of the hypothesis. On the left is a simplified understanding of autocrine and endocrine events in the ovary. On the right is a proposal for the role of white cells and cytokines in this organ.

APPENDIX I

Peroxidase Block

3% H_2O_2 in Milli Q. water

PBS, pH7.6

1) Weigh out
   NaCl 7.75g
   K_2HPO_4 1.5g
   KH_2PO_4 0.2g
2) Dilute to 1.0 litre with deionized water
3) Adjust pH as needed by addition of 3M NaOH

Antibody Diluent

PBS, pH 7.6
1% BSA
0.1% NaN_3

Stock solution of 0.5% DAB

(under fume cupboard, wear disposable gloves)
DAB 100mg
PBS 20ml
Make 0.5ml aliquots into plastic vials using autopipette, label and store at -20^oC.

PLL coated slides

1) 1mg PLL/ml of M.Q water;
2) put a small amount (approximately 5μl) at one end of the slide and draw it across, like a blood smear.
3) Leave the PLL to dry (generally a few mins);
4) Stock PLL coated slides at room temperature over silica gel until required.

APPENDIX II

Pre-treatment of tissue section or cell on chamber slide

---

1. Frozen tissue section

Cut sections of 5μm on PLL coated slides.
Place slides in a plastic slide rack and place this in a microwave oven; the rack with slides sits within a 6cm radius of the centre of the carousal of the microwave. Irradiate for 15 seconds at defrost setting (Sharp-Carousal, Model No R-7200). Slides are fixed in acetone for 5 min and then air dried.

2. Paraffin tissue section

Cut sections at 3μm on plain slides. The paraffin is melted in an incubator at 60-70^{o}C. Endogenous peroxidase is blocked with 3% aqueous H_2O_2 for 10 min and then the section is rinsed with PBS (note: do not allow slide to be dried until next step). Studies on CD3 requires proteolytic treatment for 5 min with 0.1% trypsin/0.1% $CaCl_2$ to expose the antigenic sites.

3. Cells on chamber slide

Cell culture is stopped by removal of culture media and washing twice with cold PBS. The plastic chamber is taken away from the slide. Cells on the slide are allowed air dry (<30 min), fixed in cold acetone (-20^{o}C) for 5 min and air dried again.

Staining procedures

	ABC	B-SA
NHS or NGS (33μl+2.5ml PBS)	20 min	20 min
Block excess serum (don't wash, drop off only)		
1^{o}Ab (diluted with AD)	60 min	30 min
Wash section or cells with minimum amount of PBS		
2^{o}Ab (11ul+2.5ml AD)	30 min	-
or BARI or BAMI (Link)	-	20 min
Wash section or cells with minimum amount of PBS		
ABC reagents (22μl A+22μl B	60 min	-
+2.5ml PBS) or PCS (Label)	-	20 min
Wash section or cells with minimum amount of PBS		
DAB (0.5ml stock DAB+4.5ml PBS +5μl H_2O_2)	7-10min	7min
Rinse slide with water and ammonia water		
100% ethanol	2 min x 2	2 min x 2
Xylyne	2 min x 2	2 min x 2
Mount slide with DePex		

APPENDIX III

---

^{3}H-thymidine culture media

^{3}H-thymidine was diluted in MEM-FBS (see Appendix IV). The concentration of ^{3}H-thymidine was 0.5 uCi in 0.05ml MEM-FBS for each well (96 well culture plate).

Trypsin solution

0.01% trypsin solution in PBS freshly prepared before each experiment.

APPENDIX IV

Silicone coated pipette

Disposable Pasteur pipettes (glass) were soaked in M.Q. water overnight, rinsed routinely by M.Q water and dried. Before being autoclaved, these pipettes were coated by sigmacote to form a thin film of silicone on the glass preventing cells from adhering to the pipette wall.

MEM

1. Measure out approximately 900ml M.Q. Water in a glass vessel;
2. Add 9.59 g of powdered MEM to water at room temperature (15°C to 30°C) and stir until dissolved;
3. Add 2.1g of $NaHCO_3$ per litre of medium;
4. Add 1.0g of glucose per litre of medium;
5. Add antibiotics (penicillin–G 100mg and streptomycin 25mg) per litre of medium;
6. PH should be approximately 7.4 at room temperature (normally no pH adjustment should be required);
7. Dilute to the final volume of 1 litre with M.Q. water and stir gently;
8. Sterilize immediately by membrane filtration with filter units;
9. Aseptically dispense into sterile glass bottles or plastic culture vessels with minimum final air space;
10. Label and store at 2°C to 8°C, no longer than 4 weeks.

MEM with 10% FBS

FBS was pre–heat–inactivated (56°C for 30 min). MEM with 10% FBS (MEM–FBS) was made up one day before cell culture, sterilized immediately, dispensed into sterile vessels and transferred to incubator in a humidified environment with 5% CO_2 and 95% air at 37°C overnight with loose cap on the media containing vessels.

M199

Same as the preparation of MEM except for the addition of glucose.

M199 with 0.1% BSA

M199 with 0.1% BSA (M199–BSA) was made up one day before cell culture, sterilized immediately, dispensed into sterile vessels and transferred to incubator overnight with loose cap on the media containing vessels.

APPENDIX V

Percoll stock solution

1. Stock A (100ml)

NaCl	11.862 g
KCl	0.7 g
$MgSO_4.7H_2O$	0.1 g
KH_2PO_4	0.1 g
DL–Lactic Acid Sodium Salt	7.4 ml
Glucose	1.0 g
PG	0.12 g

2. Stock B (10ml)

$NaHCO_3$	0.42 g
Phenol Red	0.002 g

3. Stock C (10ml)

Na Pyr	0.102 g

4. Stock D (10ml)

$CaCl_2.2H_2O$)	0.524 g

5. Stock E (50ml)

Hepes (Na Salt)	6.508 g
Phenol Red	0.01 g

Add 20ml M.Q Water; adjust pH to 7.5 with 1M HCl (about 25ml) and add remaining M.Q water.

* Note– Stock A, D, E, last 3 months
Stock B, C, last 2 weeks.

10X media

Stock		ml	ml	ml
	A:	10.0	15.0	20.0
	B:	1.6	2.4	3.2
	C:	0.7	1.05	1.4
	D:	1.15	1.725	2.3
	E:	8.4	12.6	16.8
		21.85	32.775	43.7

Percoll Solution

20ml of 10x media + 180ml percoll
30ml of 10x media + 270ml percoll
40ml of 10x media + 360ml percoll

* check osmolarity – adjust to 280 mOsm/l

$\frac{X \text{ mOsm}}{280} \times \text{vol (ml)} = \text{No. of ml of percoll to add.}$

HTF media

		ml	ml
Stock	A:	5.0	15.0
	B:	0.8	2.4
	C	0.355	1.065
	D	0.575	1.725
	E	4.2	12.6
	M.Q water	89.05	267.15

* check osmolarity and adjust to 280 mOsm with M.Q water.
Filter sterilize and store at 4°C for up to two weeks.

50% percoll solution

The preparation of 100ml of 50% percoll solution includes 50ml percoll solution and 50ml HTF media.

APPENDIX VI

RPMI 1640–FBS

The procedures of preparation of RPMI 1640 with 10% FBS are similar to the preparations of MEM and MEM–FBS except for the addition of glucose see Appendix IV.

Hank's Balanced Salt Solution (HBSS) pH 7.4

	g/l
$CaCl_2 . 2H_2O$	0.185
KCl	0.40
KH_2PO_4	0.06
$Mg\ Cl_2 . 6H_2O$	0.10
$Mg\ SO_4 . 7H_2O$	0.10
NaCl	8.00
$Na\ HCO_3$	0.35
$Na_2\ HPO_4$	0.48
D–glucose	1.00
Phenol red	0.01

APPENDIX VII

IL-1β RIA KIT – ASSAY PROTOCOL*

	Total Counts (TC)	Non-specific binding (NSB)	Zero standards (Bo)	Standards**	Samples
Buffer	–	200	100	–	–
Standard	–	–	–	100	–
Sample	–	–	–	–	100
Antiserum	–	–	100	100	100
	Vortex mix, cover tubes and incubate at room temperature (15–30°C) for 4 hours				
[^{125}I]IL-1β	100	100	100	100	100
	Vortex mix, cover tubes and incubate at 4°C for 16–24 hours				
Amerlex-M second antibody	–	250	250	250	250
	Vortex mix. Incubate for 10 minutes at room temperature. Separate **either** using Amerlex-M separator for 15 minutes **or** by centrifugation for 10 minutes at >1500g. Decant supernatants, drain for 5 minutes and count.				

* All volumes are microlitres.

** Standards include 0, 0.3, 0.6, 1.25, 2.5, 5, 10, 20 and 40fmol IL-1β/tube

APPENDIX VIII

IL–2 RIA KIT – ASSAY PROTOCOL*

	Total Counts (TC)	Non–specific binding (NSB)	Zero standards (Bo)	Standards**	Samples
Buffer	–	200	100	–	–
Standard	–	–	–	100	–
Sample	–	–	–	–	100
Antiserum	–	–	100	100	100
Vortex mix, cover tubes and incubate at room temperature (15–30°C) for 1 hour or for 4 hours					
[^{125}I]IL–2	100	100	100	100	100
Vortex mix, cover tubes and incubate atroom tepmerature for 3 hours or at 4°C for 16–24 hours					
Amerlex–M second antibody	–	250	250	250	250
Vortex mix. Incubate for 10 minutes at room temperature. Separate **either** using Amerlex–M separator for 15 minutes **or** by centrifugation for 10 minutes at 1500g. Decant supernatants, drain for 5 minutes and count.					

* All volumes are microlitres.

** Standards include 0, 2.5, 5, 10, 20, 40, 80, 160 and 320 fmol IL–2/tube

APPENDIX IX

TNFα RIA KIT – ASSAY PROTOCOL*

	Total Counts (TC)	Non–specific binding (NSB)	Zero standards (Bo)	Standards**	Samples
Buffer	–	200	100	–	–
Standard	–	–	–	100	–
Sample	–	–	–	–	100
Antiserum	–	–	100	100	100
	Vortex mix, cover tubes and incubate at room temperature (15–30°C) for 4 hours or overnight (16–24 hours) at 37°C				
[^{125}I]TNFα	100	100	100	100	100
	Vortex mix, cover tubes and incubate at 4°C for 16–24 hours				
Amerlex–M second antibody	–	250	250	250	250
	Vortex mix. Incubate for 10 minutes at room temperature. Separate **either** using Amerlex–M separator for 15 minutes **or** by centrifugation for 10 minutes at >1500g. Decant supernatants, drain for 5 minutes and count.				

* All volumes are microlitres.

** Standards include 0, 1, 2, 3, 9, 7.8, 15.6, 31.2, 62.5 and 125 fmol TNFα/tube

APPENDIX X

cAMPRIA KIT – ASSAY PROTOCOL*

	Total Counts (TC)	Zero standards (Bo)	Standards**	Samples
Buffer	–	100	–	–
Standard	–	–	100	–
Samples	–	–	–	100
[^{125}I]cAMP	100	100	100	100
Antiserum	–	100	100	100
	Vortex mix, cover tubes and incubate at 4^{o}C			
Amerlex–M second antibody	–	500	500	500

Vortex mix. Incubate for 10 minutes at room temperature. Separate either using Amerlex–M separator for 15 minutes or by centrifugation for 10 minutes at >1500g. Decant supernatants, drain for 5 minutes and count.

* All volumes are microlitres.

** Standards include 0, 25, 50, 100, 200, 400, 800 and 1600 fmol cAMP/tube

APPENDIX XI

BIOCLONE PROGESTERONE RIA KIT – ASSAY PROTOCOL

TUBE DESCRIPTION	STANDARD OR SAMPLE (µl)	PROGESTERONE ANTISERUM (µl)	125I PROGESTERONE (µl)	PRECIPITATING SOLUTION (µl)
TC	–	–	200	–
NSB	50 (0 STD)	–	200	500
0 nmol/l STD	50	200	200	500
1 nmol/l STD	50	200	200	500
2 nmol/l STD	50	200	200	500
5 nmol/l STD	50	200	200	500
10 nmol/l STD	50	200	200	500
20 nmol/l STD	50	200	200	500
50 nmol/l STD	50	200	200	500
100 nmol/l STD	50	200	200	500
SAMPLE	50	200	200	500

Vortex and incubate at 37°C for 30 minutes.

Incubate at room temperature for 15 minutes.
Vortex then centrifuge for 20 mins at 3290 X G.
Decant supernatant, drain, blot and count pellet.

Phosphate–BSA Buffer (PBS–BSA) 0.1M pH7.0

	g/l
$NaH_2PO_4.2H_2O$	6.1
$Na_2HPO_4.2H_2O$	10.9
NaCl	9.3
NaN_3	1.0
BSA	1.0

APPENDIX XII

SPECTRIA ESTRADIOL COATED TUBE RIA KIT – ASSAY PROTOCOL*

Procedure	Total	NSB	Standard	Control sera	Unknown
Pipette sample		100*	100	100	100
Pipette estradiol [125I]	200	200	200	200	200
Pipette estradiol antiserum			200	200	200
Pipette distilled water		200			
Mix		X	X	X	X
Incubate 3h/37°C		X	X	X	X
Decant		X	X	X	X
Wash with washing solution		1000	1000	1000	1000
Count for 1 min	X	X	X	X	X

* All volumes are given in microlitres.
** Standard include 0, 0.05, 0.15, 0.5, 1.5, 5.0 and 15.0 pnmol.

REFERENCES

Aarden, L., Lansdorp, P., and Degroot, E. (1985). A growth factor for B cell hybridomas production by human monocytes. Lymphokines **10**:175–85.

Adashi, E.Y. and Hsueh, A.J.W. (1982). Estrogens augment the stimulation of ovarian aromatase activity of follicle–stimulating hormone in cultured rat granulosa cells. J. Biol. Chem. **257**:6077–83.

Adashi, E.Y., Resnick, C.E., D'Ercole, A.J., Svoboda, M.E. and Van Wyk, J.J. (1985). Insulin–like growth factors as intraovarian regulators of granulosa cell growth and function. Endocr. Rev. **6**:400–20

Adashi, E.Y., Resnick, C.E., Svoboda, M.E. and Van Wyk, J.J. (1986). Follicle–stimulating hormone enhances somatomedin–C binding to cultured rat granulosa cells: evidence for cAMP dependence. J. Biol. Chem. **261**:3923–6.

Adashi, E.Y., Resnick, C.E., Hernandez, E.R., Svoboda, M.E. and Van Wyk, J.J. (1988a). Characterization and regulation of a specific cell membrane receptor for somatomedin–C/insulin–like growth factor I in cultured rat granulosa cells. Endocrinology **122**:194–201.

Adashi, E.Y., Resnick, C.E., Svoboda, M.E. and Van Wyk, J.J. (1988b). *In vivo* regulation of granulosa cell somatomedin–C/insulin–like growth factor I receptors. Endocrinology **122**:1383–90.

Adashi, E.Y. (1989). Editorial cytokine–mediated regulation of ovarian function: encounters of a third kind. Endocrinology 124:2043–45.

Adashi, E.Y., Resnick, C., Hernandez, E.R., Svoboda, M.E., Hoyt, E., Clemmons, D.R., Lund, P.K. and Van Wyk, J.J. (1989a). Rodent studies on the potential relevance of insulin–like growth factor (IGF–1) to ovarian physiology. In: Anne N. Hirshfield (ed.). Growth Factors and the Ovary. Plenum Press, New York and London. p95–105.

Adashi, E.Y., Resnick, C.E., Croft, C.S. and Payne, D.W. (1989b). Tumor necrosis factor α inhibits gonadotropin hormonal action in non transformed ovarian granulosa cells. J. Biol. Chem. **264**:11591–6.

Adashi, E.Y. (1990). The potential relevance of cytokines to ovarian physiology: the emerging role of resident ovarian cells of the white blood series. Endocrine. Rev. **11**:454–64.

Adashi, E.Y., Resnick, C.E., Packman, J.N., Hurwitz, B.A. and Payne, D.W. (1990). Cytokine–mediated regulation of ovarian function: tumor necrosis factor α inhibits gonadotropin–supported progesterone accumulation by differentiating and luteinized murine granulosa cells. Am. J. Obstet. Gynecol. **162**:889–99.

Alila, H.W. and Hansel, W. (1984). Induction of lymphopenia causes luteal dysfunction in cattle. Biol. Reprod. **31**:671–8.

Allen, L.S., McClure, J.E., Goldstein, A.L., Barkley, M.S. and Michael, S.D. (1984). Estrogen and thymic hormone interactions in the female mouse. J. Reprod. Immunol. **6**:25–37.

Amsterdam, A., Koch, Y., Lieberman, M.E. and Lindner, H.R. (1975). Distribution of binding sites from human chorionic gonadotropin in the preovulatory follicle of the rat. J. Cell. Biol. **67**:894–900.

Amsterdam, A. and Rotmensch, S. (1987). Structure–function relationships during granulosa cell differentiation. Endocr. Rev. **8**:309–37.

Anderson, D.J. and Hill, J.A. (1988). Cell mediated immunity in infertility. Am. J. Reprod. Immunol. Microbiol. **17**:22–30.

Andreani, C.L., Payne, D.W., Packman, J.N., Resnick, C.E., Hurwitz, A. and Adashi EY. (1991). Cytokine–mediated regulation of ovarian function. Tumor necrosis factor α inhibits gonadotropin–supported ovarian androgen biosynthesis. J. Biol. Chem. **266**: 6761–66.

Aono, T., Kitamura, Y., Fukuda, S. and Matsumoto, K. (1981). Localization of 4–ene–5α–reductase, 17β–ol–dehydrogenase and aromatase in immature rat ovary. J. Steroid Biochem. **14**:1369–77.

Arai, K., Lee, F., Miyajima, A., Miyatake, S., Arai,, N. and Yokota, T. (1990). Cytokines: co–ordinators of immune and inflammatory responses. Annu. Rev. Biochem. **59**: 783–836.

Azad, N., Emanuele, N.V., Halloran, M.M., Tentler, J. and Kelley, M.R. (1991). Presence of luteinizing hormone–releasing hormone (LHRH) mRNA in rat spleen lymphocytes. Endocrinology **128**:1679–81.

Azoulay, M., Webb, C.G. and Sachs, L. (1987). Control of hematopoietic cell growth regulators during mouse fetal development. Mol. Cell. Biol. **7**:3361–4.

Azuma, C., Tanable, T., Konishi, M., Kinashi, T., Noma, T., Matsuda, F., Yaoita, Y., Takatsu, K., Hammarstrom, L., Smith, C.I.E., Severinson, E. and Honji, T. (1987). Cloning of cDNA for human T–cell replacing factor (interleukin–5) and comparison with the murine homologue. Nucl. Acids Res. **14**:9149–58.

Bagavandoss, P., Kunkel, S.L., Wiggins, R.C. and Keyes, P.L. (1988). Tumor necrosis factor α (TNF–α) production and localization of macrophages and T lymphocytes in the rabbit corpus luteum. Endocrinology **122**:1185–7.

Bagavandoss, P., Wiggins, R.C., Kunkel, S.L., Remick, DG. and Keyes P.L. (1990). Tumor necrosis factor production and accumulation of inflammatory cells in the corpus luteum of pseudopregnancy and pregnancy in rabbits. Biol. Reprod. **42**:367–76.

Baird, A., Emoto, N., Shimasaki, S., Gonazalez, A.M., Fauser B. and Hsueh, J.W. (1989). Fibroblast growth factors as local mediators of gonadal function. In: Anne N. Hirshfield (ed). Growth Factor and the ovary. Plenum Press, New York and London. p151–60.

Banks R.E., Evans, S.W. and Whicher, J.T. (1991). Polypeptide regulatory factors in pregnancy. Contemp. Rev. Obstet. Gynaecol. **3**:22–9.

Batta, S.K., Wentz, A.C. and Channing, C.P. (1980). Steroidogenesis by human ovarian cell types in culture: influence of mixing of cell types and effect of added testosterone. J. Clin. Endocrinol. Metab. **50**:274–9.

Batticane, N., Morale, M.C., Gallo, F., Farinella, Z. and Marchetti, B. (1991). Luteinizing hormone–releasing hormone signalling at the lymphocyte involves stimulation of interleukin–2 receptor expression. Endocrinology **129**:277–86.

Beach, J.E., Smallridge, R.C., Kinzer, C.A., Bernton, E.W., Holaday, J.W. and

Fein, H.G. (1989). Rapid release of multiple hormones from rat pituitaries perifused with recombinant interleukin-1. Life Sci. 44:1-7.

Beckmann, M.W., Polacek, D., Seung, L. and Schreiber, J.R. Human ovarian granulosa cell culture: determination of blood cell contamination and evaluation of possible culture purification steps. (1991). Fertil. Steril., **56**:881-7.

Beers, W.H. (1975). Follicular plasminogen and plasminogen activator and the effect of plasmin on ovarian follicle wall. Cell **6**:379-86.

Behrman, H.R. and Armstrong, D.T. (1969). Cholesterol esterase stimulation by luteinizing hormone in luteinized rat ovaries. Endocrinology **85**: 474-80.

Ben-David, M. and Schenker, J.G. (1982). Human ovarian receptors to human prolactin: implications in infertility. Fertil. Steril. **38**:182-6.

Berkowitz, R.S., Faris, H.M., Hill, J.A. and Anderson, D.J. (1990). Localization of leukocytes and cytokines in chorionic villi of normal placenta and complete hydatidiform moles. Gynaecol. Oncol. **37**:396-400.

Beutle, B. (1988). Tumor necrosis, cachexia, shock, and inflammation: a common mediator. Ann. Rev. Biochem. **57**:505-18.

Beverley, P. Chapter 58. Human leucocyte subpopulations. In: Weir, D.M., Herzenberg, L.A., Blackwell, C., Herzenberg, Leonore A. (eds). Handbook of experimental immunology in four volumes Vol. 2: Cellular immunology. (1986). Blackwell Scientific Publications, 58.1-58.9.

Bieszczad, R.R., McClintock, J.S., Pepe, G.J. and Dimino, M.J. (1982). Progesterone secretion by granulosa cells from different sized follicles of human ovaries after short term incubation. J. Clin. Endocrinol. Metab. **55**:181-4.

Bjersing, L. (1967). Histochemical demonstration of Δ-5-3β- and 17(-hydroxysteroid dehydrogenase activities in porcine ovary. Histochem. **10**:295-304.

Bjersing, L. and Carstensen, H. (1967). Biosynthesis of steroids by granulosa cells of the porcine ovary *in vitro*. J. Reprod. Fertil. **14**:101-11.

Bjersing, L. (1977). Ovarian Histochemistry. In: Zuckerman, L., Weir, B.J. (eds). The Ovary. Vol 1. Academic Press, New York. p303.

Boehm, K.D., Kelley, M.F., Ilan, J. and Iland, J. (1989). The interleukin 2 gene is expressed in the syncytiotrophoblast of the human placenta. Proc. Natl. Acad. Sci. USA **86**:656-60.

Bogovich, K. and Richards, J.S. (1982). Androgen biosynthesis in developing ovarian follicles: evidence that luteinizing hormone regulates thecal 17α-hydroxylase and C_{17-20}-lyase activities. Endocrinology **111**:1201-8.

Bohma, M. and Donat, H. (1989). Detection of T-lymphocytes in human endometrium. Zentralbl-Gynakol (German) (Abstract in English from MEDLINE) **11**:1205-14.

Bomsel-Helmreich, O., Gougeon, A., Thebault, A., Saltarelli, D., Milgrom, E., Frydman, R. and Papiernik, E. (1979). Heathly and atretic human follicles in the preovulatory phase: differences in evolution of follicualr morphology and steroid content of follicular fluid. J. Clin. Endocrinol. Metab. **48**:686-94.

Boyd, G.S., Arthur, J.R., Beckett, G.J., Mason, J.I., Trzeciak, W.H. (1975). The role of cholesterol and cytochrome P-450 in the cholesterol side-chain cleavage reaction in adrenal cortex and corpora lutea. J. Steroid Biochem. **6**:427-36.

Braquet, P., and Rola-Pleszynski, M. (1977). Platelet-activating factor and cellular immune responses. Immunol. Today **8**:345-52.

Bukovsky, A and Caudle, M.R. (1992). Tumor necrosis factor-alpha is expressed in rat pituitary but not in rat or human ovaries. Biol Reprod. Suppl. **46**(1) Abstract 28, pp57.

Brown, K.A., Roy, F. Le, Noble, G., Bacon, K., Camp, R., Vora, A. and Dumonde, D.C. (1991). Interleukin 8 acts on monocytes to increase their attachment to cultured endothelium and enhance the expression of surface adhesion molecules. In: Westwick, J., Lindley, I.J.D. and Kunkel, S.L. (eds). Chemotactic Cytokines. Biology of the inflammatory peptide supergene family. Plenum Press, New York and London. p 161-2.

Brown, K.M., Muchmore, A.V. and Rosenstreich, D.L. (1986). Uromodulin, an immunosuppressive protein derived from pregnancy urine, is an inhibitor of interleukin 1. Immunology **83**:9119-23.

Bryant-Greenwood, G.D. (1982). Relaxin as a new hormone. Endocr. Rev. **3**:62-90.

Bulmer D. (1964). The histochemistry of ovarian macrophages in the rat. J. Anat. Lond. **98**:313-9.

Bulmer, J.N. and Johnson, P.M. (1984). Macrophage populations in the human placenta and amniochorion. Clini. Exp. Immunol. **57**: 393-403.

Bulmer, J.N. and Sunderland, C.A. (1984). Immunohistological characterization of lymphoid cell populations in the early human placental bed. Immunology **52**:349-57.

Bulmer, J.N. and Ritson, A. (1988). The decidua in early pregnancy. In: Beard, R.W. and Shar, F. (eds.), Early Pregnancy Loss: Mechanisms and Treatment. Peacock Press, Ashton-under-Lyne, England. pp171-82.

Caffrey, J.L., Fletcher, P.W., Diekman, M.A., O'Callaghan, P.L., Niswender, G.D. (1979). The activity of ovine luteal cholesterol esterase during several experimental conditions. Biol. Reprod. **21**:601-8.

Canipari, R. and Strickland, S. (1986). Studies on the hormonal regulation of plasminogen activator production in the rat ovary. Endocrinology **118**:1652-9.

Cannon, J.G. and Dinarello, C.A. (1985). Increased plasma interleukin-1 activity in women after ovulation. Science **227**:1247-9.

Carbone, A., Piantelli, M., Musiani, P., Larocca, L.M., Aiello, F.B., Maggiano, N., Scoppetta, C., Crucitti, F. and Ranelletti, F.O. (1986). Estrogen binding sites in peripheral blood mononuclear cells and thymocytes from 2 myasthenia gravis patients. J. Clin. Lab. Immunol. **21**:87-91.

Carson, R.S., Trounson, A.O., and Findlay, J.K. (1982). Successful fertilisation of human oocytes *in vitro*: concentration of estradiol-17β, progesterone and androstenedione in the antral fluid of donor follicles. J. Clin. Endocrinol. Metab. **55**:798-800.

Carswell, E.A., Old, L.J., Kassel, R.L., Green, S., Fiore, N., and Williamson, B. (1975). An endotoxin–induced serum factor that causes necrosis of tumors. Proc. Natl. Acad. Sci. USA. **72**:3666–70.

Casper, R.F. and Erickson, G.F. (1981). *In vitro* heteroregulation of LH receptors by prolactin and FSH in rat granulosa cells. Mol. Cell. Endocrinol. **23**:161–71.

Castilla, J. A., Sampalo, A., Molina, R., Samaniego, F., Mozas, J., Vergara, F., Garrido, F. and Herruzo, A.J. (1990). Mononuclear cell subpopulations in human follicular fluid from stimulated cycles. Am. J Immunol. Reprod. **22**:127–9.

Catt, K.J., Harwood, J.P., Aguilera G. and Dufau, M.L. (1979). Hormonal regulation of peptide receptors and target cell response. Nature **280**:109–16.

Caubo, B. and Tonetta, S.A. (1989). Binding sites for IGF–I identified on theca cells from large porcine follicles. In: A.N., Hirshfield (ed.). Growth Factors and the Ovary. Plenum Press. New York and London p169–74.

Cavender, J.L. and Murdoch, W.J. (1988). Morphological studies of the microcirculatory system of periovulatory ovine follicles. Biol. Reprod. **39**:989–97.

Chaiet, L. and Wolf FJ. (1964). The properties of streptavidin, a biotin–binding protein production by streptomyces. Arch. Biochem. Biophys. **106**:1–8.

Chang, R.J. and Jaffe, R.B. (1978). Progesterone effects on gonadotrophin release in women pretreated with estradiol. J. Clin. Endocrinol. Metab. **47**:119–25.

Chang, R.J. and Lee, S.H. (1986). Effects of interfeon–gamma and tumor necrosis factor–alpha on the expression of an Ia antigen on a murine macrophage cell line. J. Immunol. **137**:2853–6.

Channing, C.D. and Kammerman, S. (1974). Binding of gonadotropins to ovarian cells. Biol. Reprod. **10**:179–98.

Chegini, N., Lei, Z.M. and Rao, C.V. (1989). Light and electron microscope immunocytochemical localization of epidermal growth factors in bovine corpora lutea of pregnancy. In: A.N. Hirshfield (ed.). Growth Factors and the Ovary. Plenum Press. New York and London, p213–220.

Chen, B. D.M., and Najor, F. (1987). Macrophage activation by interferon α and β is associated with a loss of proliferative capacity. Cell. Immunol. **106**:343–54.

Chen, T.T., Bukovsky, A. and Caudle, M.R. (1992). Tumor necrosis factor–alpha is expressed in rat pituitary byt not in rat or human opvaries. Biol. Reprod. Suppl. No. 1 Vol 46 p57.

Chin, T.W., Ank, B.J., Strom, S.R. and Stiehm, E.R. (1988). Enhanced interferon production and lymphokine–activated cytotoxicity of human placental cells. Cell Immunol. **113**:1–9.

Clinton, T.K., Roby, K.F., Lyles, R. and Terranova, P.F. (1991). Effects of TNFα and prolactin: increased hCG binding in human granulosa cells; differential interaction with hCG–stimulated progesterone secretion. In: Signaling mechanisms and gene expression in the ovary (Ed. Gibori G). Serono Symposia USA. Springer–Verlag, New York pp. 453–8.

Cohen, J.H.M., Danel, L., Cordier, G., Saez, S. and Revillard, J–P. (1983). Sex steroid receptors in peripheral T cells: absence of androgen receptors and restriction

of estrogen receptors to OKT8–positive cells. J. Immunol. **131**:2767–71.

Collins, T., Lapierre, L.A., Fiers, W., Strominger, J.L., and Pober, J.S. (1986). Recombinant human tumor necrosis factor increases mRNA levels and surface expression of HLA–A,B antigens in vascular endothelial cells and dermal fibroblasts *in vitro*. Proc. Natl. Acad. Sci. USA. **83**:446–50.

Conlon, P.J. Morrissey, P.J., Nordan, R.P., Grabstein, K.H., Prickett, K.S., Reed, S.G., Goodwin, R., Cosman, D., and Namen, A.E. (1989). Murine thymocytes proliferate in direct response to interleukin– 7. Blood **74**:1368–73.

Copenhaver, W.M. (1964). The female reproductive system. In. revised by Copenhaven, W.M. (ed). Bailey's Textbook of Histology. Baltimore, Calcutta. Sans Tache Press pp516–28.

Costa, O., Mulchahey, J.J. and Blalock, J.E. (1990). Structure and function of luteinizing hormone–releasing hormone (LHRH) receptors on lymphocytes. Prog. Neuro. Endocrin. Immunol. **3**:55–60.

Crainie, M., Guilbert, L. and Wegmann, T.G. (1990). Expression of novel cytokine transcripts in the murine placenta. Biol. Reprod. **43**:999–1005.

Crisp, T.M. (1977). Hormone requirements for early maintenance of rat granulosa cell cultures. Endocrinology **101**:1286–97.

Cupps, P.T., Laben, R.C. and Meed, S.W. (1959). Histology of pituitary, adrenal and reproductive organs in normal cattle with lowered reproductive efficiency. Hilgardia **29**:383–99.

Danel, L., Souweine, G., Monier, J.C. and Saez, S. (1983). Specific estrogen binding sites in human lymphoid cells and thymic cells. J. Steroid Biochem. **18**:559–63.

Daniel, S.A.J. and Armstrong, D.T. (1980). Enhancement of follicle–stimulating hormone–induced aromatase by androgens in cultured rat granulosa cells. Endocrinology **107**:1027–33.

Daniel, S.A.J. and Armstrong, D.T. (1984). Site of action of androgens on follicle–stimulating hormone–induced aromatase activity in cultured rat granulosa cells. Endocrinology, **114**:1975–82.

Darbon, J.M., Oury, F., Laredo, J. and Bayard, F. (1989). Tumor necrosis factor–alpha inhibits follicle–stimulating hormone–induced differentiation in cultured rat granulosa cells. Biochem. Biophys. Res. Commun. **163**:1038–48.

Davies, T.F., Katikineni, M., Chan, V., Harwood, J.P., Dufau, M.L. and Catt, K.J. (1980). Lactogenic receptor regulation in hormone–stimulated steroidogenic cells. Nature **283**:863–5.

de Jong F.H. and Sharpe, R.M. (1976). Evidence for inhibin–like activity in bovine follicular fluid. Nature **263**:71–2.

Demura, R., Ono, M., Demura, H., Shizume, K. and Oouchi, H. (1982). Prolactin directly inhibits basal as well as gonadotropin–stimulated secretion of progesterone and 17β–estradiol in the human ovary. J. Clin. Endocrinol. Metab. **54**:1246–50.

Dinarello, C.A. and Mier, J.W. (1987). Lymphokines. N. Engl. J. Med. **317**:940–5.

Dinarello, C.A., Cannon, J.G., Wolff, S.M., Bernheim, H.A., Beutler, B., Cerami, A., Figari, I.S., Palladine, M.A. and O'Connor, J.V. (1986). Tumor necrosis factor (cachectin) is an endogenous pyrogen and induces production of interleukin 1. J. Exp. Med. **163**:1433–50.

Doisy, E.A., Veler, C.D and Thayer, S. (1929). Folliculin from urine of pregnant women. Am.J.Physiol. **90**:329–30.

Dorrington, J.H., Moon, Y.S and Armstrong, D.T. (1975). Estradiol–17β biosynthesis in cultured granulosa cells from hypophysectomized immature rats: stimulation by follicle–stimulating hormone. Endocrinology. **97**:1328–31.

Dorrington, J.H. and Armstrong, D.T. (1979). Effect of FSH on gonadal functions. Rec. Prog. Horm. Res. **35**:301–42.

Droesch, K., Rosenwaks, Z., Fulgham, D.L., Alexander, N.J. and Liu, H.C. (1988). Distribution of T cell subsets in follicular fluid. Fertil. Steril. **50**:618–21.

Dudley, D.J., Mitchell, M.D., Creighton, K. and Branch, D.W. (1990). Lymphokine production during term human pregnancy: differences between peripheral leukocytes and decidual cells. Am. J. Obstet. Gynecol. **163**:1890–3.

Durham, C.R., Zhu, H., Masters, B.S.S., Simpson, E.R.. and Mendelson, C.R. (1985). Regulation of aromatase activity of rat granulosa cells: induction of synthesis of NADPH–cytochrome P–450 reductase by FSH and dibutyryl cyclic AMP. Mol. Cell. Endocrinol. **40**:211–9.

Durum, S.K., Schmidt, J.A., and Oppenheim, J.J. (1985). Interleukin 1: An immunological perspective. Annu. Rev. Immunol. **3**: 263–87.

Durum, S.K. and Oppenheim, J.J. (1989). Macrophage–derived mediators interleukon 1, tumor necrosis factor, interleukin 6, interferon, and related cytokines. In: William E, Paul (ed.). Fundamental Immunology, second edition. Raven Press Ltd., New York. p639–61.

Ebaugh, M.J. and Smith, E.M. (1988). Human lymphocyte production of immunoreactive luteinizing hormone. FASEB J. **2**:A1642 (Abstr7811).

Eckstein, B. and Nimrod A. (1977). Properties of microsomal Δ–3–ketosteroid 5α–reductase in immature rat ovary. Biochem. Biophy. Acta. **499**:1–9.

Eichler, F. and Keiling, R. (1988). Variations in the percentages of lymphocyte subtypes during the menstrual cycle in women. Biomed. Pharmacother. **42**:285–8.

Emi, N., Kanzaki, H., Yoshida, M., Takakura, K., Kariya, M., Okamoto, N., Imai, K. and Mori, T. (1991). Lymphocytes stimulate progesterone production by cultured human granulosa luteal cells. Am. J. Obstet. Gynaecol. **165**:1469–74.

Emoto, N. and Baird A. (1988). The effect of tumor necrosis factor/cachectin on follicle–stimulating hormone–induced aromatase activity in cultured rat granulosa cells. Biochem. Biophys. Res. Commun. **153**:792–8.

Engels, J.A., Friedlander, R.L. and Eik–Nes, K.B. (1968). An effect *in vivo* of clomiphene on the rate of conversion of androstenedion–C^{14} to estrone–C^{14} and estradiol–C^{14} by the canine ovary. Metabolism **17**:189–98.

Erickson, G.F., Wang, C. and Hsueh, A.J.W. (1979). FSH induction of functional LH receptors in granulosa cells cultured in a chemically defined medium. Nature

279:336–8.

Erickson, G.F., Casper, R., Hofeditz, C. (1983). Role of serum–free defined medium in regulation of LH receptor in cultured rat granulosa cells. Mol. Cell. Endocrinol. **30**:37–50.

Erickson, G.F., Magoffin, D.A., Dyer, C.A. and Hoffeditz, C. (1985). The ovarian androgen producing cells: a review of structure/function relationship. Endocr. Rev. **6**:371–99.

Espey, L.L. and Lipner, H. (1965). Enzyme–induced rupture of rabbit graafian follicle. Am. J. Physiol. **208**:208–13.

Espey, L.L. (1967). Ultrastructure of the apex of the rabbit graafian follicle during the ovulatory process. Endocrinology **81**:267–76.

Espey, L.L. and Rondell, P. (1967). Estimation of mammalian collagenolytic activity with a synthetic substrate. J. Appl. Physiol. **23**:757–61.

Espey, L.L. (1971). Decomposition of connective tissue in rabbit ovarian follicles by multivesicular structures of thecal fibroblasts. Endocrinology **88**:437–44.

Espey, L.L. (1980). Ovulation as an inflammatory reaction – a hypothesis. Biol. Reprod. **22**:73–106.

Evans, G., Lischinsky, A., Daniel, S.A.J. and Armstrong, D.T. (1984). Androgen–inhibition of FSH–stimulated progesterone production by granulosa cells of pre–pubertal pig. Can. J. Physiol. Pharmacol. **62**:840–45.

Farkash, Y., Timberg, R and Orly, J. (1986). Preparation of antiserum to rat cytochrome P–450 cholesterol side chain cleavage, and its use for ultrastructural localization of the immunoreactive enzyme by protein A–gold technique. Endocrinology. **118**:1353–65.

Fanjul, L.F., de Galarreta, R. and Hsueh, A.J.W. (1983). Progestin augmentation of gonadotropin–stimulated progesterone production by cultured rat granulosa cells. Endocrinology **112**:405–7.

Favier, R., Edelman, P., Mary J–Y., Sadoul, G. and Douay, L. (1990). Presence of elevated serum interleukin–2 levels in pregnant women. New Eng. J. Med. **322**:270.

Fibbe, W.E., van–Damme, J., Billiau, A., Goselink, H.M., Voogt–PJ, van–Eeden, G. (1988). Interleukin 1 induces human marrow stromal cells in long–term culture to produce granulocyte colony–stimulating factor and macrophage. Blood **71**:430–5.

Findlay, J.K., Zhang, Z.W., Hulchinson, L.A., Carson, R.S., Burger, H.G. and Herington, A.G. (1991). Intrafollicular roles of inhibin, interferon–α, and related peptides. In: Haseltine FP and Findlay, JK (eds). Growth Factors in Fertility Regulation. Cambridge University Press, Cambridge. pp 195–208.

Fisch, B., Margara, R.A., Winston, R.M.L. and Hiller, S.G. (1989). Cellular basis of luteal steroidogenesis in the human ovary. J. Endocrinology **122**:303–11.

Fishman, J. (1982). Biochemical mechanisms of aromatization. Cancer Res. **42**:3277S–9S.

Flynn, A., Finke, J.H. and Hilfiker, M.L. (1982). Placental mononuclear

phagocytes as a source of interleukin-1. Science **218**:475-7.

Forage, R.G., Ring, J.M., Brown, R.W., McInerney, B.V., Cobon, G.S., Gregson, R.P., Robertson, D.M., Morgan, F.J., Hearn, M.T., Findlay, J.K., Wettenhall, R.E.H., Burger, H.G. and de Kretser, D.M. (1986). Cloning and sequence analysis of cDNA species coding for the two subunits of inhibin from bovine follicular fluid. Proc. Natl. Acad.. Sci. USA. **83**:3091-5.

Forbes, I.J. and Leong, A. S-Y. (1987). The lymphoid tissues. In. Forbes I.J. and Leong, A. S-Y. (eds). Essential oncology of the lymphocyte. Springer-Verlag. The Dorset Press, Dorchester. p93.

Fortune, J.E. and Hansel, W. (1979). The effects of 17β-estradiol on progesterone secretion by bovine theca and granulosa cells. Endocrinology **104**:1834-8.

Friedman, R.M. (1988). Interferons. In: Textbook of immunophysiology, J.J. Oppenheim and E.M. Shevach. Oxford Press, New York.

Fukuda, S., Terakawa, N., Sato, B., Imori, T. and Matsumoto, K. (1979). Hormonal regulation of activities of 17β-ol-dehydrogenases, aromatase and 40-ene-5α-reductase in immature ovaries. J. Steroid Biochem. **11**:1421-27.

Fukuoka, M., Mori, T., Taii, S. and Yasuda, K. (1988). Interleukin-1 inhibits luteinization of porcine granulosa cells in culture. Endocirnology **122**:367-9.

Fukuoka, M. Yasuda, K., Taii, S., Takakura, K. and Mori, T. (1989). Interleukin-1 stimulates growth and inhibits progesterone secretion in cultures of porcine granulosa cells. Endocrinology **124**:884-90.

Garman, T.D. Jacobs, K.A., Clark, S.C., and Raulet, D.H. (1987). B-cell-stimulatory factor 2 (beta2 inteferon) functions as a second signal for interleukin 2 production by murine T cells. Proc. Natl. Acad. Sci. USA. **84**:7629-33

Gauldie, J., Richards, C., Harnish, S., Lansdorp, P., and Baumann, H. (1987). Interferon beta-2/B cell stimulatory factor Type 2 shares identity with monocyte-derived hepatocyte-stimulating factor and regulates the major acute phase protein response in liver cells. Proc. Natl. Acad. Sci. USA **84**:7251-5.

Gearing, D.P., King, J.A., Gough, N.M. and Nicola, N.A. (1989). Expression cloning of a receptor for human granulocyte-macrophage colony-stimulating factor. EMBO J. **8**:3667-76.

Gibori, G. and Richards, J.S. (1978). Dissociation of two distinct luteotropic effects of prolactin: regulation of luteinizing hormone-receptor content and progesterone secretion during pregnancy. Endocrinology **102**:767-74.

Gillim, S.W., Christensen, A.K. and McLennan, C.E. (1969). Fine structure of the human menstrual corpus luteum at its stage of maximum secretory activity. Am. J. Anat. **126**:409-28.

Gillis, S. (1989). T-cell-derived lymphokines. In: William E. Paul (ed.). Fundamental Immunology, second edition. Raven Press Ltd., New York p621-38.

Glass, J.D., Fitz, T.A. and Niswender, G.D. (1984). Cytosolic receptor for estradiol in the corpus luteum of the ewe: variation throughout the estrous cycle and distribution between large and small steroidogenic cell types. Biol. Reprod. **31**:967–74.

Gleich, G.J. and Adolphson, C.R. (1986). The eosinophilic leukocyte: structure and function. Adv. Immunol. **39**:177–253.

Goff, A.K., Leung, P.C.K. and Armstrong, D.T. (1979). Stimulatory action of follicle–stimulating hormone and androgens on the responsiveness of rat granulosa cells to gonadotropins *in vitro*. Endocrinology **104**:1124–9.

Gore–Langton, R.E. and Armstrong, D.T. (1988). Follicular steroidogenesis and its control. In: E. Knobil and J. Neill et al (eds.). The Physiology of Reproduction. Raven Press. Ltd., New York. p331–85.

Gorospe, W.C., Tuchel, T. and Kasson, B.G. (1988). Gamma–Interferon inhibits rat granulosa cell differentiation in culture. Biochem. Biophys. Res. Commun. **157**:891–7.

Gorospe, W.C. and Kasson, B.G. (1989). Lymphokines from concanavalin–A–stimulated lymphocytes regulate rat granulosa cell steroidogenesis *in vitro*. Endocrinology **123**:2462–71.

Gospodarowicz, D., and Blatechi, H. (1979). Fibroblast and epidermal growth factors are mitogenic agents for cultured granulosa cells of rodent, porcine and human origin. Endocrinology **104**:757–64

Gottschall, P.E., Uehara, A., Hoffmann, S.T. and Arimura, A. (1987). Interleukin–1 inhibits follicle stimulating hormone–induced differentiation in rat granulosa cells *in vitro*. Biochem. Biophys. Res. Commun. **149**:502–9.

Gottschall, P.E., Katsuura, G,. Hoffman, S.T. and Arimura, A. (1988a). Interleukin 1: an inhibitor of luteinizing hormone receptor formation in cultured rat granulosa cells. FASEB J. **2**:2492–96.

Gottschall, P.E., Katsuura, G., Dahl, R.R., Hoffmann, S.T. and Arimura, A. (1988b) Discordance in the effects of interleukin–1 on rat granulosa cell differentiation induced by follicle–stimulating hormone or activator of adenylate cyclase. Biol. Reprod. **39**:1074–85.

Gorabstein, K.H., Urdal, D.L., Tushinnski, R.J., Mochizuki, D.Y., Price, V.L., Cantrell, M.A., Gillis, S. and Conlon, P.J. (1986). Induction of macrophage lumoricidal activity by granulocyte–macrophage colony–stimulating factor. Science **232**:506–8.

Gorabstein, K.H., Park. L.S., Morrisey, P.J., Sassenfeld, H., Price, V., Urdal, D.L., and Widmer, M.B. (1987). Regulation of murine T cell proliferation by B cell stimulatory factor–1. J. Immunol. **139**:1148–53.

Grody, W.W., Schrader, W.T. and O'Malley, B.W. (1982). Activation, transformation, and subunit structure of steroid hormone receptors. Endocr. Rev. **31**:141–63.

Guo, H., Calkins, J.H., Sigel, M.M. and Lin, T. (1990). Interleukin–2 is a potent inhibitor of leydig cell steroidogenesis. Endocrinology **127**:1234–9.

Hahlin, M., Dennefors, B., Johanson, C. and Hamberger L. (1988). Luteotropic

effects of prostaglandin E_2 on the human corpus luteum of the menstrual cycle and early pregnancy. J. Clin. Endocrinol. Metab. **66**:909–14

Hamond, J.M. (1981). Peptide regulators in the ovarian follicle. Aust. J. Biol. Sci. **34**:491–504.

Hefeneider, S.H., Henney, C.S., and Gillis, S. (1982). Natural Cell–Mediated Immunity, vol. 2, edited by R.B. Herberman. Academic Press, New York.

Henderson, K.M., Kieboom, L.E., McNatty, K.P., Lun, S., and Heath, D.A. (1984). ^{125}I–HCG binding to bovine thecal tissue from healthy and atretic antral follicles. Mol. Cell. Endocrinol. **34**:91–8.

Hernandez, E.R., Resnick, C.E., Svoboda, M.E., Van Wyk, J.J., Payne, D.W. and Adashi, E.Y. (1988). Somatomedin–C/insulin–like growth factor I as an enhancer of androgen biosynthesis by cultured rat ovarian cells. Endocrinology **122**:1603–12.

Herriot, D.M., Warnes, G.M. and Kerin, J.F. (1986). Pregnancy–related chemotactic activity of human follicular fluid. Ferti. Steri. **45**:196–201.

Herrmann, F., Schulz,G., Lindemann, A., Meyenburg, W., Oster, W., Krumwieh, D., and Metelsmann, R. (1988). Yeast–expressed granulocyte–macrophage colony–stimulating factor in cancer patients: a phase ib clinical study. Behring Ist. Mitt. **83**:107–18.

Hill, J.A., Haimovici, F. and Anderson, D.J. (1987a). Products of activated lymphocytes and macrophages inhibit mouse embryo development *in vitro*. J. Immunol. **139**:2250–4.

Hill, J.A., Barbieri, R.L. and Anderson, D.J. (1987b). Detection of T_8 (suppressor/cytotoxic) lymphocytes in human ovarian follicular fluid. Fertil. Steril. **47**:114–7.

Hillier, S.G., Knazek, R.A. and Ross, G.T. (1977). Androgenic stimulation of progesterone production by granulosa cells from preantral ovarian follicles: further *in vitro* studies using replicate cell cultures. Endocrinology **100**:1539–49.

Hillier, S.G., Zeleznik, A.J. and Ross, G.T. (1978). Independence of steroidogenic capacity and luteinizing hormone receptor induction in developing granulosa cells. Endocrinology **102**:937–46.

Hillier, S.G. (1981). Regulation of follicular oestrogen biosynthesis: a survey of current concepts. J. Endocrinol. **89**:3p–18p.

Hillier, S.G., van den Boogaard, A.J.M., Reichert, L.E. Jr., van Hall, E.V. (1981). Control of preovulatory follicular estrogen biosynthesis in the human ovary. J. Clin. Endocrinol. Metab. **52**:847–54.

Hillier, S.G. and de Zwart, F.A. (1982). Androgen/antiandrogen modulation of cyclic AMP–induced steroidogenesis during granulosa cell differentiation in tissue culture. Mol. Cell. Endocrinol. **28**:347–61.

Hillier, S.G., Fisch, B. and Wickings, E.J. (1987). Sex steroid biosynthesis in luteinized granulosa cells. In: Naftolin F, DeCherney AH (eds). The Control of Follicle Development, Ovulation and Luteal Function: Lessons From *In Vitro* Fertilization. Serono Symposia Publications from Raven Press Volume 35. New York., p65–78.

Hillier, S.G., Yong, E.L., Illingworth, P.J., Baird, D.T., Schwall, R.H. and Mason, A.J. (1991). Effect of recombinant inhibin on androgen synthesis in cultured human thecal cells. Mol. Cell Endocrinol. **75**:R1–6.

Ho, P.C., Tang, G.W., Fu, H.K., Fan, M.C., Lawton, J.W. (1988). Immunologic studies in patients with premature ovarian failure. Obstet. Gynecol. **71**:622–6.

Hoff, J.O., Quigley, M.E. and Yen, S.S.C. (1983). Hormonal dynamics at midcyle: a reevaluation. J. Clin. Endocrinol. Metab. **57**:792–6.

Hoffman, M.K., Gilber, K.M., Hirst, J.A., and Scheid, M. (1987). An essential role for interleukin 1 and a dual function for interleukin 2 in the immune response of murine B lymphocytes to sheep erythrocytes. J. Mol. Cell. Immunol. **3**:29–36.

Hsu, S–M., Raine, L. and Fanger, H. (1981). Use of avidin–biotin–peroxidase complex (ABC) in immunoperoxidase techniques: a comparison between ABC and unlabelled antibody (PAP) procedures. J. Histochem. Cytochem. **29**:577–80.

Hsueh, A.J.W., Welsh, Jr. T.H. and Jones, P.B.C. (1981). Inhibition of ovarian and testicular steroidogenesis by epidermal growth factor. Endocrinology **108**:2002–4.

Hsueh, A.J., Adashi, E.Y., Phillip, B.C., Jones, B.C. and Weish, T.H. Jr. (1984). Hormonal regulation of the differentiation of cultured ovarian granulosa cells. Endocr. Rev. **5**:76–127.

Hsueh, A.J., Dahl, K.D., Vaughan, J., Tucker, E., Rivier, J., Bardin, C.W. and Vale, W. (1987). Heterodimers and homodimers of inhibin subunits have different paracrine action in the modulation of luteinizing hormone–stimulated androgen biosynthesis. Proc. Natl. Acad. Sci. USA. **84**:5082–6.

Hume, D.A., Halpin, D., Charlton, H. and Gordon, S. (1984). The mononuclear phagocyte system of the mouse defined by immunohistochemical localization of antigen F4/80: macrophages of endocrine organs. Proc. Natl. Acad. Sci. USA. **81**:4174–7.

Hunt, J.S. (1989). Macrophages in human uteroplacental tissues: a review. Am. J. Reprod Immunol. **21**:119–22.

Hunter, M.G. and Armstrong, D.T. (1986). Estrogens inhibit steroid production by dispersed porcine thecal cells. Biol. Reprod. (Suppl. 1) **34**:196, (Abstr. 293).

Hurwitz, A., Ricciarelli, E., Botero, L., Rohan, R.M., Hernandez, E.R. and Adashi, E.Y. (1991). Endocrine– and autocrine–mediated regulation of rat ovarian (theca–interstitial) interleukin–1β gene expression: gonadotropin–dependent preovulatory acquisition. Endocrinology **129**: 3427–9.

Hylka, V.W., Caubo, B. and Tonetta, S.A. (1989). Analysis of binding sites for IGF–I on membranes from granulosa cells of small, medium, and large porcien follicles. In: A.N. Hirshfield (ed.). Growth Factors and the Ovary. Plenum Press. New York and London, p163–8.

Ireland, J.J. and Richards, J.S. (1978a). Acute effects of estradiol and follicle–stimulating hormone on specific binding of human [^{125}I]–iodo–follicle stimulating hormone to rat ovarian granulosa cells *in vivo* and *in vitro*. Endocrinology **102**:876–83.

Ireland, J.J.and Richars, J.S. (1978b). A previously undescribed role for luteinizing

hormone (LH:hCG) on follicular cell differentiation. Endocrinology **102**:1458–65.

Ito, A., Hirro, D., Sakyo K. and Mori. Y. (1987). The role of leukocyte factors on uterine cervical ripening and dilation. Biol. Reprod. **37**:511–7.

Ito, A., Hiro, D., Ojima, Y. and Mori, Y. (1988). Spontaneous production of IL–1–like factors from pregnant rabbit uterine cervix. Am. J. Obstet. Gynaecol. **159**:261–5.

Jäättelä, M., Kuusela, P. and Saksela, E. (1988). Demonstration of tumor necrosis factor in human amniotic fluids and supernatants of placental and decidual tissues. Lab. Invest. **58**:48–52.

Jacobs, B.R., Suchocki, S., Smith, R.G. (1980). Evidence for human ovarian progesterone receptor. Am. J. Obstet. Gynecol. **138**:332–6.

Jaiswal, K., Krishna, A. and Pandey, L.K. (1987). Human ovarian mast cells: distribution and degranulation pattern. Proceedings of the XVII Annual Conference of the Endocrine Society of India Vranasi p20 (Abstract).

Jensen, C.E. and Zacharie, F. (1958). Studies on the mechanism of ovulation. II. Isolation and analysis of acid mucopolysaccharides in bovine follicular fluid. Acta Endocrinol. **27**:356–68.

Johnson, E.M., Jones, L.A. and Kozak, R.W. (1992) Expression and function of insulin–like growth factors receptors on anti–CD3–activated human T lymphocytes. J. Immunol. **148**:63–71.

Jones, R.E., Duvall, D. and Guillette, L.J. Jr. (1980). Rat ovarian mast cells: distribution and cyclic changes. Anat. Rec. **197**:489–90.

Jones, P.B.C. and Hsueh, A.J.W. (1981). Direct stimulation of ovarian progesterone metabolizing enzyme by gonadotropin–releasing hormone in cultured granulosa cells. J. Biol. Chem. **256**:1248–54.

Jones, P.B.C. and Hsueh, A.J.W. (1982). Pregnenolone biosynthesis by cultured granulosa cells: modulation by follicle–stimulating hormone and gonadotropin––releasing hormone. Endocrinology **111**:713–21.

Jones, P.B.C., Welsh, Jr. T.H. and Hseuh A.J.W. (1982). Regulation of ovarian progestin production by epidermal growth factor in cultured rat granulosa cells. J. Biol. Chem. **257**:11268–73.

Junqueir, L.C., Carneiro, J. and Long, J.A. (1986). The female reproductive system. In: Junqueira, L.C., Carneiro, J. and Long, J.A. (eds). Basic Histology, Lange Medical Publications. Los. Altos. California, p491.

Kameda T., Matsuzaki, N., Sawai, K., Okada, T., Saji, F., Matsuda, T., Hirano, T., Kishimoto, T. and Tanizawa, O. (1990). Production of interleukin–6 by normal human trophoblast. Placenta **11**:205–13.

Karakawa, T., Karachi, K., Aono T. and Matsumoto, K. (1976). Formation of 5α–reduced C_{19}–steroids from progesterone *in vitro* by a pathway through 5α–reduced C_{21}–steroids in ovaries of late prepubertal rats. Endocrinology **98**:571–9.

Kase, N.G. (1990). The hormones of reproduction and how they work. In: Kase, N.G., Weingold, A.B. and Gershenson D.M. (eds). Principles and Practice of Clinical Gynaecology. Second Edition. Churchill Livingstone Inc. New York. p93.

Kasson, B.G.and Gorosope, W.C. (1989). Effects of interleukin-1, 2 and 3 on follicle-stimulating hormone-induced differentiation of rat granulosa cells. Mol. Cell. Endocrol. **62**:103-11.

Katabuchi, H., Fukumatsu, Y. and Okamura, H. (1989). Immunohistochemical and morphological observations of macrophages in the human ovary. In: A.N. Hirshfield (ed.). Growth Factors and the Ovary. Plenum Press, New York and London, p409-13.

Kauma, S., Matt, D., Strom, S., Eierman, D. and Turner T. (1990). Interleukin-1β, human leukocyte antigen HLA-DRα and transforming growth factor-β expression in endometrium, placenta, and placental membranes. Am. J. Obstet. Gynaecol. **163**:1430-7.

Kehrl, J.H., Miller, A., and Fauci, A.S. (1987). Effect of tumor necrosis factor alpha on mitogen-activated human B cells. J. Exp. Med. **166**:786-91.

Kelly, R.W., Deam, S., Cameron, M.J. and Seamark, R.F. (1986). Measurement by radioimmunoassay of prostaglandins as their methyl oximes. Prostaglandins Leukotr. Med. **24**:1-14.

Kennedy, T.G. (1987). Interactions of eicosanoids and other factors in blastocyst implantation. In Eicosanoids and Reproduction MTP Press Lancaster, p. 77-88.

Kerin, J.F., Warnes, G.M., Quinn, P., Kirby, C.A., Jeffrey, R., Matthews, C.D., Seamark, R.F. Texler, K., Antonas, B. and Cox, L.W. (1984). *In vitro* fertilization and embryo transfer program, Department of Obstetrics and Gynaecology, The University of Adelaide at The Queen Elizabeth Hospital, Woodville, South Australia.. J. In Vitro Fertil. and Embryo Transfer. **1**:63-71.

Killick, S., and Elstein, M. (1987). Pharmacologic production of luteinized unruptured follicles by prostaglandin synthetase inhibitors. Fertil. Steril. **47**:773-7.

King, A., Birkby, C. and Loke, Y.W. (1989). Early human decidual cells exhibit NK activity against K562 cell line but not against first trimester trophoblast. Cell Immunol. **118**:337-44.

Kirsch, T.M., Friedman, A.C., Vogel, R.L. and Flickinger, G.L. (1981). Macrophages in corpora lutea of mice: characterization and effects on steroid secretion. Biol. Reprod. **25**:629-38.

Kirsch T.M., Vogel, R.L. and Flickinger, G.L. (1983). Macrophages: a source of luteotropic cybernins. Endocrinology **113**:1910-12.

Kishimoto, T. (1985). Factors affecting B-cell growth and differentiation. Ann.Rev. Immunol. **3**:133-57.

Knecht, M. and Catt, K.J. (1983). Modulation of cAMP-mediated differentiation in ovarian granulosa cells by epidermal growth factor and platelet-derived growth factor. J. Biol. Chem. **258**:2789-94.

Knecht, M., Darbon, J.M., Ranta, T., Baukal, A.J. and Catt, K.J. (1984). Estrogens enhance the adenosine 3', 5'-monophosphate-mediated induction of follicle stimulating hormone and luteinizing hormone receptors in rat granulosa cells. Endocrinology **115**:41-9.

Knobil, E., Plant, T.M., Wildt, L., Belchetz, P.E. and Marshall, G. (1980). Control of the rhesus monkey menstrual cycle: permissive role of hypothalamic gonadotropin-releasing hormone. Science **207**:1371-3.

Koos, R.O. and Clark, M.R. (1982). Production of 6-keto-prostaglandin F1α by rat granulosa cells *in vitro*. Endocrinology **111**:1513-8.

Koppelman, M.C. and Dufau, M.L. (1982). Prolactin receptors in luteinized rat ovaries: unmarking of specific binding sites with detergent treatment. Endocrinology **111**:1350-7.

Krishna, A. and Terranova, P.F. (1985). Alterations in mast cell degranulation and ovarian histamine in the proestrous hamster. Biol. Reprod. **32**:1211-17.

Krishna, A., Beesley, K. and Terranova, P.F. (1989). Histamine, mast cells and ovarian function. J. Endocrinology **120**:363-71.

Kupper, T.S. (1991). Immune and inflammatory processes in cutaneous tissues. Mechanisms and speculations. J. Clin. Invest. **86**:1783-9.

Kuribayashi, K., Gillis, S., Kern, D.E. and Henney, C.S. Murine NK cell cultures: effects of interleukin-2 and interferon on cell growth and cytotoxic reactivity. (1981). J. Immuno. **126**:2321-7.

Lala, P.K., Kennedy, T.G. and Parhar, R.S. (1988). Suppression of lymphocyte alloreactivity by early gestational human decidua. II. Characterization of the suppressor mechanisms. Cell Immunol. **116**:411-22.

Lee, C., Keyes, P.L. and Jacobson, H.I. (1971). Estrogen receptor in the rabbit corpus luteum. Science **173**:1032-3.

Lee, G., Namen, A.E., Gillis,S., Ellingsworth, L.R., and Kincade, P.W. (1989). Normal B cell precursors responsive to recombinant murine IL-7 and inhibition of IL-7 activity by transforming growth factor-beta. J. Immunol. **142**:3875-83.

Lei, Z.M., Chegini, N. and Rao, CH.V. (1991). Quantitative cell composition of human and ovine corpora lutea from various reproductive states. Biol. Reprod. **44**:1148-56.

Leung, P.C.R., Goff, A.K., Kennedy, T.G., and Armstrong, D.T. (1978). An intraovarian inhibitory action of estrogen and androgen production *in vivo*. Biol. Reprod. **19**:641-7.

Leung, P.C.R. and Armstrong, D.T. (1979). Estrogen treatment of immature rats inhibits ovarian androgen production *in vitro*. Endocrinology **104**:1411-17.

Leung, P.C.R. and Armstrong, D.T. (1980). Further evidence in support of a short-loop feedback action of estrogen on ovarian androgen production. Life Sci. **27**:415-20.

Lieberman, M.E., Barnea, A., Bauminger, S., Tsafriri, A., Collins, W.P. and Lindner, H.R. (1975). LH effect on the pattern of steroidogenesis in cultured Graafian follicles of the rat: dependence on macromolecular synthesis. Endocrinology **96**:1533–42.

Lieberman, S., Greenfield, N.J and Wolfson, A. (1984). A heuristic proposal for understanding steroidogeneic processes. Endocr. Rev. **5**:128–48.

Ling, N., Ying, S., Ueno, N., Esch, F., Denoroy, L. and Guillemin, R. (1985). Isolation of partial characterization of a Mr 32000 protein with inhibin activity from porcine follicular fluid. Proc. Natl. Acad. Sci. U.S.A. **82**:7217–21.

Lipner, H. (1971). Ovulation from histamine depleted ovaries. Proc. Soc. Exp. Biol Med. **136**:111–4.

Lischinsky, A., Evans, G. and Armstrong, D.T. (1983). Site of androgen inhibition of FSH–stimulated progesterone production in porcine granulosa cell. Endocrinology **113**:1999–2003.

Lobb, D.K, Skinner, M.K and Dorrington, J.H. (1988). Rat theca/interstitial cells produce a mitogenic activity that promotes the growth of granulosa cells. Mol. Cell Endocrinol. **55**:209–17.

Lobb, D.K. and Dorrington, J.H. (1989). Bovine thecal cells secrete transforming growth factor α and β. In: Anne N. Hirshfield (ed.). Growth Factors and the Ovary. Plenum Press, New York and London. p199–203.

Loukides, J.A., Loy, R.A., Edwards, R., Honig, J., Visintin, I. and Polan ML. (1990). Human follicular fluids contain tissue macrophages. J. Clin. Endocrinol. Metab. **71**:1363–7.

Low, B.G. and Hansen, P.J. (1988). Actions of steroids and prostaglandins secreted by the placenta and uterus of the cow and ewe on lymphocyte proliferation *in vitro*. Am. J. Reprod. Immunol. Microbiol. **18**:71–5.

Madej, E. (1980). Effects of exogenous hormones on the activity of Δ 5–3β–hydroxysteroid dehydrogenase in cultured granulosa cells from proestrous and preovulatory rat ovarian follicles. Acta Histochem. **67**:253–60.

Magoffin, D.A., and Erickson, G.F. (1981). Mechanism by which 17β–estradiol inhibits ovarian androgen production in the rat. Endocrinology **108**:962–9.

Magoffin, D.A. and Erickson, G.F. (1982a). Primary culture of differentiating ovarian androgen–producing cells in defined medium. J. Biol. Chem. **257**:4507–13.

Magoffin, D.A. and Erickson, G.F. (1982b). Direct inhibitory effects of estrogen on LH–stimulated androgen synthesis by ovarian cells cultured in defined medium. Mol. Cell Endocrinol. **28**:81–9.

Makris, A. and Ryan, K.J. (1980). The source of follicular androgens in the hamster follicle. Steroids, **35**:53–64.

Mannel, D.M., Mizel, S.B., Diamanstein, T., and Falk, W. (1985). Induction of interleukin 2 responsiveness in thymocytes by synergistic action in interleukin 1 and interleukin 2. J. Immunol. **134**:3108–10.

Marchetti, B., Guarcello, V. and Scapagnini, U. (1988). Luteinizing hormone-releasing hormone agonist (LHRH-A) binds to lymphocytes and modulates the immune response. In. Castagnetta L, Nenci I (eds) Biology and Biochemistry of Normal and Cancer cell Growth. Harwood Acadamic Press, London., 149-52.

Marchetti, B., Guarcello, V., Morale, M.C., Bartoloni, G., Farinella, Z. and Scapagnini, U. (1989). Luteinizing hormone-releasing hormone-binding sites in the rat thymus: characteristics and biological functions. Endocrinology **125**:1025-36.

Marden, M.L., Channing, C.P., Schwartz, N.B. (1977). Suppression of follicle stimulating hormone in intact and acutely ovariectomized rats by porcine follicular fluid. Endocrinology **101**:1639-42.

Mason, A.J., Hayflick, J.S., Ling, N., Esch, F., Ueno, N., Ying, S-Y., Guillemin, R., Niall, H. and Seeburg, P.H. (1985). Complementary DNA sequences of ovine follicular fluid inhibin show precursor structure and homology with transforming growth factor-β. Nature **318** 6047:659-63.

Masuhiro, K., Matsuzaki, N., Nishino, E., Taniguchi, T., Kameda, T., Li, Y., Saji, F. and Tanizawa, O. (1991). Trophoblast-derived interleukin-1 (IL-1) stimulates the release of human chorionic gonadotropin by activating IL-6 and IL-6-receptor system in first trimester human trophoblasts. J. Clin. Enedocrinol. Metab. **72**:594-601.

Mathur, S., Jerath, R.S., Mathur, R.S., Williamson, H.O. and Fudenberg, H.H. (1980). Serum immunoglobulin levels, auto-immunity and cell-mediated immunity in primary ovarian failure. J. Reprod. Immunol. **2**:83-92.

Martinat, N and Combarnous, Y. (1983). The release of plasminogen activator by rat granulosa cells is highly specific for FSH activity. Endocrinology **113**:433-5.

Matthews, N. and Neale, M.L. (1987). Cytotoxicity assays for tumor necrosis factor and lymphotoxin. In: Clemens MJ, Morris AG and Gearing AJH (eds). Lymphokines and inteferons; A Practical Approach. Oxford IRL Press; 221-5.

May, J.V., McCarty, Jr. K. Reichert, L.E. Jr. and Schomberg, D.W. (1980). Follicle-stimulating hormone-mediated induction of functional luteinizing hormone/human chorionic gonadotropin receptors during monolayer culture of porcine granulosa cells. Endocrinology **107**:1041-9.

Mendelson, C.R., Durham, C., Evans, C. and Simpson, E.R. (1985). The induction of aromatase activity in estrogen-producing cells is mediated by the increased synthesis of aromatase cytochrome P-450. 67th Annual Meeting of The Endocrine Society, p.77 (Abstr. 307).

Midgley, Jr. A.R. (1973). Autoradiographic analysis of gonadotropin binding to rat ovarian tissue sections. Adv. Exp. Med. Biol. **36**:365-78.

Ming, W.J., Bersani, L. and Mantovani, A. (1987). Tumor necrosis factor is chemotactic for monocytes and polymorphonuclear leukocytes. J. Immunol. **138**:1469-74.

Miyama, M., Umesaki, N., Kawabata M., Sugawa, T. and Duan, J.S. (1991). Production of cytokines in decidual tissue and its significance in maintenance of pregnancy. Inter. J. Obstet. Gynaecol. XIII World Congress of Gynaecology and Obstetrics (FIGO) 1991, p28.

Miyamoto, K., Hasegawa, Y., Fukuda, M., Nomura, M., Igarashi, M., Kangawa,

K. and Matsuo, H. (1985). Isolation of porcine follicular fluid inhibin of 32k daltons. Biochem. Biophys. Res. Commun. **129**:396–403.

Mondschein, J.S. and Schomberg, D.W. (1981a). Platelet–derived growth factor enhances granulosa cell luteinizing hormone receptor induction by follicile–stimulating hormone and serum. Endocrinology **109**:325–7.

Mondschein, J.S. and Schomberg, D.W. (1981b). Growth factors modulate gonadotropin receptor induction in granulosa cell cultures. Science **211**:1179–80.

Moon, Y.S., Tsang, B.K., Simpson, C. and Armstrong, D.T. (1978). 17β–Estradiol biosynthesis in cultured granulosa and thecal cells of human ovarian follicles: stimulation by follicle–stimulating hormone. J. Clin. Endocrinol. Metab. **47**:263–7.

Morale, M.C., Batticane, N., Bartoloni, G., Guarcello, V., Farinella, Z., Galasso, M.G. and Marchetti, B. (1991). Blockade of central and peripheral luteinizing hormone–releasing hormone (LHRH) receptors in neonatal rats with a potent LHRH–antagonist inhibits the morphofunctional development of the thymus and maturation of the cell–mediated and humoral immune responses. Endocrinology **128**:1073–85.

Moscatelli, D. (1987). High and low affinity binding sites for basic fibroblast growth factor on cultured cells: absence of a role for low affinity binding in the stimulation of plasminogen activator production by bovine capillary endothelial cells. J. Cell. Physiol. **131**:123–30.

Mule, J.J., Shu, S., and Rosenberg, S.A. (1985). The anti–tumor efficacy of lymphokine–activated killer cells and recombinant interleukin 2 *in vivo*. J. Immunol. **135**:646–52.

Mule, J.J., Smith, C.A., and Rosenberg, S.A. (1987). Interleukin 4 (B cell stimulatory factor 1) can mediate the induction of lymphokine–activated killer cell activity directed against tumor cells. J. Exp. Med. **166**:792–7.

Murdoch, W.J. (1987). Treatment of sheep with prostaglandin F2α enhances production of a luteal chemoattractant for eosinophils. Am. J. Reprod. Immunol. Microbiol. **15**:52–6.

Murdoch, W.J. and McCormick, R.J. (1989). Production of low molecular weight chemoattractants for leukocytes by periovulatory ovine follicles. Biol. Reprod. **40**:86–90.

Murdoch, W.J. and Steadman, L.E. (1991). Investigations concerning the relationship of ovarian eosinophilia to ovulation and luteal function in the sheep. Am. J. Reprod. Immuno. **25**:81–7.

Nakamura, Y., Kato, H. and Terranova, P.F. (1990). Interleukin–1α increases thecal progesterone production of preovulatory follicles in cyclic hamsters. Biol. Reprod. **43**:169–73.

Nakayama, E., Asano, S., Kodo, H. and Miwa, S. (1985). Suppression of mixed lymphocyte reaction by cells of human first trimester pregnancy endometrium. J. Reprod. Immunol. **8**:25–31.

Naritoku, W.Y. and Taylor, C.R. (1982). A comparative study of the use of monoclonal antibodies using three different immunohistochemical methods: an evaluation of monoclonal and polyclonal antibodies against human prostatic acid phosphatase. J. Histoch. Cytochem. **30**:253–60.

Navickis, R.J., Jones, P.B., Hsueh, A.J.W. (1982). Modulation of prolactin receptors in cultured rat granulosa cells by FSH, LH and GnRH. Mol. Cell. Endocrinol. **27**:77–88.

Neufeld, G. and Gospodarowicz, D. (1985). The identification and partial characterization of the fibroblast growth factor receptor of baby hamster kidney cells. J. Biol. Chem. **260**:13860–8.

Neufeld, G., Ferrara, N., Schweigerer, L., Mitchell, R., Gospodarowicz, D. (1987). Bovine granulosa cells produce basic fibroblast growth factor. Endocrinology **121**:597–603.

Nimrod, A., Erickson, G.F., Ryan, K.J. (1976). A specific FSH receptor in rat granulosa cells: properties of binding *in vitro*. Endocrinology **98**:56–64.

Nimrod, A. (1977). Studies on the synergistic effect of androgen on the stimulation of progesterone secretion by FSH in cultured rat granulosa cells: a search for the mechanism of action. Mol. Cell. Endocrinol. **8**:201–11.

Nimrod, A., Lamprecht, S.A. (1980). Hormone–induced desensitization of cultured rat granulosa cells to FSH. Biochem. Biophys Res. Commun. **92**:905–11.

Nimrod, A. (1981). On the synergistic action of androgen and FSH on progestin secretion by cultured rat granulosa cells. Cellular and mitochondrial cholesterol metabolism. Mol. Cell. Endocrinol. **21**:51–62.

Nishikawa, K., Saito, S., Morii, T., Hamada, K., Ako, H., Narita, N. and Ichijo M., Kurahayashi, M., Sugamura, K. (1991). Accumulation of CD 16–CD56$^+$ natural killer cells with high affinity interleukin 2 receptors in human early pregnancy decidua. Int. Immunol. **3**:743–50.

Nishino, E., Matsuzaki, N., Masuhiro, K., Kameda, T., Taniguchi, T., Takagi, T., Saji, F. and Tanizawa, O. (1990). Trophoblast–derived interleukin–6 (IL–6) regulates human chorionic gonadotropin release through IL–6 receptor on human trophoblasts. J. Clin. Endocrinol. Metab. **71**:436–41.

Norman, R.J., Warnes, G.M., Wang, X., Kirby, C.A. and Matthews, C.D. (1991). Differential effects of gonadotrophin–releasing hormone agonists administered as desensitization or flare protocols on hormonal function in the luteal phase of hyperstimulated cycles. Human Reprod. **6**:206–13.

Nothnick, W.B. and Pate, J.L. (1990). Interleukin–1β is a potent stimulator of prostaglandin synthesis in bovine luteal cells. Biol. Reprod. **43**:898–903.

Odell, W.D. (1979). The reproductive system in women. In: DeGroot, L.J. *et al* (eds) Endocrinology volume 3. Academic Press, London. p1387.

Olsson, J–H., Carlsson, B. and Hillensjö, T. (1990). Effect of insulin–like growth factor I on deoxyribonucleic acid synthesis in cultured human granulosa cells. Fertil. Steril. **54**:1052–7.

Ortaldo, J.R., and Herberman, R.B. (1986). Augmentation of Natural Killer Activity in Immunobiology of Natural Killer Cells, CRC Press, Boca Ration, FL. Vol. II, p145.

Paavola, L.G. (1979). The corpus luteum of the guinea pig. Fine structure of macrophages during pregnancy and postpartum luteolysis and the phagocytosis of luteal cells. Am. J. Anat. **154**:337–64.

Parhar, R.S., Yagel, S. and Lala, P.K. (1989). PGE_2-mediated immunosuppression by first trimester human decidual cells blocks activation of maternal leukocytes in the decidua with potential anti-trophoblast activity. Cell Immunol. **120**:61-74.

Peters, H. and McNatty, K. (1981). The Ovary. St Albans, England, Granada Press, p45.

Petraglia, F., Garuti, G.C., Ramundo, B.D., Angioni, S., Genazzani, A.R. and Bilezikjian, L.M. (1990). Mechanism of action of interleukin-1β in increasing corticotropin-releasing factor and adrenocorticotropin hormone release from cultured human placental cells. Am. J. Obstet. Gynecol. **163**:1307-12.

Piacsek, B.E. and Huth, J.E. (1971). Changes in ovarian venous blood flow following cannulation: effect of luteinizing hormone (LH) and antihistamine. Proceedings of the Society for Experimental Biology and Medicine. **138**:1022-4.

Plunkett, E.R., Moon, Y.S., Zamecnik, J. and Armstrong, D.T. (1975). Preliminary evidence of a role for prostaglandin F in human follicular function. Am. J. Obstet. Gynaecol. **123**:391-7.

Pober, J.S., Gimbrone, M.A. Jr., Lapierre, L.A., Mendrick, D.L., Fiers, W., Rothlein, R. and Springer, T.A. (1986). Overlapping patterns of activation of human endothelial cells by interleukin 1, tumor necrosis factor and immune interferon. J. Immunol. **137**:1893-6.

Polan, M.L., Laufer, N., Dlugi, A.M., Tarlatzis, B.C., Haseltine, F.P., De Cherney, A.H. and Behrman, H.R. (1984). Human chorionic gonadotropin and prolactin modulation of early luteal function and luteinizing hormone receptor-binding activity in cultured human granulosa-luteal cells. J. Clin. Endocr. Metab. **59**:773-9.

Polan, M.L., Daniele, A. and Kuo, A. (1988). Gonadal steroids modulate human monocyte interleukin-1 (IL-1) activity. Fertil. Steril. **49**:964-8.

Polan, M.L., Loukides, J., Nelson, P., Carding, S., Diamond, M., Walsh, A. and Bottomly, K. (1989). Progesterone and estradiol modulate interleukin-1β messenger ribonucleic acid levels in cultured human peripheral monocytes. J. Clin. Endocrinol. Metab. **69**:1200-6.

Polan, M.L., Kuo, A., Joukides, J. and Bottomly, K. (1990). Cultured human luteal peripheral monocytes secrete increased levels of interleukin-1. J. Clin. Endocrinol. Metab. **70**:480-4.

Pollet, R.J., Levey, G.S. (1980). Principles of membrane receptor physiology and their application to clinical medicine. Ann. Intern. Med. **92**:663-80.

Rabinovici, J., Rosenthal, S., Dandekar, P., Martin, M.C. and Angle, M.J. (1990). Insulin-like growth factor I (IGF-I) levels in follicular fluid from human preovulatory follicles: correlation with serum IGF-I levels. Fertil. Steril. **54**:428-33.

Rani, C.S., Salhanick, A.R. and Armstrong, D.T. (1981). Follicle-stimulating hormone induction of luteinizing hormone receptor in cultured rat granulosa cells: an examination of the need for steroids in the induction process. Endocrinology **108**:1379-85.

Rebar, R.W., Morandini, I.C., Erickson, G.F. and Petze, J.E. (1979). The hormonal basis of reproductive defects in athymic mice: Diminished gonadotropin

concentrations in prepubertal females. Endocrinology **108**:120–6.

Reddi, K., Norman, R.J., Deppe, W.M., Kemp,M., Machattie, T. and Joubert, S.M. (1987). Amniotic membrane production of prostaglandin F2 alpha is reduced in dysfunctional human labour–results of *in vivo* studies. J. Clin. Endocrinol. Metab. **65**:1000–5.

Reichert, L.E.Jr. (1962). Endocrine influences on rat ovarian proteinase activity. Endocrinology **70**:697–700.

Rettori, V., Jurcovicova, J. and McCann, S.M. (1987). Central action of interleukin–1 in altering the release of TSH, growth hormone, and prolactin in the male rat. J. Neurosci Res. **18**: 179–83.

Richards, J.S. (1975). Estradiol receptor content in rat granulosa cells during follicular development modification by estradiol and gonadotropins. Endocrinology **97**:1174–84.

Richards, J.S. and Williams, J.J. (1976). Luteal cell receptor content for prolacitn (PRL) and luteinizing hormone (LH): Regulation by LH and PRL. Endocrinology **99**:1571–81.

Richards, J.S., Ireland, J.J., Rao, M.C., Bernath, G.A., Midgley, Jr. A.R. and Reichert, Jr. L.E. (1976). Ovarian follicular development in the rat: hormone receptor regulation by estradiol, follicle–stimulating hormone and luteinizing hormone. Endocrinology **99**:1562–70.

Richards, J.S. (1979). Hormonal control of ovarian follicular development: a 1978 perspective. Rec. Prog. Horm. Res. **35**:343–68.

Richardson, M.C., Masson, G.M. and Sairam, M.R. (1984). Inhibitory action of chemically deglycosylated human chorionic gonadotrophin on hormone–induced steroid production by dispersed cells from human corpus luteum. J. Endocrinol. **101**:327–32.

Rifkin, R.M. Hersh, E.M., and Salmon, S.E. (1988). A phase I study of therapy with recombinant granulocyte–macrophage colony–stimulating factor administered by IV bolus or continuous infusion. Behring Inst. Mitt. **83**:125–33.

Robb, R.J., Munck, A., and Smith, K.A. (1981). T cell growth factor receptor. Quantitation, specificity and biological relevance. J. Exp. Med. **154**:1455–74.

Robertson, D.M., Foulds, L.M., de Vos, F., Leversha, L. and de Kretser. D.M. (1990). Identification of inhibin and inhibin–related proteins in human follicular fluid. Reprod. Fertil. Dev. **2**:327–35.

Roby, K.F. and Terranova, P.F. (1988). Tumor necrosis factor alpha alters follicular steroidogenesis *in vitro*. Endocrinology **123**:2952–4.

Roby, K.F. and Terranova, P.F. (1989). Localization of tumor necrosis factor (TNF) in rat and bovine ovary using immunocytochemistry and cell blot: Evidence for granulosal production. In: Hirshfield A N, ed. Growth factors and the ovary. New York: Plenum Press; p273–8.

Roby, K.F., Lyles, W.R. and Terranova, P.F. (1990). Immunological evidence for a human ovarian tumor necrosis factor–α. J. Clin. Endocrinol. Metab. **71**:1096–102

Roby, K.F.and Terranova P.F. (1990). Effects of tumor necrosis factor α *in vitro* on

steroidogenesis of healthy and atretic follicles of the rat: theca as a target. Endocrinology 126:2711–8.

Rolland, R. and Hammond, J.M. (1975). Demonstration of a specific receptor for prolactin in porcine granulosa cells. Endocr. Res. Commun. **2**:281–98.

Romero, R., Avila, C., Santhanam, U. and Sehgal, P.B. (1990). Amniotic fluid interleukin 6 in preterm labour. Association with infection. J. Clin. Invest. **85**:1392–400.

Rosenberg, S.A., Mule, J.J., Spiess, P.J., Reichart, C.M., and Schwarz, S.L. (1985). Regression of established pulmonary metastases and subcutaneous tumor mediated by the systemic administration of high–dose recombinant interleukin 2. J. Exp. Med. **161**:1169–88.

Rosenwasser, L.J. Dinarello, C.A., and Rosenthal, A.S. (1979). Adherent cell function in murine T–lymphocyte antigen recognition. IV. Enhancement of murine T–cell antigen recognition by human leukocytic pyrogen. J. Exp. Med. **150**:709–14.

Ross, R. and Vogel, A. (1978). The platelet–derived growth factor. Cell. **14**:203–10.

Rothchild, I. (1981). The regulation of the mammalian corpus luteum. Rec. Prog. Horm. Res. **37**:183–298.

Rouabhia, M., Deschaux, P.A. and Othmane, O. (1987). Neuroimmunodulation of the immune response by the endocrine system: effect of luteinizing hormone (LH) on the proliferative response of lymphocytes. Jpn. J. Med. Sci. Biol. **40**: 147–51.

Rouabhia, M., Chakir, J. and Deschaux, P. (1991). Interaction between the immune and endocrine systems: immunomodulatory effects of luteinizing hormone. Progress in Neuro. Endocrin. Immunology. 4:86–91.

Rubin, B.L., Deane, H.W., Hamilton, J.A. and Drike, E.C. (1963). Changes in Δ 5–3β–hydroxysteroid dehydrogenase activity in the ovaries of maturing rats. Endocrinology **72**:924–30.

Saji, F., Azuma, C., Kimura, T., Koyama, M., Ohashi, K. and Tanizawa, O. (1990). Gene expression of macrophage colony–stimulating factor and its receptor in human placenta and decidua. Am. J. Reprod. Immuno. **24**:99–104.

Sakakura, T. and Nishizuka, Y. (1972). Thymic control mechanism in ovarian development: reconstitution of ovarian dysgenesis in thymectomized mice by replacement with thymic and other lymphoid tissues. Endocrinology **90**:431–7.

Sancho–Tello, M. and Terranova, P.F. (1991). Involvement of protein kinase C in regulating tumor necrosis factor α––stimulated progesterone production in rat preovulatory follicles *in vitro*. Endocrinology **128**:1223–8.

Sancho–Tello, M., Imakawa, K. and Terranova, P.F. (1991). A novel expression of tumor necrosis factor–α mRNA in the rat ovary. 24th annual meeting Society for the study of reproduction. Suppl. No. 1. Biol. Reprod. **44**:98.

Sanderson, C.J. Campbell, H.D. and Young, I.G. (1988). Molecular and cellular biology of eosinophil differentiation factor (interleukin–5) and its effects on human and mouse B cells. Immunol. Rev. **102**:29–50.

Sasano, H., Mori, T., Sasano, N., Nagura, H. and Mason JI. (1990).

Immunolocalization of 3β–hydroxysteroid dehydrogenase in human ovary. J. Reprod. Fert. **89**:743–51.

Sato, E., Ishibashi, T. and Iritani, A. (1982). Meiotic arresting substance separated from porcine ovarian granulosa cells and hypothetical arresting mechanism of meiosis. Adv. Exp. Med. Biol. **147**:161–73.

Scalzo, C.M. and Michael, S.D. (1988). Source of high testosterone levels associated with autoimmune ovarian dysgenesis in neonatally thymectomized B6A mice. Biol. Reprod. **38**:1115–21.

Scambia, G., Ranelletti, F.O., Panici, P.B., Baiocchi, G., Piantelli, M., Capelli, A. and Mancuso, S. (1990). Type II estrogen binding sites in human peripheral blood mononuclear cells: variations during the menstrual cycle. Gynecol. Endocrinol. **4**:15–23.

Scheurich, P., Thoma, B., Ucer, U., and Pfizenmaier, K. (1987). Immunoregulatory activity of recombinant human tumor necrosis factor (TNF–alpha). Induction of TNF receptors on human T cells and TNF–alpha–mediated enhacement of T cell responses. J. Immunol. **138**:1786–90.

Schmidt, G., Owman, C. and Sjoberg, N.O. (1986). Histamine induces ovulation in the isolated perfused rat ovary. J. Reprod. Fertil. **78**:159–66.

Schmidt, G., Ahren, K., Brannstrom, M., Kannisto, P., Owman, Ch., Sjoberg, N–O. and Tenenbaum, A. (1987). Histamine stimulates progesterone synthesis and cyclic adenosine 3' 5'–monophosphate accumulation in isolated preovulatory rat follicles. Neuroendocrinology **46**:69–74.

Schmidt, G., Owman, C. and Sjoberg, N.O. (1988). Cellular localization of ovarian histamine, its cyclic variations and histaminergic effects on ovulation in the rat ovary perfused *in vitro*. J. Reprod. Fertil. **82**:409–17.

Schomberg, D.W., Coudert, S.P., Short, R.V. (1967). Effects of bovine luteinizing hormone and human chorionic gonadotropin on the bovine corpus luteum *in vivo*. J. Reprod. Fertil. **14**:277–85.

Schreiber, J.R. and Hsueh, A.J.W. (1979). Progesterone "receptor" in rat ovary. Endocrinology **105**:915–19.

Schreiber, J.R., Nakamura, K. and Erickson, G.F. (1980). Progestins inhibit FSH–stimulated steroidogenesis in cultured rat granulosa cells. Mol. Cell. Endocrinol. **19**:165–73.

Schreiber, J.R., Nakamura, K. and Erickson, G.F. (1981). Progestins inhibit FSH–stimulated granulosa estrogen production at a post–cAMP site. Mol. Cell. Endocrinol. **21**:161–70.

Schreiber, J.R., Makamura, K. and Weinstein, D.B. (1983). Androgen and FSH synergistically stimulate rat ovary granulosa cell utilization of rat and human lipoproteins. In: Factors regulating Ovarian Function, edited by G. Greenwald and P.F. Terranova. Raven Press, New York. p311–315. .

Schuler, L.A., Toaff, M.E. and Strauss III, J.F. (1981). Regulation of ovarian cholesterol metabolism: control of 3–hydroxy–3–methylglutaryl coenzyme A reductase and acyl coenzyme A: cholesterol acyltransferase. Endocrinology **108**:1476–86.

Schwartz, N.B. and Channing, C.P. (1977). Evidence of ovarian "inhibin": suppression of the secondary rise in serum follicle stimulating hormone levels in proestrous rats by injection of porcine follicular fluid. Proc. Natl. Acad. Sci. USA. **74**:5721–4.

Semer, D., Reisler, K., MacDonald, P.C. and Casey, M.L. (1991). Responsiveness of human endometrial stromal cells to cytokines. Ann. N.Y. Acad. Sci. **622**:99–110.

Shemesh, M. and Ailenberg, M. (1977). The effect of androstenedione on progesterone accumulation in cultures of bovine ovarian follicles. Biol. Reprod. **17**:499–505.

Sherwood, O.D. and Downing, S.J. (1983). The chemistry and physiology of relaxin. In: Greenwald, G.S., Terranova, P.F. (eds). Factors Regulating Ovarian Functions. Raven Press, New York, p381.

Shimoya, K., Matsuzaki, N., Taniguchi, T., Jo, T., Neki, R., Saji, F. and Tanizawa, O. (1991). Trophoblast–derived interleukin 1 and tumor necrosis factor–α stimulated release of human chorionic gonadotropin from human trophoblasts. Inter. J. Obstet. Gynecol. XIII World Congress of Gynaecology and Obstetrics (FIGO). p257.

Shire, J.G.M. and Pantelouris, E.M. (1974). Comparison of endocrine function in normal and genetically athymic mice. Comp. Biochem. Physiol. **47A**:93–100.

Skinner, M.K., Lobb, D.K and Dorrington, J.H (1987). Ovarian theca/interstitial cells produce an epidermal growth factor–like substance. Endocrinology **121**:1892–9.

Skinner, M.K. (1989). Transforming growth factor production and action in the ovarian follicle: theca cell–granulosa cell interactions. In: Anne N. Hirshfield (ed.). Growth Factors and the Ovary. p141–150.

Smith, K.A. (1980). T–cell growth factor. Immunol. Rev. **51**:337–57.

Soubiran, P., Zapitelli, J.P. and Schaffar, L. (1987). IL–2–like material is present in human placenta and amnion. J. Reprod. Immunol. **12**:225–34.

Spangelo, B.L., Judd, A.M., Isakson, P.C. and MacLeod, R.M. (1989). Interleukin–6 stimulates anterior pituitary hormone release *in vitro*. Endocrinology **125**:575–7.

Standaert, F.S., Zamora, C.S. and Chew, B.P. (1991). Quantitative and qualitative changes in blood leukocytes in the porcine ovary. Am. J. Reprod. Immunol. **215**:163–8.

Starkey, P.M., Sargent, I.L. and Redman, C.W. (1988). Cell populations in human early pregnancy decidua: characterization and isolation of large granular lymphocytes by flow cytometry. Immunology **65**:129–34.

Stimson, W.H. (1988). Oestrogen and human T–lymphocytes: presence of specific receptors in the T–suppressor/cytotoxic subset. Scand. J. Immunol. **28**:345–50.

Stites, D.P., Bugbee, S. and Siiteri, P.K. (1983). Differential actions of progesterone and cortisol on lymphocyte and monocyte interaction during lymphocyte activation–relevance to immunosuppression in pregnancy. J. Reprod. Immunol. **5**:215–8.

Strauss III, J.F., Schuler, L.A., Rosenblum, M.F., and Tanaka, T. (1981).

Cholesterol metabolism of ovarian tissue. Adv. Lipid Res. **18**:99–157.

Strickland, S. and Beers, W.H. (1976). Studies on the role of plasminogen activator in ovulation. *In vitro* response of granulosa cells to gonadotropins, cyclic nucleotides and prostaglandins. J. Biol. Chem. **251**:5694–702.

Sueldo, C.E., Kelly, E., Montoro, L., Subias, E., Baccaro, M. and Swanson, J.A. (1990). Effect of interleukin–1 on gamete interaction and mouse embryo development. J. Reprod. Med. **35**:868–72.

Sugarman, B.J., Aggarwal, B.B., Hass, P.E., Figari, I.S., Palladina, M.A. and Shepard, H.M. (1985). Recombinant human tumour necrosis factor–α. Effects on proliferation of normal and transformed cells *in vitro*. Science **230**:943–5.

Tabibzadeh, S. (1990). Evidence of T–cell activation and potential cytokine action in human endometrium. J. Clin. Endocrinol. Metab. **71**:645–9.

Tabibzadeh, S., Kaffka, K.L., Satyaswaroop, P.G. and Killian, P.L. (1990a). Interleukin–1 (IL–1) regulation of human endometrial function: presence of IL–1 receptor correlates with IL–1–stimulated prostaglandin E2 production. J. Clin. Endocrinol. Metab. **70**:1000–6.

Tabibzadeh, S., Kaffka, K.L., Kilian, P.L. and Satyaswaroop, P.G. (1990b). Human endometrial epithelial cell lines for studying steroid and cytokine actions. In Vitro. Cell Dev. Biol. **26**:1173–9.

Talini, B. and Taylor, C.R. (1983). New developments in immunoperoxidase techniques and their application. Arch. Pathol. Lab. Med. **107**:105–17.

Tamatani, T., Tsunoda, H., Iwasaki, H., Kaneko, M., Hashimoto, T. and Onozaki, K. (1988). Existence of both IL–1 alpha and beta in normal human amniotic fluid: unique high molecular weight form of IL–1 beta. Immunology **65**:337–42.

Tan, C.H. and Armstrong, D.T. (1984). FSH–stimulated 3β–HSD activity of porcine granulosa cells: inhibition by androgens. Proceedings of the 3rd Joint Meeting of the British Endocrine Societies, Edinburgh, Abstr. 12.

Takakura, K., Shunzo, T., Fukuoka, M., Yasuda, K., Tagaya Y., Yodoi, J., Mori, T. (1989). Interleukin–2 Receptor/p55 (Tac)–Inducing Activity in Porcine Follicular Fluids. Endocrinology **125**:618–23.

Tartakovsky, B., Kovacs, E.J., Takacs, L. and Durum, S.K. (1986). T cell clone producing an IL–1–like activity after stimulation by antigen–presenting B cells. J. Immunol. **137**:160–6.

Taylor, C.R. (1986). Theoretical and practical aspects of the different immunoperoxidase techniques. In: Immunomicroscopy: a diagnostic tool for the surgical pathologist. W.B. Saunders Company, p23–42.

Thanki, K.H. and Channing, C.P. (1978). Effects of follicle–stimulating hormone and estradiol upon progesterone secretion by porcine granulosa cells in tissue culture. Endocrinology **103**:74–80.

Thibault, G., Degenne, D., Girard, A.C., Guillaumin, J.M., Lacord, M. and Bardos, P. (1991). The inhibitory effect of human syncytiotrophoblast plasma membrane vesicles on *in vitro* lymphocyte proliferation is associated with reduced interleukin 2 receptor expression. Cell Immunol. **138**:165–74.

Thompson, E.A. Jr. and Siiteri, R.K. (1976). Partial resolution of the placental microsomal aromatase complex. J. Steroid Biochem. **7**:635–9.

Tietz, N.W. and Finley, P.R. (1983). Section I – General clinical tests. In Clinical Guide to Laboratory Tests. W.B. Saunders Company, Philadelphia, p174.

Tindall, V.R. (1987). Ovarian function. In. revised by V.R. Tindall (ed) fifth edition, Jeffcoate's Principles of Gynaecology. Butterworths and Co (publishers) Ltd. p53–7.

Toaff, M.E., Strauss III, J.F. and Hammond, J.M. (1983). Regulation of cytochrome P–450 scc in immature porcine granulosa cells by FSH and estradiol. Endocrinology **112**:1156–8.

Too, C.K., Bryant–Greenwood, G.D. and Greenwood, F.C. (1984). Relaxin increases the release of plasminogen activator collagenase and proteoglycanase from rat granulosa cells *in vitro*. Endocrinology **115**:1043–52.

Triebwasser, W. F., Clark, M.R., LeMaire, W.J and Marsh, J.M. (1978). Localization and *in vitro* synthesis of prostaglandins in components of rabbit preovulatory Graafian follicles. Prostaglandins. **16**:621–31.

Tsafriri, A., Bar–Ami, S. (1982). Oocyte maturation inhibitor: A 1981 new perspective. Adv. Exp. Med. Biol. **147**:145–59.

Tsai–Morris, C.H., Ghosh, M., Hirshfield, A.N., Wise, P.M. and Brodie, A.M.H. (1983). Inhibition of ovarian aromatase by prolactin *in vivo*. Biol. Reprod. **29**:342–6.

Tsang, B.K., Moon, Y.S., Simpson, C.W., and Armstrong, D.T. (1979). Androgen biosynthesis in human ovarian follicles: cellular source, gonadotropic control, and andenosine 3' 5'–monophospyate mediation. J. Clin. Endocrinol. Metab. **48**:153–8.

Tsuji, M., Terada, N., Sato, B. and Matsumot, K. (1982). 5β– and 5α–reductases from 4–ene–3–ketosteroids in golden hamster ovaries at different stages of development. J. Steroid Biochem. **16**:207–13.

Uilenbroek, J.T.J. and van der Linden R. (1983). Changes in gonadotrophin binding to rat ovaries during sexual maturation. Acta Endocrinol. (Copenh.) **104**:413–9.

Unanue, E.R. (1989). Macrophages, antigen–presenting cells and the phenomena of antigen handling and presentation. In William E. Paul (ed.) Fundamental Immunology, Second edition, Raven Press Ltd., New York. p95–115.

Van Damme, J., Cayphas, S., Opdenakker, G., Billiau, A., and Van Snick, J. (1987a). Interleukin 1 and poly (rI)–poly(rC) induce production of a hybridoma growth factor by human fibroblasts. Eur. J. Immunol. **17**:1–7.

Van Damme, J., Opdenakker, G., Simpson, R., Rubira, M., Cayphas, S., Vink, A., Billiau, A., and Van Snick, J. (1987b). Identification of the human 26–kD protein, interferon beta–2, as a B cell hybridoma/plasmacytoma growth factor induced by interleukin 1 and tumor necrosis factor. J. Exp. Med. **165**:914–9.

Varga, B., Acs, Z., Papp, M. and Start, E. (1967). Effect of some biogenic amines on adrenal,ovarian and thyroid blood flow. Endocrinologia Experimentalis **1**:173–9.

Veldhuis, J.D., Klase, P., and Hammond, J.M. (1980). Divergent effects of prolactin upon steroidogenesis by porcine granulosa cells *in vitro*: influence of

cytodifferentiation. Endocrinology **107**:42–6.

Veldhuis, J.D., Klase, P.A., Strauss III, J.F. and Hammond, J.M. (1982). Facilitative interactions between estradiol and luteinizing hormone in the regulation of progesterone production by cultured swine granulosa cells: relation to cholesterol metabolics. Endocrinology **111**:441–7.

Veldhuis, J.D. (1985). Bipotential actions of estrogens on progesterone biosynthesis by ovarian cellls. II. Relation of estradiol's stimulatory actions to cholesterol and progestin metabolism in cultured swine granulosa cells. Endocrinology **117**:1076–83.

Veldhuis, J.D., Garmey, J.C., Urban, R.J., Demers, L.M. and Aggarwal BB. (1991) Ovarian actions of tumor necrosis factor–α (TNFα). Pleiotropic effect of TNFα on differentiated functions of untransformed swine granulosa cells. Endocrinology **129**:641–8.

Virelizier, J.L., Perez, N., Arenzana–Seisdedos, F. and Devos, R. (1984). Pure interferon gamma enhances class II HLA antigens on human monocyte cell lines. Eur. J. Immunol. **14**:106–8.

Vogel, S.N., Finbloom, D.S., English, K.E., Rosenstreich, D.L., and Langreth, S.G. (1982). Interferon–induced enhancement of macrophage Fc receptor expression: β–interferon treatment of C3H/HeJ macrophages results in increased numbers and density of Fc receptors. J. Immunol. **130**:1210–4.

Wallach, E.E., Wright, K.H. and Hamada, Y. (1978). Investigation of mammalian ovulation with an *in vitro* perfused rabbit ovary preparation. Am. J. Obstet. Gynecol. **132**:728–38.

Wang, A.M., Creasey, A.A., Ladner, M.B., Lin, L.S., Strickler, J., Van Arsdell, J.N., Yamamoto, R., and Mark, D.F. (1985). Molecular cloning of the complementary DNA for human tumor necrosis factor. Science **228**:149–54.

Wang, C., Hsueh, A.J.W. and Erickson, G.F. (1979). Induction of functional prolactin receptors by follicle–stimulating hormone in rat granulosa cells *in vivo* and *in vitro*. J. Biol. Chem. **254**:11330–6.

Wang, C., Hsueh, A.J.W. and Erickson, G.F. (1980). Prolactin inhibition of estrogen production by cultured rat granulosa cells. Mol. Cell. Endocrinol. **20**:135–44.

Wang, C., Hsueh, A.J.W. and Erickson, G.G. (1981). LH stimulation of estrogen secretion in cultured granulosa cells. Mol. Cell. Endocrinol. **24**:17–28.

Wang, C. and Chan, V.C. (1982). Divergent effects of prolactin on estrogen and progesterone production by granulosa cells of rat Graafian follicles. Endocrinology **110**:1085–93.

Wang, C. and Leung, A. (1982). Gonadotropins regulate plasminogen activator production by rat granulosa cells. Endocrinology **112**:1201–7.

Wang, C., Hsueh, A.J.W. and Erickson, G.F. (1982). The role of cyclic AMP in the induction of estrogen and progestin synthesis in cultured granulosa cells. Mol. Cell. Endocrinol. **25**:73–83.

Wang, J.M., Bersani, L. and Manlovani, A. (1987). Tumor necrosis factor is chemotactic for monocytes and polymorphonuclear leukocytes. J. Immunol. **138**:1469–74.

Warren, D.J., and Moore, M.A.S. (1988). Synergism among interleukin 1, interleukin 3, and interleukin 5 in the production of eosinophils from primitive hemopoietic stem cells. J. Immunol. **140**:94–9.

Weiss, A. (1989). T lymphocyte activation. In: Fundamental Immunology, Second Edition, edited by William E. Paul, Raven Press Ltd., New York, p359–84.

Weiss, T.J., Armstrong, D.T., McIntosh, J.E.A. and Seamark, R.F. (1978). Maturational changes in sheep ovarian follicles: gonadotrophic stimulation of cyclic AMP production by isolated theca and granulosa cells. Acta Endocrinol. (Copenh.) **89**:158–65.

Welsh, Jr. J.H. Jones, P.B.C., Ruiz de Galaretta, G.M., Fanjul, L.F. and Hsueh, A.J.W. (1982). Androgen regulation of progestin biosynthetic enzymes in FSH–treated rat granulosa cells *in vitro*. Steroids **40**:691–700.

Welsh, Jr. T.J., Jia, X–C., Jones, P.B.C., Zhuang, L–Z. and Hsueh, A.J.W. (1984). Disparate effects of triphenylethylene antiestrogens on estrogen and progestin biosynthesis by cultured rat granulosa cells. Endocrinology **115**:1275–82.

Westergaard, L.G. and Andersen, C. Y. (1989). Epidermal growth factor (EGF) in human preovulatory follicles. Human Reprod. **3**:257–60.

Weusten, J.J., Blankenstein, M.A., Gmelig–Meyling, F.H., Schuurman, H.J., Kater, L. and Thijssen, J.H. (1986). Presence of oestrogen receptors in human blood mononuclear cells and thymocytes. Acta Endocrinol. (Copenhagen) **112**:409–14.

Wheelock, E.F. (1965). Interferon–like virus–inhibitor induced in human leukocytes by phytohemagglutinin. Science **149**:310.

White, S.S. and Ojeda, S.R. (1981). Changes in ovarian luteinizing hormone and follicle–stimulating hormone receptor content and in gonadotropin–induced ornithine decarboxylase activitiy during prepubertal and pubertal development of the female rat. Endocrinology **109**:152–61.

Wickings, E.J., Hillier, S.G. and Jr. Reichert, L.E. (1986). Gonadotrophic control of steroidogenesis in human granulosa–lutein cells. J. Reprod. Fert. **76**:677–84.

Winter, J.S., Gow, K.W., Perry, Y.S., Greenberg, A.H. (1990). A stimulatory effect of interleukin–1 on adrenocortical cortisol secretion mediated by prostaglandins. Endocrinology **127**:1904–9.

Wolf, R.L., Ilekis, J. and Benvenister, R. (1983). Characterization of an immune suppressor from transformed human trophoblastric JEG–3 cells. Cell Immunol. **78**:356–67.

Xiao, S. and Findlay, J.K. (1991). Interferon gamma modulates follicle–stimulating hormone action: studies on hormone release and sites of action in cultured rat granulosa cells. Proc. Aust. Soc. Reprod. Biol. Sydney, Vol 23, Abstr. No. 31.

Yagel, S., Parhaer, R.S. and Lala, P.K. (1989). Trophic effects of first–trimester human trophoblasts and human chorionic goandotropin on lymphocyte proliferation. Am. J. Obstet. Gynecol. **160**:946–53.

Yanagishita, M., Rodbard, D and Hascall, V.C. (1979). Isolation and characterization of proteoglycans from porcine ovarian follicular fluid. J. Biol. Chem.

254:911–20.

Yasuda, K., Kuoka, M., Taii, S., Takakura, K. and Mori, T. (1990). Inhibitory effects of interleukin–1 on follicle–stimulating hormone induction of aromatase activity, progesterone secretion, and functional luteinizing hormone receptors in cultures of porcine granulosa cells. Biol. Reprod. **43**, 905–12.

Yelavarthi, K.K., Chen, H–L., Yang, Y., Cowley, Jr. B.D., Fishback, J.L. and Hunt, J.S. (1991). Tumor necrosis factor–α mRNA and protein in rat uterine and placental cells. J. Immunol. **146**:3840–8.

Yen, S.S.C., Tsai, C.C., Naftolin, F., Vandenberg, G., Ajabor, L. (1972). Pulsatile patterns of gonadotrophin release in subjects with and without ovarian function. J. Clin. Endocrinol. Metab. **34**:671–5.

Yen, S.S.C. and Lei, A. (1976). The apparent paradox of the negative and positive feedback control system on gonadotrophin secretion. Am. J. Obstet. Gynecol. **126**:942–54.

Zachariae, F. (1959). Acid mucopolysaccharides in the ovary and their role in the mechanims of ovulation. Acta Endocrinol. (Suppl) **47**:33–8.

Zeleznik, A.J., Midgley, Jr. A.R. and Reichert, Jr. L.E. (1974). Granulosa cell maturation in the rat: increased binding of human chorionic gonadotropin following treatment with follicle–stimulating hormone *in vivo*. Endocrinology **95**:818–25.

Zhang, Z.W., Carson, R.S. Herington, A.C., Lee, V.W. and Burgar, H.G. (1987). Follicle–stimulating hormone and somatomedin–C stimulate inhibin production by rat granulosa cells *in vitro*. Endocrinology **120**:1633–8.

Zhou, M.H. and Galway, A.B. (1991). Inhibitory effect of interleukin–1 beta on follicle stimulating hormone (FSH) induced estrogen production by cultured rat granulosa cells. Sheng Li Hsueh Pao (Chinese) **43**:67–72.

Zhuang, L.Z., Adashi, E.Y. and Hsueh, A.J.W. (1982). Direct enhancement of gonadotropin–stimulated ovarian estrogen biosynthesis by estrogen and clomiphene acitrate. Endocrinology **110**:2219–1.

Zmigrod, A., Lindner, H.R. and Lamprecht, S.A. (1972). Reductase pathways of progeterone metabolism in the rat ovary. Acta Endocrinol. (Copenh.) **69**:141–52.

Zolti, M., Meirom, R., Shemesh, M., Wollach, D., Mashiach, S., Shore, L. and Rafael, Z.B. (1990). Granulosa cells as a source and target organ for tumor necrosis factor–α. FEBS. **261**:253–5.

Zolti, M., Bider, D., Ben–Rafael, Z., Mashiach, S., Meirom, R., Apte, R.M. and Shemesh, M. (1991). Cytokine involvement in oocytes and early embryo. Fertil. Steril. **56**:265–72.