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AN INVESTIGATION INTO THE IMMUNOMODULATORY ACTIVITIES OF HUMAN PLACENTAL PROTEIN 14 (PP14)

BY

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Publications

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ABSTRACT

An Investigation into the Immunomodulatory Activities of Human Placental Protein 14 (PP14).

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PP14 has been shown to suppress the incorporation of ^{3}H-Thymidine into both mitogenically and allogeneically stimulated lymphocytes in a dose dependent manner. The suppressive activity was shown to be specific, in that PP14 did not affect cellular viability, nor interact with the mitogen phytohaemagglutinin (PHA). Flow cytometric analysis indicated that PP14 had no effect on the expression of the Tac antigen, the transferrin receptor or HLA-DR molecules on the surface of stimulated lymphocytes. Neither did PP14 affect the interaction of interleukin-2 (IL-2) with its cell surface receptor. The suppressive activity was partially reversed by the addition of exogenous IL-2. PP14 inhibited the production of IL-2 from mitogenically stimulated lymphocytes and led to a small, but significant reduction in soluble IL-2 receptor release. Radiolabel binding studies and IL-2 dose response curves indicated that PP14 affected the affinity of the IL-2 receptor on PHA stimulated lymphocytes. This was supported by the observation that PP14 increased the level of cell surface-associated IL-2 on stimulated lymphocytes. There was a small inhibition of gamma interferon levels early in the culture period. PP14 had no effect on the CD4/CD8 ratio following stimulation and was not found to be associated with the cell surface, nor mask cell surface expression of the CD2 antigen.

These data suggest that the immunosuppressive activity of PP14 may, in part, be mediated via a modulation of the functional, high affinity IL-2 receptor. It is not known as yet whether such an activity is effective at the level of induction of the receptors or whether the primary control is at another level of the response. PP14 may have implications in the study of implantation and fertility and prove of wider interest in the field of transplantation biology and the control of the immune response.

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ADF adult T cell leukaemic (ATL)-derived factor

AFP Alpha foeto protein

ATP Adenoside triphosphate

ala alanine

Ag Antigen

AT III Antithrombin III

BCGF B cell growth factor

BLG Beta lactoglobulin

BSA Bovine serum albumin

°C degrees Celsius

Ca^{2+} calcium ion

CA III carbonic anhydrase III

CD3 component of T cell antigen receptor (cf T3)

CD4+ T helper cell

CGF Cytotoxic cell generating factor

Ci/mmol Curies/millimole

cm centimetre (10^{-2} metre)

C5a component of complement system

CNBr-S4B cyanogen bromide activated Sepharose-4B

CO_2 carbon dioxide

Con A concanavalin A

CSF-GM colony stimulating factor granulocyte macrophage

CV coefficient of variation

DMEM Dulbecco's modification of Eagle's medium

DMEM+ antibiotic-supplemented DMEM

DNA Deoxyribonucleic acid

cDNA complementary DNA

EDTA Ethylenediaminetetracetic acid

ELISA Enzyme linked immunoad sorbent assay

EPF Early pregnancy factor

E Receptor sheep red blood cell receptor

FACS Fluorescence activated cell sorter

Fig figure

FITC Fluoroscein isothiocyanate

g gram

x g relative centrifugal force

GTP Guanidine triphosphate

h hour

H^+ hydrogen ion

hCG Human chorionic gonadotrophin

HLA Human leucocyte antigen

HLA-A,B,C class I antigens (human)

HLA-D class II antigens (human)

hPL human placental lactogen

HRP horseradish peroxidase

^{3}H-Tdr tritiated thymidine

^{125}I iodine-125

Ia murine class II antigen

IFN interferon

IgE immunoglobulin E

IgG immunoglobulin G

IL-1 Interleukin-1

IL-1R Interleukin-1 receptor

IL-2 Interleukin-2

rIL-2 recombinant IL-2

IL-2R Interleukin-2 receptor

sIL-2R soluble IL-2R

iU international units

IVF *In vitro* fertilisation

K^+ potassium ion
Kd dissociation constant
kDa kilo Dalton
kg kilogram
KI potassium iodide
l litre
log logarithm to base 10
LPS lipopolysaccharide
M Molar
m^2 square metre
mA milliamp (10^{-3} amp)
mCi millicurie (10^{-3} Curie)
mg milligram (10^{-3} g)
MHC Major histocompatibility complex
ml millilitre
MLR Mixed lymphocyte reaction
mM millimolar
mm millimetre
MSA Mitogen stimulation assay
Na^+ sodium ion
ng nanogram (10^{-9} g)
NK cell Natural killer cell
nm nanometre (10^{-9} metre)
pNPP p-nitrophenyl phosphate
OPD o-phenylenediamine
PAF Platelet activating factor
PAPP-A Pregnancy associated plasma protein-A
PBS phosphate buffered saline
PBS/FCS 1% FCS in PBS
PEG Polyethylene glycol
PHA phytohaemagglutinin
PKC Protein kinase C
pM picomole (10^{-12} M)
PMA phorbol myristate acetate
[ ] concentration
PMC Peripheral blood mononuclear cells
PMSF phenyl methyl sulphonyl fluoride
PP Placental protein
PP14 Placental protein 14
PWM pokeweed mitogen
PZP Pregnancy zone protein
RIA radioimmunoassay
RIF Receptor inducing factor
RNA Ribonucleic acid
mRNA messenger RNA
rpm revolutions per minute
S phase proliferative phase of cell cycle
ser serine
SF1+ antibiotic-supplemented SF1 growth medium
SP1 Pregnancy specific beta1 glycoprotein
T3 component of T cell antigen receptor
Ti component of T cell antigen receptor
Th T helper cell
Tcyt T cytotoxic cell
TCA trichloroacetic acid
TCGF T cell growth factor (IL-2)
TFR Transferrin receptor
TLX Trophoblast lymphocyte cross reactive
uCi microcurie (10^{-6} Curie)
ug microgram (10^{-6} g)
ul microlitre (10^{-6} l)
v/v volume to volume ratio
w/v weight to volume ratio
< less than

CHAPTER ONE

INTRODUCTION-THE ESTABLISHMENT OF PREGNANCY.

1.1 Implantation.

Three to four days after fertilisation of the human oocyte by the male spermatozoon, the morula arrives at the uterus through the tubal isthmus. At this stage it is important for the blastocyst to implant into the maternal endometrium as nutrients are rapidly exhausted. The zona pellucida is shed due to trophoblastic activity which enables the trophoblast to come into direct contact with the endometrium allowing attachment. The presence of the zona pellucida until this stage prevents premature attachment of blastomeres to the tubal mucous membrane. The extent of the trophoblastic penetration subsequent to attachment determines the thickness of the membrane (placental membrane) interposed between the embryonic (later foetal) blood stream and that of the mother and across which the exchanges between mother and foetus must take place. The extent of trophoblastic invasion varies between species, from the situation where trophoblastic penetration is minimal, leaving the chorion (trophoblast plus supporting vascular mesenchyme) lying merely in contact with the endometrium epithelium (EPITHELIOCHORIAL placentation) to the situation in the human and some other species, where the trophoblast destroys the maternal epithelium and connective tissue stroma and also the endothelium of the maternal blood vessels to leave the maternal blood bathing the chorion directly and to form a HAEMOCHORIAL placental structure (Grosser et al., 1927).

1.2 The Decidua/Decidual Reaction

From the time of the seventh day after fertilisation, two simultaneous events begin to occur, the embedding of the fertilised ovum and the formation of the decidua. The term decidua was first used for that part of the uterine tissue which is shed on parturition (the gestational endometrium). Ultrastructurally the metabolic activity of the decidual cells appears to be maximal by the seventieth day of pregnancy and begins to decline after the hundredth day (Wynn 1974).

As the embedding of the ovum is in progress changes occur in the secretory endometrium which results in its becoming transformed into the decidua:-

1) The endometrium hypertrophies, reaching 6mm to 8mm in thickness.
2) The stroma becomes increasingly vascular and oedematous.
3) The stroma cells swell and enlarge with the result that they become closely packed together in the superficial part of the functional layer. They now form what is known as the compact layer. These decidual cells are polygonal in shape as a result of the mutual pressure they exert upon each other.

The deciduum is thicker, richer and more vascular than the secretory endometrium. The decidual cells are a characteristic feature of pregnancy, rarely occuring apart from it. The development of the deciduum results from an increased output of progesterone from the corpus luteum. The stimulus to maintain the corpus luteum and the increased production of progesterone is chorionic gonadotrophin

which is released from the invading trophoblast. As a result of the embedding of the ovum into the decidual lining of the uterus, the deciduum becomes divided into three parts:-

1) The decidua basalis, being that part of the decidua which lies between the developing ovum and the myometrium.
2) The decidua capsularis, which covers the ovum and separates it from the uterine cavity.
3) The decidua vera (parietalis) which lines the remainder of the cavity of the uterus (Fig 1.1).

1.3 Development of the Trophoblast

Whilst the blastocyst is becoming embedded in the decidua, a continuous process of growth and development is progressing in trophoblast cells which proliferate and differentiate into three layers:-

1) An outer layer of fused cells known as the syncytium or the syncytiotrophoblast.
2) An inner layer known as Langhans' layer or the cytotrophoblast, composed of single cells with complete cell membranes (Langhans' cells) lying beneath the syncytium.
3) Below the cytotrophoblast lies a layer of loose connective tissue, known as primitive mesenchyme.

Digestive enzymes secreted by the trophoblast are responsible for the invasion of the decidua. All cells in the decidua are invaded by the trophoblast including the walls of the smaller blood vessels leading to the syncytial meshwork which fills with circulating maternal blood. Finger like projections of trophoblast (primitive villi) containing all three layers grow in all directions in the decidua, and are bathed on their outer surface by circulating

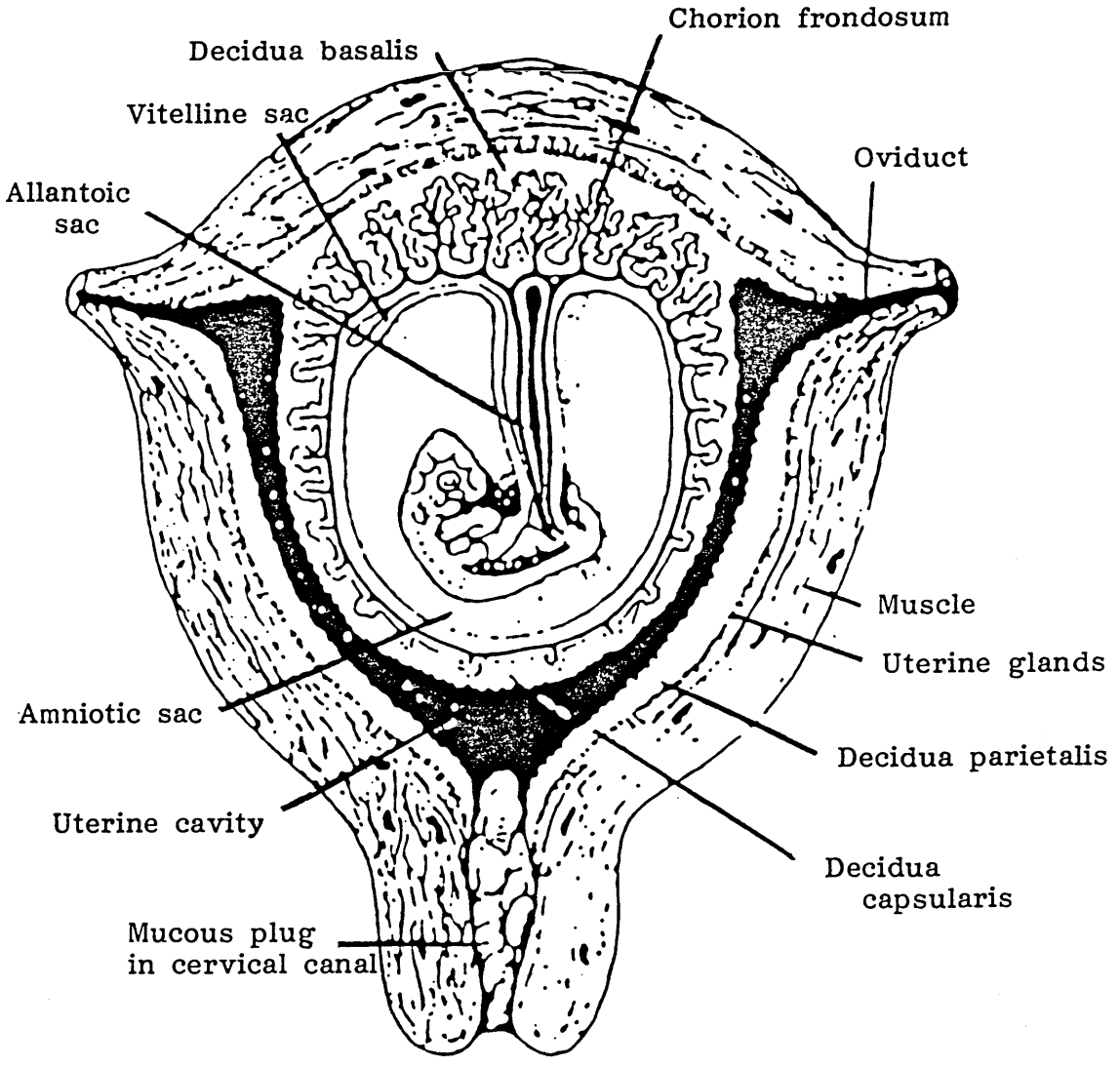


Figure 1.1 Relationships of structures in the uterus at the end of the seventh week of pregnancy (Benson 1977).

maternal blood. The trophoblast becomes the primitive chorion.

Three weeks after fertilisation, the villi begin branching to form a tree-like structure, some floating freely in the maternal blood lakes, others passing deeply into the endometrium and acting as anchoring villi. Each villus is bathed in maternal blood and still contains the same three layers. The blood filled spaces between the villi are now known as the intervillous spaces.

1.4 The Immunological Implications of Pregnancy

Pregnancy in animals imposes dramatic physiological and biological changes on the mother. Many of their implications have been studied, however the immunological consequences of the presence of the foreign foetal allograft still remain obscure. From an immunological standpoint, for a successful pregnancy to occur the maternal immune response must be modulated at a number of sites.

From the time of implantation human trophoblast possibly exhibiting foreign paternal antigens is exposed directly to the maternal tissues. This leads to the possibility of soluble antigens of foetal origin being released into the maternal circulation. The trophoblast may be regarded as a semi-foreign allograft which would normally institute a classical graft rejection reaction. When it is realised that the total area of chorion exposed directly to the maternal blood and tissue is around $14m^2$ it is remarkable that no allograft rejection occurs. In the course of the decidual reaction an immunological response does occur, as observed by the infiltration of small lymphocytes into the endometrium surrounding the implanting conceptus, however this reaction does not normally lead to foetal rejection.

SECTION A

THE ALLOGRAFT REJECTION RESPONSE

1.5 The Mechanism of Graft Rejection.

Graft rejection between unrelated individuals has been recognised for many years, however it is only in the last 30 years that this has been shown to be mediated via the adaptive immune system, exhibiting the two key features of adaptive immunity namely memory and specificity. Only graft sites which are accessible to the immune system exhibit graft rejection and privileged sites exist in the body where allogeneic grafts can survive indefinitely.

The major histocompatibility complex (MHC) is the genetic region found in all mammals which codes for two biochemically and functionally distinct classes of cell surface histocompatibility antigens. In man the cell surface histocompatibility antigens are known collectively as HLA antigens with the MHC region being found on chromosome 6. Class I MHC alloantigens include the glycoprotein products of the gene loci HLA-A, -B and -C and are involved as target antigens in the allograft responses. Class I MHC antigens are 45 kDa glycoproteins non-covalently associated at the cell surface with a 12 kDa polypeptide beta$_2$-microglobulin (encoded in chromosome 15).

The class II MHC antigens (including products of the HLA-D region) are surface glycoproteins consisting of a 33-34 kDa heavy chain together with a 28-29 kDa light chain and display a more restricted tissue distribution than class I MHC products. They appear to be essential for antigen presentation to the

immunocompetent cells which regulate immune responses. The class II transplantation antigen is a simple receptor containing a single binding site for peptides and functions to position the antigen uniquely for T cell receptor binding (Guillet et al., 1986).

The products of the MHC are responsible for the rapid rejection of grafts between individuals and function in the signalling between lymphocytes and the cells expressing antigen. They are the major obstacle to successful graft survival. In addition to the MHC there are a number of independently segregating loci the products of which in some cases are sufficient to cause graft rejection, however graft rejection arising from such allogeneic differences can usually be overcome by immunosuppressive therapy.

1.6 The Cellular Components of the Adaptive Immune System.

The cellular components of the adaptive immune system are the T and B lymphocytes and the antigen presenting cells of the mononuclear phagocytic system including macrophages, whose role is to present antigen to specific antigen sensitive lymphocytes. The major accessory functions of macrophages are believed to be interleukin-1 (IL-1) production and the cross-linking of the T cell antigen receptor (T3-Ti) complex (Williams et al., 1985). T lymphocytes are mainly responsible for the rejection of solid grafts and are sufficient alone to cause graft rejection. Histological studies show infiltration by mononuclear cells, mainly small lymphocytes, preceding graft destruction by several days. Although the main cause of graft rejection is via the cell-mediated immune system, antibody can cause rejection, but to a lesser degree. Such a role for

antibody is enhanced in situations where the recipient is sensitised to particular donor antigens or in reactions to haemopoietic cells.

It was originally thought that graft rejection was effected by cytotoxic T cells, however later work in mice showed that graft destruction did not correlate with cytotoxic T cell development and other studies have shown helper cells to be the effectors in rejection reactions due to MHC differences. These observations have led to the proposal that graft rejection may be a response related to delayed hypersensitivity reactions with graft rejection being mediated by monocytes and macrophages recruited to the site. In summary there are two proposed mechanisms which may mediate graft destruction:-

1) Foreign MHC class II antigens on the graft stimulate host T helper cells (Th) to help cytotoxic cells (Tcyt) destroy the target graft cell. Tcyt cells recognise the graft cell via the foreign MHC class I antigens.
2) The cells reacting to the graft release lymphokines which stimulate macrophages to enter the graft and destroy it.

The rejection of foreign allografts has no physiological basis, however it is analogous to the <u>in vivo</u> recognition and rejection of virally infected cells. The HLA molecule on the surface of the virally infected cell acts as a code guiding the cytotoxic T cell to its target.

1.7 Regulation of the Immune Response

Once an immune response has been initiated there is a capability for amplification. The primary regulator of an immune response is the antigen causing it, however there is also a need for regulation by intrinsic components of that response.

Antigen specific regulation of the immune response appears to be mediated by sub-sets of the T lymphocytes which are distinguishable by surface antigen markers, namely the suppressor T cell (Ts, $CD8^+$) and the helper or inducer T cell (Th, $CD4^+$) (Cantor and Boyse 1977). T suppressor cells appear to be essential for the regulation of the immune response (Green et al.,, 1983), however the nature of the antigen receptor on these cells is unclear. It has been reported that the activity of at least some human $CD8^+$ suppressor cells is likely to be mediated by the T cell receptor (Modlin et al., 1987). The suppressor T cells only function if continually stimulated, however the helper T cell which activates the Ts cell is itself inhibited by the suppressor cell thus facilitating a feedback response. The interaction of regulatory T cells with other T cells may be mediated by the recognition of foreign antigen which has become attached to the surface of the interacting cell (Mitchison et al., 1970).

Very little is known about the molecular mechanisms by which lymphocytes communicate with one another, one possibility is that they interact directly via antigen bridging allowing short range membrane bound or secreted molecules to act. Many immunologists now believe that lymphocytes may communicate by secreting specific signalling molecules which operate over relatively long distances

(Tada and Okumura 1979). A variety of soluble protein regulatory factors have been isolated from T lymphocytes, termed helper and suppressor factors, which have the same antigen specificity and activity as the cells which produced them (Germain and Benacerraf 1980). It is still uncertain, however, whether antigen specific factors normally function in soluble form, as it is possible that they represent membrane bound receptors which have been shed from cells.

In addition to antigen specific regulatory molecules there also exist non antigen specific mechanisms of regulation, the non-specific regulation being centred around the secretion of regulatory molecules termed lymphokines from certain T cells. A number of these molecules have been described (Table 1.1).

TABLE 1.1 Lymphokines and their activities.

Regulation of other lymphocytes (non antigen-specific)	
Interleukin-2	IL-2
Interleukin-3	IL-3
Interferons alpha and gamma	IFN
Soluble immune response suppressor	SIRS
Inhibitor of DNA synthesis	DDS
Allogenic effector factor	AEF
T cell replacing factor	TRF
Regulation of other lymphocytes (antigen specific factors)	
Assorted antigen-specific helper factors	T_HF
Assorted antigen-specific suppressor factors	T_SF

The regulatory polypeptides secreted by T cells affect both lymphoid and other haemopoietic cells including erythroid, megakarocytic, granulocytic and macrophage precursors and mast cells. T cells themselves are subject to regulatory influences by other cytokines such as interleukin-1 (Gillis 1983).

1.8 Interleukin-1 (IL-1)

Macrophages are activated by the stimulation of resting cells by a number of soluble or particulate stimuli. The activation is accompanied by the expression of class II histocompatibility molecules on the cell surface and the secretion of a range of soluble products such as the multipotent cytokine interleukin-1 (IL-1) (Unanue 1981). It is suggested that IL-1 may be a key intercellular messenger for host defence and has a critical role in the clonal expansion of T lymphocytes (Wood 1984) and in B lymphocyte activation (Aarden et al., 1979). IL-1 appears to be a biologically fundamental molecule; this concept being supported by the fact that IL-1 in evolutionary terms is old (estimated at 400 million years) and evolved before lymphocytes and immunoglobulins. The most potent soluble inducer of IL-1 from monocytes and macrophages appears to be endotoxin or lipopolysaccharide (LPS) from Gram negative bacteria (Munoz et al., 1987).

1.8.1 The biochemistry of Interleukin-1

IL-1 has been purified to apparent homogeneity in many different laboratories (Mizel and Mizel 1981, Pacak and Siegert 1982) and the activity has been found to be associated with a small protein whose

molecular weight is usually estimated as 12-15 kDa. In almost all species it has been found that IL-1 activity is mediated by several distinct proteins with differing molecular weights ranging from 2-75 kDa (Kimball et al., 1984). In addition to molecular weight heterogeneity of IL-1 there is also a charge heterogeneity with two peaks of activity being observed at isoelectric points of approximately pH 7 and 5 and other peaks reported between the two (Lachman et al., 1977).

The identity of the 'real' interleukin-1 has been a controversial issue for many years and it is now generally accepted that the smaller molecular weight species are proteolytic breakdown products and the 75 kDa represents an aggregated form of the molecule (Dinarello et al., 1984). Auron et al. (1984) isolated a human IL-1 cDNA clone from peripheral blood monocytes encoding a 269 amino acid precursor lacking a signal peptide, which is cleaved to a product with most of the reported biological activities of natural IL-1 of pI 7.0. A second human IL-1 gene was discovered when March et al., (1985) isolated two distinct but distantly related cDNA clones from a human macrophage DNA library encoding proteins sharing IL-1 activity. The proteins were termed IL-1 alpha and IL-1 beta with the latter showing considerable homology to the human pI 7.0 cDNA.

1.8.2 The production and release of Interleukin-1

Although both species of IL-1 are produced by a population of macrophages in response to the same stimulant, it is not known whether a single cell is capable of producing both IL-1 alpha and beta simultaneously. The absence of a characteristic signal sequence

which is usually a feature of secreted proteins transported from an intracellular to an extracellular environment has raised questions as to whether IL-1 is truly a secretory protein. Gery and Lepe-Zunige (1984) have proposed that perforation of the plasma membrane provides one mechanism for the release of IL-1 from intracellular sites. Alternatively IL-1 may be released from non-injured cells by a sequence of intracellular enzymatic cleavages.

In addition to a secreted form of IL-1, a plasma membrane associated form which stimulates the proliferation of thymocytes has been described on murine peritoneal macrophages. The degree of stimulation of T cell lines and clones is quantitatively related to the amounts of membrane IL-1 expressed as well as class II antigens (Kurt-Jones et al., 1985a). It has been suggested that the antigen presenting/accessory functions of the macrophage are mediated by IL-1 which is only present on the plasma membrane of stimulated macrophages (Kurt-Jones et al., 1986). The concept of a membrane-associated form of IL-1 may be exciting, however it has not been demonstrated using either iodination with lactoperoxidase of stimulated cells nor by immunoprecipitation analysis (Mizel 1987).

1.8.3 Mechanism of action of Interleukin-1

The effects of IL-1 on T cells are summarised in Table 1.2

TABLE 1.2 Effects of IL-1 on T cells

Comitogenic with lectins or Ag + Ia
Increased lymphokine secretion (IL-2, BCGF, CSF etc)
Increased expression of IL-2 receptors
Increased stability of E-rosettes
Chemotaxis

The exact mechanism of the activating action of IL-1 on T cells is not yet known but cell cycle studies have shown that IL-1 is not required for lectins to induce the G_0 to G_{1a} blast transformation, however, having entered G_{1a} cells can then respond to IL-1 by proceeding through G_{1b} to the S phase of the cell cycle (Kristensen et al., 1982). These events appear to involve the induction of S phase by the intermediate formation of interleukin-2 (IL-2).

IL-1 appears to regulate IL-2 in two ways, firstly at the level of production, and secondly at the level of action, this proposal being based on the following evidence:-

1) IL-1 induces IL-2 production by lectin activated T cells (Larsson et al., 1980).
2) IL-1 increases the expression of structures recognised by anti-IL-2 receptor antibodies and induces receptivity to IL-2, possibly by increasing the receptor affinity rather than by increasing receptor number (Oppenheim et al., 1986).

1.8.4 Does T cell activation depend on IL-1?

Macrophages are required at the time of the primary signal for T cell activation otherwise early (IL-2 production, IL-2 receptor expression and RNA synthesis) and late (DNA synthesis) T cell responses are prevented (Williams et al., 1985). IL-1 can partially restore T cell responses (Williams et al., 1985), but full activation seems to require the accessory cell membrane, presumably to present antigen in the context of class II MHC (Unanue et al., 1984). Such

data suggest an enhancing role, as opposed to an obligatory role for IL-1 in T cell activation, with IL-1 having a limited ability to activate T cells in the absence of accessory cells (Koide et al., 1987).

The following working model for T cell activation has been proposed by Mizel (1987):

In the presence of T cell mitogen or antigen, T cells and accessory cells are mutually stimulatory. The accessory cell is induced to begin IL-1 synthesis and secretion, the induction involving a complex of processed antigen, class II MHC and T cell receptor. The T cells initiate IL-2 and IL-2 receptor synthesis and expression and are stimulated to produce IL-1 receptors to become fully responsive to IL-1 (Shirakawa et al., 1987). IL-1 responsiveness confers a heightened ability of T cells to produce IL-2 and IL-2 receptors in response to antigen/class II MHC antigen complexes or mitogen. The interaction of IL-2 and its receptor then results in T cell proliferation. It may also be that activated T cells produce a lymphokine which augments the ability of the accessory cells to produce IL-1 giving rise to a secondary amplification loop in the interactions of accessory cells and T cells.

1.8.5 The Interleukin-1 receptor (IL-1R)

The murine IL-1 receptor was originally reported to be a 79.5 kDa T cell plasma membrane protein which bound the 17.5 kDa form of human IL-1 beta with high affinity (Dower et al., 1985). Subsequent studies have shown that a similar IL-1 receptor is present on a

variety of cell types of both human and murine origin. The existence of two distantly related species of interleukin-1 (IL-1 alpha and IL-1 beta) raises the question as to which form of IL-1 can bind to cells. The two forms of IL-1 have been shown to share a common receptor (Dower et al., 1986 a,b). One presumes that the two forms of the hormone were produced by a gene duplication event followed by evolutionary divergence, however both gene products have conserved the receptor binding function and it may be that some advantage is to be gained from the existence of two distinct molecules with an identical spectrum of activities.

1.9 Interleukin-2 (IL-2)

Interleukin-2 was originally termed T cell growth factor (TCGF) or T cell derived glycoprotein hormone and was initially thought to be strictly a T cell trophic growth factor, stimulating replication by binding to specific cell surface receptors. However highly purified IL-2 can cause the proliferation of natural killer cells (Hefneider et al., 1983, Henney et al., 1981) and other cytotoxic effector cells (Merluzzi et al., 1983, Grimm et al., 1983). IL-2 receptors have been described on B cells activated by pokeweed mitogen (PWM) (Waldmann et al., 1984). IL-2 also induces or enhances the production of a variety of other cytokines such as B cell growth factor (IL-4), colony stimulating factor and interferons (Farrar et al., 1982, Pearlstein et al., 1983) and appears to play a central role in the regulation of immune function.

Resting T cells have been reported to be unresponsive to the effects of IL-2 although IL-2 receptors appear to be present (Robb et

al., 1981, 1984; Depper et al., 1984). Using recombinant IL-2 (rIL-2), resting T cells have been shown to respond by an increase in DNA synthesis, an increased IL-2 receptor (Tac protein) expression and the production of interferon and morphologic blast transformation (Hammer and Gillis 1986). Given the potential for therapeutic administration of rIL-2 (Lotze et al., 1985) effects on resting T cells may become increasingly important.

Normal human monocytes also express receptors for IL-2 on their surface (Herrmann et al., 1985) which indicates that IL-2 is not exclusively a lymphocytotrophic hormone (Benveniste and Merril 1986). The biological significance of the effect of IL-2 on monocytes has yet to be determined.

The detectable levels of IL-2 in culture supernatants of lymphocytes stimulated with either PHA or inactivated mumps virus peaks at 18-24 hours and 48 hours respectively. A decrease in IL-2 levels in the culture supernatant after day 2 is paralleled by an increased expression of IL-2R. The maximal T cell proliferation occurs shortly after or simultaneously with the maximal expression of IL-2R (Bruserud et al., 1986).

The major physiological role of IL-2 is thought to be the activation of resting T lymphocytes (in G_1 phase) to progress through the proliferative phases (S_1, G_2 and M) of the cell cycle with IL-2 mediating its effects on T cells via hormone-like interactions with surface receptors.

Production of IL-2 has been shown to increase the rate of synthesis of RNA for the transferrin receptor which is essential for the iron uptake required for cellular growth and proliferation

(Blazsek and Mathe 1984) and for the cells to enter the S phase of cell division. IL-2 has also been reported to cause the secretion of gamma interferon (Miedema et al., 1985) and tumour necrosis factors alpha and beta (Nedwin et al., 1985), but does not alter T cell antigen receptor mRNA levels.

How IL-2 binding leads to a biochemical response is unknown. The cytoplasmic domain of the receptor, which is very short (13 amino acids), or the transmembrane domain may bind a subunit protein which functions as a signal transducing unit (Watson 1986).

1.9.1 Biochemistry of Interleukin-2

Human IL-2 is a 133 amino acid glycoprotein (Lowenthal et al., 1985) with a molecular weight range of 14.5-17.0 kDa, depending on the stimulators and/or co-stimulators used in its production and has a secondary structure predominantly of alpha helix (Liang et al., 1985). The observed heterogeneity is a result of differential glycosylation (Welte et al., 1982) which has no apparent effect on IL-2 action on purified T cell populations.

The role of the carbohydrate may reside in the mechanism of clearance (Robb and Smith 1981). The rapid removal of circulating desialylated glycoproteins is well documented and is mediated by a carbohydrate recognition system present in hepatocytes (Neufeld and Ashwell 1979). From the potential immunotherapeutic standpoint, recombinant IL-2 which is non-glycosylated might be expected to have a privileged life span in vivo.

1.9.2 The Interleukin-2 receptor (IL-2R)

The interleukin-2 receptor gene is localised on chromosome 10 and codes for a 272 amino acid glycoprotein with a cytoplasmic domain of 13 amino acids (Greene et al., 1985b). Using monoclonal antibodies to the IL-2R (anti-Tac) the receptor has been shown to contain a 55 kDa glycoprotein (Tac antigen). The binding of antigen to specific receptors present on the surface of resting T cells in the presence of macrophage-derived IL-1 triggers the de novo synthesis and secretion of interleukin-2 (Morgan et al., 1976), the IL-1 modulating/enhancing the transient expression of high and low affinity IL-2R. The continuous expression of the receptors on the cell surface and consequently IL-2-dependent cell proliferation requires repeated stimulation by the antigen (Diamantstein and Osawa 1984, Reske-Kunz et al., 1984). The IL-2-dependent proliferation of cells without external stimuli is either due to the presence of a small number of IL-2 receptor-bearing cells in the respective population or to a small number of IL-2 receptors present on the surface of cells.

1.9.3 High and low affinity IL-2 receptors

The evidence for high and low affinity IL-2 receptors came from early IL-2 receptor binding assays using radiolabelled IL-2 and radiolabelled anti-Tac antibodies. Anti-Tac antibodies react equivalently with both high and low affinity types of IL-2R. The number of IL-2 receptors on the surface of PHA-activated lymphoblasts has been reported to be between 2000 and 6000 (Robb et al., 1981; Depper et al., (1984). Robb et al., (1984) demonstrated a large

pool of IL-2 receptors with 1000 to 10000 fold lower apparent affinity, this finding was supported by Lowenthal et al (1986) although these workers reported a 100 fold affinity difference. For the high affinity IL-2R the affinity for IL-2 is relatively high (Kd = 5-20 pM) with the IL-2 receptors fully saturated at concentrations around 10^{-10} M (1.5 ng/ml). The sensitivity with which a T cell responds to IL-2 is not determined solely by the number of high affinity receptors it bears. Using cDNA transfection experiments it appears that the Tac protein is only part of the high-affinity functional IL-2R molecules and not necessarily the most important part (Greene et al., 1985b, Sabe et al., 1986).

Investigators have now turned their attention to a search for the so-called "converter" protein that would confer high affinity IL-2 binding on the Tac antigen protein. A 75 kDa protein completely separate and distinguishable from the high affinity receptors and low affinity binding sites has been identified (Teshigawara et al., 1987) which is easily distinguishable from the 55 kDa Tac antigen protein. Clones expressing the high affinity receptors have been shown to clearly display both IL-2 binding proteins, suggesting both proteins may be involved in the formation of fully functional, high affinity receptors (Smith 1988). High affinity IL-2 binding results in the occupation of sites on two distinct proteins with the binding site cooperating to form biologically relevant high affinity IL-2 receptors. The 75 kDa chain has been referred to as the alpha-chain, the 55 kDa (Tac antigen) as the IL-2 receptor beta-chain (Fig 1.2). Tac antigens expressed in the absence of 75 kDa chains cannot trigger a cell growth response. The 75 kDa protein can mediate rapid

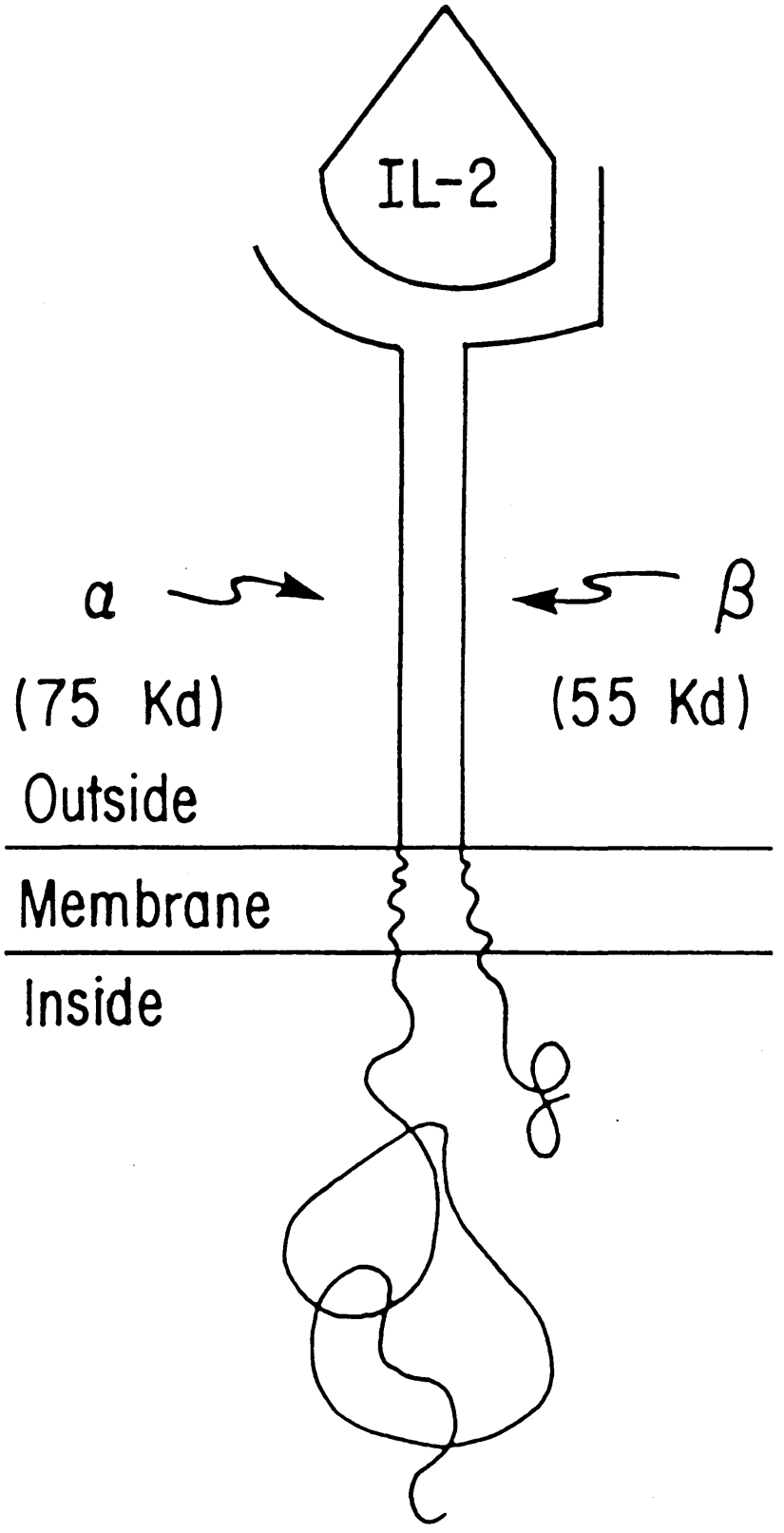


Figure 1.2 The Proposed Structure of High-Affinity Interleukin-2 Receptors (from Smith 1987).

internalisation of membrane bound IL-2 ($t_{1/2}$ = 15 minutes) whereas internalisation after binding to the Tac antigen is negligable (Robb and Greene 1987). Unlike many receptors composed of multiple subunits the 75 kDa and Tac antigen proteins appear to be able to be transported to and displayed on the surface of cells in the absence of any other protein.

There are three possible mechanisms by which IL-2 binding may stimulate T cell proliferation. The binding of IL-2 to Tac antigens may be stimulatory, however this possibility is unlikely as saturation of the Tac low affinity binding sites does not affect the rate of cell proliferation. Binding to 75 kDa chains alone can stimulate signal transduction, but the concentrations required to stimulate cell growth are 100 fold higher than those required on binding to the 75 kDa - Tac heterodimer. It therefore appears that the 75 kDa subunit is the biologically relevant moiety with the Tac antigen appearing to function as a helper binding protein having no signalling capacity of its own (Smith 1988).

Kinetic binding studies comparing cells which solely express 75 kDa chains or Tac antigens with cells expressing both chains have shown that:

1) IL-2 dissociates from the Tac antigen rapidly (1.7 s).
2) IL-2 binds to the 75 kDa chain 2 - 3 orders of magnitude more slowly than to the Tac antigen ($k = 3.8 \times 10^5/M.s$).
3) Hybrid binding characteristics are observed in cells expressing both chains.

(Wang and Smith 1987)

IL-2 binding to high affinity receptors takes on the characteristics of binding to the Tac antigen for the forward reaction, associating rapidly to the Tac - 75 kDa dimer. The reverse reaction follows the behaviour of IL-2 as if it were binding to the 75 kDa chain and exhibits a slow dissociation ($t_{1/2}$ 50 minutes) as compared to the dissociation from the Tac antigen ($t_{1/2}$ 1.7 s). There is no covalent linkage between alpha and beta chains and it is a non-covalent association which is responsible for the formation of high affinity binding sites, such interaction is likely to be dynamic and subject to association and dissociation.

The incubation of cells with anti-Tac antibody causes a shift towards intermediate affinity and leads to an abrogation of cellular responses to low levels of IL-2 (Wang and Smith 1987) by preventing the binding of IL-2 to the Tac antigen and disrupting IL-2 binding to the alpha/beta heterodimer.

The observation of the generation of high affinity receptors from the two distinct low affinity binding proteins is of great importance. This system appears to have been selected and conserved (the murine IL-2R appears to be similar) and appears to be operative in other polypeptide hormone receptor systems, with interleukin-3 and nerve growth factor receptors comprising two distinct binding proteins (Nicola and Peterson 1986, Hosang and Shooter 1985).

1.9.4 The stimulation of IL-2 receptor expression

Once expressed on the cell surface high affinity IL-2R, representing around 10% of the IL-2R are at least partially internalised as a consequence of IL-2 binding (Smith and Cantrell 1985). However it is uncertain whether the receptor-mediated

endocytosis is involved in signal transduction. The interaction of IL-2 with its high affinity membrane receptor triggers the expression of additional receptors on the cell surface (Depper et al., 1985), stimulates an expansion of the antigen reactive clonal T cell population and generates specific effector cells which mediate helper, suppressor and cytotoxic T cell functions.

The activation of resting T cells with phytohaemagglutinin (PHA) leads to the induction of IL-2R expression which peaks at 48-72 hours (Depper et al., 1984). IL-2R levels decline after extended periods in culture with 80-90% of the IL-2R being lost after 10-12 days (Depper et al., 1984), possibly indicating an important regulatory role in the termination of the T cell immune response. Such cultured non-proliferating T cells are still capable of restimulation of IL-2R expression and a second round of cellular proliferation (Depper et al, 1985), with mitogenic lectin or antigen producing up-regulation of IL-2R expression (Kaplan et al., 1984). The greatest increase in receptor number on restimulation is obtained by re-exposure to mitogenic lectin (Depper et al., 1984) which causes a 2 to 10 fold increase in IL-2R number in 24 hours. The increase is dependant on de novo RNA and protein synthesis and is not solely the result of the mobilisation of preformed intracellular receptors to the cell surface (Greene and Leonard 1986).

Phorbol myristate acetate (PMA) also increases receptor expression as does phospholipase C (Depper et al., 1984) which suggests that activation of protein kinase C may play an important role in IL-2R expression. Of the two types of receptor, the low affinity receptors are preferentially induced in re-expression,

however the functional high affinity receptors must also be induced as proliferation is observed.

In the absence of cell cycle progression the number of IL-2R positive cells increases as does receptor density suggesting an initial threshold of IL-2R density to be required for the commitment to cell cycle progression as IL-2R expression precedes the onset of DNA synthesis (Smith 1984). After cell division in response to IL-2, daughter cells must be triggered by antigen once again to express IL-2R. In vivo it may be supposed that once antigen is cleared T cell clones lose IL-2R and revert to resting (G_0) cells. IL-2R binding reaches equilibrium within 15 minutes with several hours of IL-2R interaction being required to initiate cell cycle progression. It may be that it is an accumulation of a critical concentration of intracellular signals which ultimately determines the quantal response of the resulting DNA replication.

1.9.5 Interleukin-2 receptor release

On day 2 of culture, IL-2R can be detected in the supernatant of Con A stimulated murine splenocytes, the released IL-2R representing the majority of IL-2R present on the cell surface (possibly the low affinity type). Released IL-2R can also be detected in the culture supernatants of stimulated human lymphocytes, the isolated soluble IL-2R is of lower molecular weight than the Tac antigen isolated from the cells (Rubin et al., 1985). It has been suggested that the receptors are cleared from the cell by cell-associated proteases which may represent a method by which an immune response is terminated or it may serve as an immunoregulatory signal by competing

for available IL-2 (Rubin et al., 1985). Such observations are not in vitro artefacts as soluble IL-2R are detectable in the serum of mice bearing IL-2 binding protein positive tumour cell lines (Osawa et al., 1986a). The soluble proteins detected have anti IL-2R reactive epitopes and at least part of them can react with IL-2 (Osawa et al., 1986b).

In the human, levels of soluble IL-2R have been shown to be elevated in a number of pathological conditions. The released protein may be of significant prognostic value in patients with a number of lymphomas and leukaemias (Wagner et al., 1987). Significant elevations of soluble IL-2R have also been documented in transplant recipients experiencing rejection episodes (Colvin et al., 1987; Cornaby et al., 1987) and in persons with viral infections such as acquired immune deficiency syndrome (AIDS) (Durno et al., 1986).

1.10 The Temporal Sequence of Gene Activation Following Mitogenic Stimulation.

Analysis of RNA levels have shown that PHA, PMA and IL-2 augment IL-2R mRNA production (Depper et al., 1985). The temporal sequence of gene activation by PHA/PMA in human T cells has been defined (Kronke et al., 1985). IL-2 receptor gene transcription occurs at a high level 3 hours after activation and continues at a high level for 24 hours before declining. An increase in transcription accounts for an initial increase in IL-2R mRNA levels, however mRNA levels appear to fall before there is a significant decline in IL-2R nuclear transcription. Observed post transcriptional rates of mRNA processing or degradation may be important for the regulation of

IL-2R gene expression. The lag between peak mRNA levels (around 24 hours) and the peak receptor expression at the cell surface (48-72 hours) is currently unexplained.

The transferrin receptor gene is not expressed until 24 hours after mitogen activation and does not reach peak levels until 48 hours. The transcription of the transferrin receptor gene is dependent on protein synthesis which suggests that its expression is mediated by prior interaction of IL-2 with its cellular receptor (Neckers and Cossman 1983).

1.11 IL-2 Regulation of the T Cell Immune Response

Due to the feature of the IL-2 hormone-receptor system that both the secretion of the ligand and the cell surface expression of the receptor must each be specifically induced by antigen or mitogen, the external signals and pathways which ultimately lead to expression of the two gene products in T cells should represent one level by which T lymphocyte clonal expansion is regulated. T cells possess differential requirements for induction of IL-2R, although all murine T cells express IL-2R after Con A stimulation, T cell-accessory cell interactions might be necessary for IL-2 expression by some T lymphocytes (Malek et al., 1985). Such differential activation requirements for IL-2R expression may represent an important level of immunoregulation since it may promote a selective or sequential growth of functional T cell subsets.

In addition, the ability of IL-2 to regulate IL-2R number and perhaps subsequent signal transduction may have important implications relating to regulation of a T cell immune response, as

IL-2R upregulation should permit T cells recently activated by antigen to proliferate solely in response to IL-2 without the necessity for each daughter cell to encounter antigen. Obviously such a response must be controlled and three levels of regulation may exist:-

1) IL-2 deprivation of the cells, possibly by competition with soluble IL-2R for available IL-2.
2) Action of specific suppressor cells.
3) Activated T cells may require an increasing number of occupied IL-2R for proliferation, ie a higher local concentration of IL-2 (Malek et al., 1986).

The mechanisms by which such control would be mediated are as yet unknown.

1.12 T Cell Antigen Receptor Triggering.

The T cell antigen receptor is a molecular complex comprising at least five polypeptide chains (Meuer et al., 1983). Two of these chains are highly variable disulphide linked Ti alpha and beta subunits (90 kDa) which are non-covalently associated with the three monomorphic CD3 (T3) subunits which are believed to mediate signal transduction when the T cells are activated by receptor binding. Most CD3-Ti alpha beta type T cell receptors recognise foreign antigens in association with cell surface molecules encoded by the MHC (Benacerraf and McDevitt 1972). This phenomenon, termed MHC restriction, distinguishes T cell recognition from the direct antigen specificity of the immunoglobulin receptor on most B cells. Such MHC restriction is learned or selected for during T lymphocyte ontogeny

in the thymus (Zinkernagel and Doherty 1975). Some Ti alpha beta heterodimers recognise target structures without binding to MHC (Ritz et al., 1985). In addition to MHC restriction, T cells also respond to an MHC-encoded protein from another individual in the absence of antigen; it is this effect that accounts for the rejection of grafted or transplanted tissue (Marrack and Kappler 1986).

The precise molecular nature of the antigenic determinant recognised by the T cell receptor of the $CD4^+$ cells has not been completely resolved. Werdelin (1987) has suggested that Ia molecules serve to bind antigen fragments and stabilise them in the membrane of presenting cells, shielding them from proteolytic degradation and permitting T cells to bind the epitopes so displayed. It is this antigen-Ia complex which is recognised by the T cell receptor (Fig 1.3). The effective interaction between T cells and their target antigens is not exclusively controlled by the affinity and density of T cell receptors, rather it is the result of multiple contributions made by a combination of surface receptors and ligands. Results strongly indicate that CD4:HLA-DR binding occurs and this interaction augments T cell activation (Gay et al., 1987), the CD4 molecule serving an accessory function and stabilising cell interaction (Fig 1.3). Anti CD4 antibodies inhibit antigen-induced in vitro proliferation (Biddison et al., 1982) and lymphokine release (Swain et al., 1983). Work has shown there to be a physical association of the T cell receptor complex and CD4 (Saizawa et al., 1987) with the CD4 antigen migrating together with the T cell receptor into the contact site between the T cell and the antigen presenting cell (Kupfer et al., 1987).

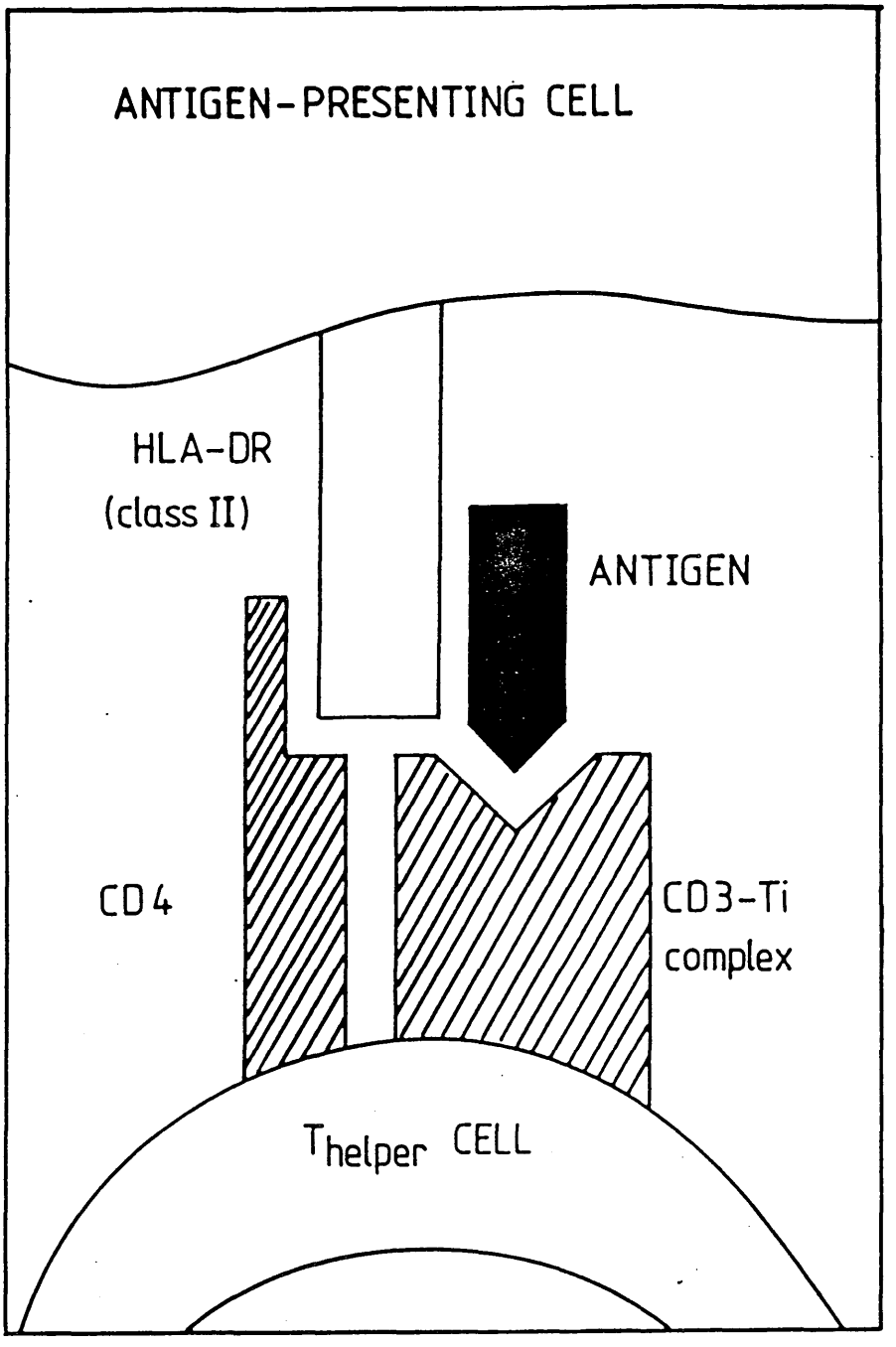


Figure 1.3 Recognition of Antigen by T cells. T helper cells are stimulated by antigen in association with MHC class II molecules on the surface of antigen-presenting cells. (Male et al., 1987).

The binding of antigen or mitogen to the T cell receptor complex leads to a calcium flux which is then followed by general activation. The binding of soluble or monovalent ligands to the T cell receptor is not sufficient to initiate the process of T cell activation. A second signal is required from macrophages which does not involve class II gene products and is possibly IL-1. That differential stimulatory requirements exist regarding growth and lymphokine release underlines the critical role of macrophages in T cell activation. Proliferation of resting T cells in response to IL-1 is only observed after triggering of the T cell receptor complex with surface linked anti-CD3 (ie antigen). One proposed mechanism is that interaction of antigen with the CD3 complex induces IL-2R expression which is then up-regulated by IL-2 (Smith and Cantrell 1985, Welte et al., 1984, Reem and Yeh 1984). This supports the idea that regulation of IL-2R expression on both resting and presensitised lymphocytes is ultimately linked with the T cell receptor for antigen (Meuer et al., 1984a). This linkage, in concert with ligand induced down-regulation of IL-2R, provides an essential mechanism to prevent uncontrolled growth in the IL-2 hormonal system and may represent the basis of clonal T cell selection by antigen.

There are two pathways to T cell activation, either via the CD3-Ti antigen receptor complex (macrophage and IL-1 dependent) or via an 'alternative' pathway mediated through a 50 kDa glycoprotein which occurs in the absence of accessory cell interaction. The binding of monoclonal antibodies specific for the E (sheep red blood cell) receptor (designated CD2, T11) seems to initiate an alternative antigen independent activation (Meuer et al., 1984b) or suppression

pathway (Wilkinson and Morris 1984) depending on the epitope bound.

One of the signals required for T cell activation through the alternative pathway is provided by the interaction of CD2 with a naturally occuring 42 kDa complementary cell-surface molecule (Hunig et al., 1987). The molecule is expressed on all blood cells and some other tissues (Hunig 1986), however this signal is insufficient to trigger T cell activation. It may be possible that there is as yet an undefined additional signal involved in T cell macrophage interaction which after multimeric antigen receptor ligation is directed from the responding T cell towards the accessory cell to engage IL-1 production, possibly requiring physical contact between T cells and the macrophages (Meuer and Meyer 1986).

An antigen-independent pathway of T cell activation has also been reported to be mediated via a novel 103 kDa T cell-specific activation antigen which mediates an autocrine IL-2 dependant mechanism (Fleischer 1987). These observations suggest the existence of antigen-independant pathways of T cell activation that can be regulated via other cell surface structures.

1.13 Signal Transduction

Much work has been done to investigate the results of T cell activation such as the production of IL-2 and other lymphokines, cellular proliferation and cytolysis of target cells. Surface markers of cellular activation such as the production of receptors for IL-2 and transferrin have also been identified. All of these require de novo activation of genes and hence a signalling system effective in transducing signals originating at the cell surface to

the nucleus. It is still unclear whether the different T cell subsets have differing signalling requirements to activate their individual functions or whether multiple activators mediate their actions through the same signal transduction mechanism.

Increased intracellular Ca^{2+} concentration is a critical component of signal transduction by various cell-surface receptors and plays an important role in T cell triggering via the antigen receptor. Only the initial phases of the T cell antigen receptor-mediated increase in intracellular [Ca^{2+}] is due solely to the release of Ca^{2+} from intracellular stores which suggests that the perturbation of the antigen receptor must activate a Ca^{2+} transporter or open a Ca^{2+} channel within the plasma membrane (Weiss et al., 1986). IL-2/IL-2R interaction does not trigger a change in [Ca^{2+}] (Mills et al., 1985).

It has been shown that different signalling requirements are needed for the induction of the IL-2R and IL-2 genes and for the activation of T cell help or cytotoxicity functions (Isakov and Altman 1985, Truneh et al., 1985). Using the phorbol ester PMA and calcium ionophore A23187 to stimulate lymphocytes, it has been suggested that the gene controlling IL-2 and IL-2R have different intracellular [Ca^{2+}] concentration requirements for their maximal expression (Chopra et al., 1987). Work has shown that IL-2R expression does not appear to require an increase in intracellular [Ca^{2+}] (Gelfand et al., 1986).

1.14 Protein Kinase C (PKC)

In vitro evidence using TPA, a stimulator of protein kinase C, suggests the protein kinase C pathway to be triggered in T cell

activation. PKC activation induces the expression of the IL-2 receptor and the disappearance of the antigen receptor complex (CD3-Ti) from the surface of T cells (Ando et al., 1985). PKC activation is not a sufficient signal for IL-2 secretion, the IL-2 gene requires both activated PKC and a Ca^{2+} signal (Isakov et al., 1987). Activated PKC cannot substitute for the IL-2 dependant triggering of the IL-2R (Albert et al., 1985).

PKC phosphorylates a wide range of substrates in vivo or in cell-free systems, in T cells the relevant proteins being IL-2R which is phosphorylated at ser 247 (Shackelford and Trowbridge 1984) and the CD3 complex. On the activation of human T cells with tumour promoters such as phorbol esters which can directly activate PKC in vitro, no transcription of the IL-2 gene or secretion of IL-2 is detectable (Isakov et al., 1985a) and the proliferation cannot be blocked by anti IL-2 antibody (Isakov et al., 1985b). These observations suggest the presence of an IL-2 independent pathway of cellular proliferation possibly via the activation of PKC and indicate that IL-2 may not be a universal signal for the proliferation of all T cells (Isakov et al., 1987).

One of the main functions of accessory cells in the immune response appears to be the activation of PKC in T cells by providing a matrix for the cross-linking of T cell antigen receptors (Droge 1986). It has been shown that PKC in T lymphocytes may be activated by arachidonic acid or other lipid components from accessory cells, the former almost as effectively as diacylglycerol (McPhail et al., 1984) and the transfer of free fatty acids from macrophages to lymphocytes may explain at least some of the cooperative effects between these cell types (Kroner et al., 1981).

The net effects of PKC activation on T cell proliferation is due to at least five different and partly counterproductive mechanisms:

1) Enhanced G_0 to G_1 transition
2) Retarded G_1 to S phase progression
3) Augmented expression of IL-2 receptors
4) Augmented IL-2 production by helper T cells resulting in enhanced G_1 to S phase progression in cells with IL-2 receptors
5) Down regulation of the antigen receptor and consequently of the antigen driven proliferation

1.15 In Vitro Investigation of the Allograft Rejection Response

Investigations into the mechanism and control of the cell-mediated graft rejection response centre around the capacity of human blood lymphocytes to proliferate in culture. The stimulants for such a proliferative response may be either sugar binding proteins (lectins) or allogeneic MHC antigens. The appearance of lymphoblasts in cultures containing lymphocytes from two unrelated individuals is termed the allogeneic mixed lymphocyte reaction and is recognised to be the *in vitro* correlate of the *in vivo* allograft rejection response. The degree of proliferation can be determined by the incorporation of radioactive precursors into the stimulated cells. Alternatively other aspects of the response such as cell surface antigen expression and/or soluble mediator release can be investigated.

The gross morphological changes and biochemical events occuring in lectin stimulated lymphocytes *in vitro* resemble many of the

antigen-induced reactions that take place *in vivo*.

1.15.1 The mitogenic stimulation of lymphocytes by the plant lectin phytohaemagglutinin (PHA).

The polyclonal activation of T lymphocytes *in vitro* can be achieved by the substitution of antigen-MHC stimulation by a variety of agents including the plant lectin phytohaemagglutinin (PHA). Whilst such activation is unrelated to the *in vivo* situation, mitogenic stimulation provides one of the best models for dissecting the control of mammalian cell growth and proliferation. How mitogens work remains obscure, but such *in vitro* activation may involve a two-step signalling system analogous to the *in vivo* situation. The initial signal may arise from the binding of the polyclonal mitogen and the second signal by IL-2 before commitment to DNA replication is achieved (Robb 1984, Palacios 1982).

The phytohaemagglutinins (PHA) obtained from the red kidney bean, *Phaseolus vulgaris*, have been shown to be a mixture of five glycoproteins that are heterogeneous by several physicochemical and biological criteria (Allen *et al.*, 1969). One of these proteins is a potent leukoagglutinin with a low haemagglutinin activity (L-PHA). L-PHA consists of four identical covalently linked subunits which have a high affinity for lymphocyte membrane receptors but a low affinity for red cell membrane receptors (Yachnin and Svenson 1972).

It has been proposed that mitogens increase cytoplasmic $[Ca^{2+}]$ which subsequently acts as a trigger for the cascade of intracellular processes necessary for proliferation (Metcalf *et al.*, 1980). PHA induces a rapid increase in intracellular $[Ca^{2+}]$ which reaches a

peak in less than 1 minute and then falls to maintain a sustained plateau. Whilst it appears that the initial peak observed is due to the release of Ca^{2+} from intracellular stores, it is Ca^{2+} uptake which may be essential for the response induced by PHA (Gelfand et al., 1987a). Data indicate that PHA triggers a membrane potential sensitive event, namely Ca^{2+} entry into the cell and this change in intracellular [Ca^{2+}] regulates IL-2 secretion (Gelfand et al., 1987b). The changes in intracellular [Ca^{2+}] are not mediated by the opening of K^{+} channels (Gelfand et al., 1987b).

PHA has been shown to bind to the CD3 (Valentine et al 1985) and the Ti (Kanellopoulos et al., 1985) structures at the lymphocyte membrane. Blocking of the CD2 molecule by monoclonal antibodies has also been shown to abolish PHA stimulated Ca^{2+} fluxes (O'Flynn et al., 1985).

In addition mitogenic lectins such as PHA and Con A are potent activators of phosphatidyl inositol turnover and PHA appears to regulate the channel opening through the generation of inositol polyphosphates (Kuno and Gardner 1987). It appears that PHA may activate lymphocytes via different cell surface structures with the different pathways having different requirements for accessory cells and factors. Mire et al., (1985) have shown that lymphocytes extensively depleted of monocytes were capable of undergoing a single round of cell division, further proliferation was dependent on exogenous IL-1 and IL-2. The IL-1 is presumably required for subsequent phases of the response.

1.15.2 The allogeneic mixed lymphocyte reaction (MLR).

The co-cultivation of lymphocytes from unrelated individuals leads to blast transformation and proliferation similar to that observed upon the addition of mitogens, the participating cells being peripheral blood lymphocytes. The MLR is a relatively simple test yet it exists in many variants. Macrophages are required for an MLR to proceed as the reaction does not occur or its strength is sharply reduced when all the adherent cells are removed from the mixed culture.

Genetic analysis has revealed that it is the HLA-D locus that codes for the determinants which stimulate the allogeneic MLR. The activation signal is provided in the main by cell surface molecules of B lymphocytes, macrophages and dendritic cells and probably some T lymphocytes. The responding cells in the MLR are of the T helper ($CD4^+$) subclass. This conclusion is supported by the finding that HLA-D antigens are expressed on non-T lymphocytes and monocytes, but are absent or weakly expressed on resting cells (Mann and Sharrow 1980). The proliferative response of the MLR is followed by the generation of cytotoxic T lymphocytes which express specificity for the allogeneic gene products of the HLA-A, -B, or -C loci. The HLA-D locus is distinct, but closely linked to the HLA-A, -B, and -C loci. The allogeneic MLR possesses the essential attributes of an immune response: memory and specificity.

The stimulated cells enter into blastogenesis with the help of macrophages and macrophage-produced factors and secrete soluble mediators into the medium. Allogeneic MHC antigens are highly immunogenic and do not need to be recognised in association with self

MHC molecules for T cell stimulation to occur. They provide the dual recognition signal of self MHC plus antigen.

The nature of the alloantigen recognising receptor is unknown. The receptor may be distinct from or the same as the receptor for other antigens, a third possibility is that the receptor is a compromise between the two. The T cell receptor may be able to recognise both self and non-self and treats differential requirements as non-self and the shared determinants as self. The allogeneic MHC appears to the responder cells as antigen altered self MHC.

SECTION B

THE IMMUNOBIOLOGY OF THE FOETO-MATERNAL RELATIONSHIP

1.16 Trophoblast and Class I MHC Antigens

Although MHC antigens are absent (or perhaps present at extremely low density) from the trophoblast tissues in direct contact with maternal blood (Billington and Bell 1983 a,b), other major trophoblast populations opposing maternal decidual tissue have now been shown to express class I MHC antigens and examination of minor trophoblast subpopulations in the placental bed, in contact with both maternal uterine cells and blood, may reveal these too to be antigenic.

Extravillous (or non-villous) trophoblast (comprising the interstitial and endovascular trophoblast in the placental bed, the cytotrophoblast in the decidua, the cell islands and in the amniochorion) has been shown to express class I antigens during the first, second and third trimesters (Redman et al., 1984, Sunderland et al., 1981b). The trophoblast class I MHC antigen expressed on extravillous cytotrophoblast cells is different to that of adults in that it has a heavy chain of about 40 kDa and does not appear to express the polymorphic HLA-A, -B, or -C determinants (Ellis et al., 1986).

Paternally encoded class I MHC antigens have been described on the spongy zone trophoblast layer of the placenta in the rat. In this case the survival of the dependent foetus must therefore be

assured by maternal immunoregulatory factors (Billington and Burrows 1986). The major line of evidence to suggest maternal recognition of paternally-inherited foetal alloantigens is the detection of HLA-A specific antibody in the serum of pregnant women and also in a variety of different species (Terasaki et al., 1970).

1.17 Trophoblast and Class II MHC Antigens

The major barrier to allogeneic transplantation is the presence of MHC class II products (HLA-DR). HLA-DR has not been found on human syncytiotrophoblast at term nor on immature villous trophoblast, trophoblast cell columns or the cytotrophoblast of the amniochorion (Sunderland et al., 1981a). HLA-DR positive cells are found in the human placenta, but they are not trophoblast and are thought to be macrophages (Sutton et al., 1983). These cells are separated from contact with the maternal blood and tissue and are not present in first trimester human material. Recently it has been reported that cytotrophoblast cells in the term amniochorion and first trimester chorionic villi can bind antibody specific for the MHC class II antigen HLA-DP (Starkey 1987). Weak binding has also been observed in 24 week and term syncytiotrophoblast.

1.18 Other Antigens on Trophoblast

The trophoblast surface membrane has a complex structure with some antigens being shared by both trophoblasts and lymphocytes (TLX, Trophoblast Lymphocyte cross reactive). The TLX antigens are thought to be allotypic (McIntyre and Faulk 1982) and there is the possibility that TLX expression on trophoblast may modulate maternal

lymphocyte function directly or indirectly and be important as stimuli of maternal immune responses. Heteroantisera reactive against human trophoblast membrane may inhibit *in vivo* mixed lymphocyte culture reactions (McIntyre and Faulk 1979a) as does a solubilised trophoblast membrane protein fraction (McIntyre and Faulk 1979b). Such observations suggest the presence of potent immunoregulatory properties associated with trophoblast membrane molecules (Pavia and Stites 1981).

Faulk and McIntyre (1981) have proposed a working hypothesis suggesting that recognition of non-self TLX antigens by maternal immunocompetent cells in pregnancy may lead to a protective response, whereas recognition of 'self' TLX antigens in association with trophoblast-specific isoantigen by maternal T cells may lead to early abortion. Evidence points towards a form of alloimmunisation by trophoblast which underpins successful pregnancy to term.

1.19 The Transferrin Receptor

The primary role for the expression of the transferrin receptor must be in the cellular iron uptake essential for growth or proliferation. The receptor is a 190 kDa disulphide-bonded dimeric glycoprotein containing two identical binding sites for transferrin (Loh *et al.*, 1980) and is encoded for by a gene on chromosome 3 which also contains the gene for transferrin itself (Enns *et al.*, 1982). The expression of the receptor is a feature of some trophoblast populations (placental villous syncytiotrophoblast), but not others - non-villous cytotrophoblast (Johnson and Molloy 1983) and trophoblast in amniochorion tissues (Hsi *et al.*, 1982).

The presence of transferrin receptors on the surface of syncytiotrophoblast may markedly restrict the amount of available iron (as iron-loaded transferrin) in placental intervillous spaces. This competition possibly impairs local proliferative responses of maternal blood lymphocytes and may suggest a role of immunobiological relevance for the trophoblast transferrin receptor.

1.20 The Presence of Immunocompetent Cells at the Foeto-Maternal Interface.

Work has identified the presence of infiltrating T lymphocytes in human uterine decidua. The decidual T lymphocytes do not generally express the CD3 antigen (Bulmer and Sunderland 1984) or the IL-2R (Bulmer and Johnson 1986), but do express T11 (CD2, sheep red blood cell receptor) molecules. The absence of IL-2R may be as a result of IL-2 inhibitors produced by the maternal decidua or an inherent inability of the lymphocytes to express the receptor (Bulmer and Johnson 1986). Alternatively the inhibition of IL-2R expression may be mediated by prostaglandins (Nakayama et al., 1985).

Maternal macrophages have been identified within the maternal decidualised endometrium (Bulmer and Johnson 1984). Foetal macrophages have also been identified within the placental chorionic villous mesenchyme and within the mesenchyme of the term amniochorion. The phenotype of the macrophage population is consistent with phagocytic, pinocytic and degradative functions which confirms previous observations of macrophages containing immune complexes within placental connective tissue (Johnson et al., 1977). The possible role of the macrophages as accessory cells is still to be elucidated.

Introduction

A number of mechanisms have been proposed to account for the continued survival of the implanted trophoblast despite the fact that it is a foreign tissue in a potentially hostile immunological environment.

Data indicate that approximately 50% of *in vivo* fertilised human eggs are aborted with a substantial proportion being due to lethal genes and chromosome anomalies (Clark *et al.*, 1986a). The idea of abortion being a form of graft rejection has come from a number of observations. Women who abort lack factors in their serum which are capable of blocking the reactivity of the lymphocytes against placental and/or paternal leucocyte antigens (Beer *et al.*, 1986). In addition *in vitro* fertilised embryos aborting in the human uterus are infiltrated by maternal mononuclear cells (Nebel *et al.*, 1985). It has been suggested that there must be a block of both the afferent (recognition) and efferent (effector) arm of the maternal immune response in order to prevent rejection of the foetus (Sargent *et al.*, 1987).

It is becoming apparent that maternal immune recognition of foetal alloantigens directly contributes to improved placental function and foetal survival. In pregnancy there seems to be a deviation of T cell function towards an immunotrophic or growth promoting role. The trophoblast may be dependent on maternal growth factors for the induction of embryonic gene expression for placental development with lymphocyte products such as interleukin-3 and CSF-GM

having been found to stimulate trophoblast growth (Athanassakis et al., 1987). A deficiency of such factors may lead to poor placentation and abortion. The suppression of the standard immune allorecognition events would be a necessary concommitant of this deviation in order to prevent a runaway immune reaction.

1.21 The Uterus as an Immunologically Privileged Site

It is possible that the uterus may be an immunologically privileged site, in that the arms of the cell mediated immune system are ineffective in the uterine environment. However there are rare abdominal ectopic pregnancies which do proceed to term and a variety of local immune responses can be induced within the uterus of laboratory animals (Cohen and Werrett 1975). There is lymphatic drainage of the deciduum and myometrium and lymphocyte infiltration can be observed near the trophoblast in human pregnancy. Work has demonstrated (Head and Gaede 1986) that the virgin rat uterus is well-endowed with Ia antigen bearing cells, with the Ia antigen expression being hormonally controlled. Most of the Ia^+ cells in the uterine endometrium appear to be dendritic shaped cells, suggesting the presence of a significant potential for local antigen processing in the uterus.

1.22 The Placenta as an Immunological Barrier

The placenta could conceivably prevent immunogenic foetal cells from reaching the maternal circulation or protect the foetus from maternal lymphocyte attack. The placenta is an incomplete barrier in this respect and it is well known that small amounts of foetal blood

containing red cells, leucocytes and lymphoid cells enter the maternal circulation prior to the termination of pregnancy (Adinolfi 1975, Schroder 1975). Cytotoxic antibodies to foetal antigens are absent in neonatal blood, possibly due to sequestration in the placenta (Carlson and Wegmann 1978, Morisada et al., 1976).

1.23 General Maternal Immunocompetence

During pregnancy the various components of the maternal immune system are basically intact with only minor alterations. There appears to be a redistribution of the T cells into different lymphoid compartments as seen by an apparent reduction in circulating total T lymphocytes in early pregnancy (up to 20 weeks of gestation) with a compensatory increase in B cells (Strelkauskas et al., 1975). A decrease in the helper and suppressor subsets has been found in mothers immediately after delivery with cord blood manifesting a high suppressor activity on maternal lymphocytes (Shohat et al., 1986, Vanderbeeken et al., 1982).

Humoral and cellular immune responses are normal and the production of specific antibodies to environmental antigens is unaltered which suggest these aspects of the T cell and B cell function to be generally intact during pregnancy. Using a variety of in vitro techniques, the normal reactivity of maternal lymphocytes is only observed in the presence of normal and not autologous maternal serum. Studies in the mouse have suggested that the suppression of maternal cell mediated sensitisation may occur locally in the decidua (Clarke et al., 1984) leaving a systemic immune system unaffected and able to function normally.

Antibody dependent cellular cytotoxicity is also normal, as seen by cytotoxicity between maternal lymphocytes and IgG coated trophoblast tissue (Taylor and Hancock 1975).

1.24 The Presence of Maternal Immunological Tolerance

A certain degree of immunological tolerance to paternal antigens may be produced in the mother due to the initial presence of sperm antigens expressed on the foetus. The observation that paternal skin allografts are rejected normally when transplanted during pregnancy has indicated that there is no apparent induction of tolerance and has discounted this theory.

1.25 The Masking of Cell Surface Alloantigens on Trophoblast

Trophoblast surface alloantigens may be masked by the presence of a fibrinoid layer of sulphated glycoprotein nature, although recent evidence has severely weakened the proposed role played by such a mucoprotein layer and it is not thought to be responsible for foetal allograft survival (Beer and Sio 1982).

Alternative mechanisms of alloantigen masking have been proposed by a number of workers. In the rabbit, uteroglobulin, a 15.8 kDa protein synthesised in the uterus in early pregnancy, crosslinks with $beta_2$-microglobulin, a component of class I MHC antigens, on the embryonic cell surface (Mukherjee et al, 1982). The cross-linking is mediated by the enzyme transglutaminase which has a four to five fold increased activity during early pregnancy, resulting in a masking of the MHC antigens on the cell surface. The masking of trophoblast antigens can also occur via antigen-antibody complexes which bind

specifically or non-specifically via Fc receptors to the surface of trophoblast cells. An alternative proposal may be that the trophoblast escapes maternal immune rejection by coating itself with maternal transferrin.

1.26 Lack of MHC Antigen Expression

The trophoblast may be particularly poorly immunogenic *in vivo* due to the lack of class II MHC antigen expression, although other foetal cells possessing MHC antigens can be released into the maternal circulation and hence only a delaying effect is possible. There is a possibility that antigenic modulation may occur within the utero-placental bed. It has been shown that factor(s) are present in retroplacental sera which inhibits the expression of class II major histocompatibility antigens induced on the human myeloid macrophage cell line U937 by recombinant gamma interferon. The inhibitory activity was shown to be due to the masking of the antigenic determinants rather than an inhibition of induction (Nicholas *et al.*, 1986). It is not known whether this mechanism occurs *in vivo* though this would of course, account for the absence of MHC antigens on trophoblast. The absence of HLA-A, -B, and -C transplantation antigens cannot explain the absence of an immune reaction *in vivo*. During pregnancy, components of the maternal system encounter the trophoblastic endovascular interface which bear these antigens en route to the maternal blood filled intervillous spaces of the placenta (Faulk and McIntyre 1983).

The decidual placental barrier theory postulating non-antigenicity of the trophoblast is now untenable in the face of

the clear cut demonstration of class I or class I like MHC antigen expression on placental elements in direct contact with maternal tissues in all species studied so far (Redman et al., 1984). It has been suggested that the non-expression of MHC antigen seen at the time of implantation is of no immunological significance and is merely a reflection of unrelated developmental or more specific trophoblast differentiation events (King et al., 1987).

1.27 The Presence of 'blocking' Antibodies

There are a number of strong candidates for contributors to blocking antibody activity including the involvement of the TLX system (Faulk and McIntyre 1983), of non-cytotoxic antibodies to an MHC linked antigen (Power et al., 1983) and of auto anti-idiotypic antibodies reactive with maternal T cell receptors for paternal alloantigens (Sucui-Foca et al., 1983).

In the human there is a claim that blocking antibody is essential for normal gestation and absent from the serum of spontaneous aborters (Rocklin et al., 1979), indeed allo-antibodies have been frequently implicated as specific blocking factors in pregnancy serum. However there is little evidence for the presence of cytotoxic T cells against which antibody would be required (Gottesman and Stutman 1980, Pavia and Stites 1981). It is possible that they may not be directed against MHC antigens (Morris and Ting 1982) and may instead be directed against the generation of cytotoxic cells, though evidence suggests that this may be a result of the activity of suppressor cells localised in the uterine decidual tissue (Bell 1983).

The absence of allo-antibodies from the serum of the majority of primiparous women argues against an essential role in maternal acceptance of the foetal allograft. The lack of circulating antibody is not due to placental bound antibody (Bell and Billington 1983).

It has been reported that pregnant female mice produce predominantly non-complement fixing IgG_1 antibodies against the foetus during pregnancy (Bell and Billington 1980), however no isotype bias was observed in mice by Chaouat et al., (1983). Smith and Sternlicht (1982) have observed no isotype bias in rats where the antibodies have been shown to be complement fixing.

Blocking antibodies may have a role in pregnancy as recurrent spontaneous abortion has been treated by immunisation with paternal cells (Mowbray et al., 1983). Such a stimulation of blocking antibodies is associated with a reduction in the risk of subsequent abortion (Mowbray 1986). This situation is analogous to the observation that organ allograft rejection is diminished by the previous immunisation with a blood transfusion. The majority of women in the spontaneous abortion group, unlike normal multiparous women do not have detectable cytotoxic antibody against paternal lymphocytes (Tongio et al., 1972).

1.28 Impaired Maternal Cellular Responses

It may be that cytotoxic cellular responses to the trophoblast antigen are impaired in vivo since recognition of trophoblast antigen will be in conjunction with either non-self MHC antigens or an absence of MHC antigens on the foetal sub-populations.

1.29 The Presence of Decidua Associated Suppressor Cells.

During normal successful allogeneic pregnancy in both mice and humans, suppressor cells have been shown to accumulate in the uterine decidua (Clark et al., 1986b). These cells are non-T cells which secrete soluble factors of molecular weights 43 kDa and 21 kDa which block the action of IL-2. In addition a second population of larger sized factor-releasing suppressor cells has also been identified (Clark et al., 1986c).

1.30 The Presence of Immunoregulatory Molecules

An intriguing and attractive explanation is that of local immunosuppression of graft rejection reactions within the utero-placental bed which is mediated by the presence of local suppressor substances. It would appear that immunoregulation must normally occur in the suppression of the recognitive and/or generative phase of the rejection response as there is no evidence for a maternal cell-mediated anti-foetal immune response in the human (Sargent and Redman 1985).

In the human a number of proteins have been identified as being secreted either uniquely (pregnancy-specific proteins) or at a greatly enhanced rate (pregnancy-associated proteins) during pregnancy. The pattern of secretion of these factors falls into two groups:

Pattern A: Those present at maximum level during the first few weeks of gestation, eg human chorionic gonadotrophin (hCG, Braunstein 1976).

Pattern B: Those the secretion of which appears to relate to the overall size of the placenta and whose levels increase throughout gestation, often reaching a plateau a few weeks before term, eg human placental lactogen (hPL).

The possibility that proteins which may have immunosuppressive properties may be secreted by the placenta/decidualising endometrium has aroused much interest both as a possible explanation of foetal survival or non-survival and in the wider general field of transplantation biology.

Any proposal suggesting that suppression of the maternal immune system explains the lack of rejection of the foetal allograft must also explain the normal systemic immunocompetence observed in pregnancy. This may be explained by the presence of specific suppressant molecules or cells such as antibody, anti-idiotype antibody or suppressor T cells directed at anti-allotype (anti-paternal) responses. Alternatively non-specific mechanisms with relatively short half lives may operate locally at the foeto-maternal interface.

1.31 Placental Proteins

The first report of the presence of a placental protein was by Ascheim and Zondek (1927) who described their discovery of a gonad-stimulating substance in the urine of pregnant women now known as human chorionic gonadotrophin and which still forms the basis of human pregnancy tests. Thirty five years later a prolactin-like substance present in the human placenta (now termed human placental

lactogen hPL) was isolated from pregnancy serum and placenta (Josimovich and MacLaren 1962). Like all other placental proteins, its physiological function defies precise definition, and it may be involved in the control of the flow of energy supplying material to the foetus.

Since the early seventies a number placental proteins have been reported, however very few of these fit the definition of a protein peculiar to pregnancy, i.e. synthesised uniquely by the placenta and secreted by it into the maternal circulation. The placental proteins have specific activities in connection with the specialised functions of the placenta such as the maintenance of pregnancy, the development of the foetus or to prepare the mother for delivery.

1.32 Placental Tissue Proteins

Around 40 proteins have been extracted from human placental tissue and have been termed soluble placental tissue proteins (PP's). Most have been observed to occur mainly in the placental tissue with very little being secreted into the maternal bloodstream (usually present at less than 1mg/l). The biological functions of only a few of these proteins have been elucidated. PP5, PP10, PP11, PP12, PP13 and PP17 cannot be detected in extracts of other human tissues and appear to be more or less 'specific' to the placenta whereas PP7, PP8 and PP9 appear to be ubiquitous in human tissues (Bohn 1985). In addition to the soluble placental tissue proteins, some proteins (PP4, PP19 and PP21) were shown to be only partly extracted with saline, total extraction requiring the use of solubilising agents, suggesting them to be at least partly associated with membranes.

Due to the number and variety of functions attributed to placental/pregnancy proteins, only those proteins/molecules which have, or have had immunosuppressive activity associated with them are reviewed.

1.33 Pregnancy-Associated Plasma Protein A (PAPP-A)

PAPP-A is a 750 kDa glycoprotein originally described by Lin et al., (1974) and since shown to be a large zinc glycoprotein (Sinosich et al., 1983). PAPP-A appears to be localised in the syncytiotrophoblast (Lin and Halbert 1976) and was initially thought to be exclusively a product of the trophoblast. In vitro studies have indicated that both trophoblast and decidua are capable of producing PAPP-A (Bischof et al., 1984) under the regulation of a specific factor present only in the circulation of pregnant women. These results have been confirmed using an in vivo study (Bischof et al., 1985).

An anticoagulatory function for PAPP-A is implied from its ability to bind to heparin (Sinosich et al., 1981), and various anticoagulants have a marked effect on PAPP-A measurements (Toop and Klopper 1983, Pinto-Furtado et al., 1984). PAPP-A has been shown to act on antithrombin III (AT III) to inhibit fibrin formation from fibrinogen (Bischof et al., 1983). In addition to its anticoagulatory effects, and due to its localisation at the foeto-maternal interface, PAPP-A has been investigated for immunomodulatory activity, and has been shown to inhibit lectin-induced lymphoblastogenesis (Bischof et al., 1982), however suppression was not reported by Sinosich et al. (1984). PAPP-A

exhibited no suppressive activity on mixed lymphocyte cultures (McIntyre et al., 1981). The reasons for these apparently contradictory results are as yet unknown, but may relate to sequestering of the lectin by PAPP-A rather than a direct effect on lymphoproliferation.

1.34 Pregnancy Zone Protein (PZP)

PZP is a 360 kDa alpha$_2$ glycoprotein (von Schoultz and Stigbrand 1973) originally reported by Smithies (1959) and appears in the literature under several synonyms. It is present in the circulation of both men and women with a dramatic rise (10-500 fold) in serum levels being observed during pregnancy, with levels reaching a plateau in the second and third trimesters. It is not synthesised by the foeto-placental unit rather it is synthesised under oestrogen induction mainly by the liver and exerts its action upon the placenta. PZP inhibits lymphocyte responses to both mitogenic and allogeneic stimulation however only at relatively high and non-physiological concentrations which casts doubt on its biological significance (von Schoultz et al., 1973, Damber et al., 1975, Stimson 1976). It may be that the suppression reflects a non-specific withdrawal of nutritional factors of importance for T cell growth in general rather than a specific immunomodulatory activity (von Schoultz and Stigbrand 1982).

1.35 Early Pregnancy Factor (EPF)

The existence of Early Pregnancy Factor is highly contentious, however it has been suggested to be a pregnancy associated protein

which causes a significantly higher rosette inhibition titre as compared with a non-pregnant control with a standard anti-lymphocyte serum (ALS) in the rosette inhibition test. The inhibition is not species specific. The results suggest EPF to be an immunomodulator possibly affecting cellular immunity. Using the delayed hypersensitivity reaction it has been shown that EPF is able to suppress the allergic reaction (effector arm), but not the sensitisation stage (Fabris 1973). In the mouse, EPF has been shown to consist of two components termed EPF-A and EPF-B, both of which are required for EPF activity. The oviduct initially produces EPF-A and the ovary produces EPF-B under the influences of ovum factor, OF, which is released by the embryo, and prolactin from the pituitary. Production in this manner continues until day 7.

From day 4 a second source of EPF develops which persists until day 15 when production ceases. EPF is present for at least the first half of pregnancy. Although this is the situation in mice, there is some evidence to suggest that it is also the case in some other species including humans (Morton et al., 1982).

EPF appears in the serum in all species within 24 hours of the estimated time of fertilisation. Its continued presence is dependant on a viable embryo and has provided a means of early diagnosis and monitoring of pregnancy (Nancarrow et al., 1979).

1.36 Platelet Activating Factor (PAF).

Alterations to platelet physiology occur immediately following conception in the human (O'Neill et al., 1984). A mild thrombocytopenia induced by the production of a platelet activating factor(s) (PAF) by the fertilised ovum has been observed. This

factor has been partially purified and shown to have similar biochemical and physiological properties to the potent platelet activating factor 1-0-alkyl-2-acetyl-sn-glyceryl -3-phosphocholine (PAF acether). PAF acether was first discovered by the observation that leucocytes sensitised with specific IgE antibody and challenged in vitro, released a potent substance which caused platelet activation (Benveniste et al., 1972). PAF is produced by a wide range of cell types, has a relatively simple biosynthetic pathway (Ninio et al., 1982) and is potent at very low concentrations (Camussi et al., 1983). Such attributes make PAF a candidate for an early embryonic message.

Results suggest that embryo-derived PAF may be the ovum factor responsible for the triggering of the generation of serum EPF activity during the preimplantation stages of pregnancy (Orozco et al., 1986), however as yet there is no evidence to suggest a direct immunosuppressive role for PAF.

1.37 Pregnancy Specific beta$_1$-glycoprotein (SP1).

SP1 is a 90-120 kDa glycoprotein with a carbohydrate content of 28% (Horne and Towler 1978) and has been demonstrated to be a potent suppressor of both mitogenically and allogeneically stimulated lymphocytes at physiological concentrations. When stimulated by phytohaemagglutinin (PHA) SP1 suppressed lymphocyte proliferation both in the G_1 phase of the cell cycle as well as the period of DNA synthesis (S phase), but only at the very early mitosis phase when pokeweed mitogen (PWM) was used. SP1 is synthesised by placental trophoblast, trophoblastic tumours (Heikinheimo et al., 1981,

Tatarinov et al., 1976) and certain types of non-trophoblastic tumours and fibroblasts in vitro (Rosen et al., 1979, Azer et al., 1980). SP1 is detectable in the serum of normal males and non-pregnant females and levels rise in maternal serum with advancing gestation to a peak as high as 330 mg/l (Bohn 1972).

1.38 Alpha Foeto Protein (AFP).

AFP is a glycoprotein synthesised in the yolk sac and foetal liver and is present at high levels in blood and amniotic fluid. Conflicting reports have appeared regarding immunosuppressive properties of both murine and human AFP. In the human it has been suggested that AFP has variable suppressive properties, being suppressive in mitogen and antigen-induced proliferation and antibody synthesis, but not in mitogen and alloantigen-induced cytotoxicity or migration inhibitory factor production (Littman et al., 1977). There is both in vivo and in vitro evidence to suggest that alpha foeto protein can act directly on antigen presenting cells to inhibit cell surface antigen expression (Lu et al., 1984).

The variability of results has led some workers to suggest that AFP is not a biologically significant immunoregulator. Both highly purified rat AFP (Parmely and Thompson 1976) and human AFP (Goecken and Thompson 1977) have been reported to lack the suppressive activities described earlier. Such variability of results may be due to a number of reasons, which have been reviewed by Tomasi (1978), with one proposal being that the suppressive activity is, in fact, due to the presence of contamination in the preparations.

Certain T cells express receptors for AFP with binding possibly being mediated by sialic acid residues (Dattwyler et al., 1975). AFP may mediate its proposed immunosuppressive activity by the induction of suppressor T cells (Bankhurst et al., 1978).

1.39 Human Chorionic Gonadotrophin (hCG)

HCG is a 38 kDa glycoprotein with a carbohydrate content of 30% which is higher than any other human hormone. HCG consists of two dissimilar non-covalently linked subunits designated alpha and beta with hCG beta being localised only in syncytiotrophoblast whereas hCG alpha is localised both in the cytotrophoblast and the syncytiotrophoblast (Tojo et al., 1982). HCG has a strong luteotropic function in the human female and plays an important role in maintaining the function of the corpus luteum during the early stage of pregnancy.

HCG is detectable in normal pregnancy as early as six to nine days following presumed conception (Braunstein et al., 1973). Levels rise logarithmically to a peak at 10 gestational weeks, followed by a decline and nadir at 17 weeks with relatively constant levels being maintained through the rest of pregnancy (Braunstein et al., 1976).

In addition to its role as a luteotropic hormone, rescuing the corpus luteum from its normal decline during the last week of the menstrual cycle, it has been suggested that hCG has immunosuppressive properties which may play a role in preventing the rejection of the foetus in pregnancy. Material extracted from pregnancy urine inhibited phytohaemagglutinin and antigen induced lymphocyte stimulation and mixed lymphocyte reactivity (Han 1974), however

purified hCG preparations were much less inhibitory than crude preparations suggesting other molecules were involved (Morse et al., 1976). Rolfe et al., (1983) have demonstrated that EPF contamination of some commercial hCG preparations accounts for its immunosuppressive activity, suggesting that there is unlikely to be a direct role for immunosuppression by the pure hormone during pregnancy.

The manner by which hCG is secreted differs from other hormones in that hCG is not packaged in secretory granules and released by exocytosis. Rather the carbohydrate portion of the hormone is completed on the cell membrane from which the hormone diffuses away. The resulting close association between hCG and the syncytiotrophoblast plasma membrane may have an effect on surface sialylation. One mechanism may be that the negatively charged trophoblast and similarly charged maternal lymphocytes would result in mutual electrostatic repulsion and thus interfere with maternal effector lymphocyte cytotoxicity (Loke 1986).

1.40 Placental Protein 15 (PP15)

Placental protein 15 was first isolated by Bohn et al., (1980) and shown to be a 30.7 kDa glycoprotein with 3.3% carbohydrate content. PP15 is composed of two identical subunits held together by non-covalent bonds. On investigating its effect on lymphocyte transformation in the mixed lymphocyte culture system, a significant inhibitory activity was demonstrated suggesting a possible role as an immunomodulatory protein during pregnancy. Studies on PP15 have been hampered by its poor antigenicity which hinders antiserum production (Bohn et al., 1982).

1.41 Placental Protein 14 (PP14)

Placental protein 14 was originally isolated from term placentae (Bohn et al., 1982), but subsequently found to be synthesised by decidua. PP14 appears to be a major component of decidual tissue, indeed, by the seventh week of gestation PP14 comprises some 10% of the total soluble protein extracted from this tissue (Julkunen et al., 1985). PP14 has been reported as a 42-43 kDa glycoprotein with a carbohydrate content of 17.5% (Bohn et al., 1980) and is one of a group of proteins independently described by a number of workers which exhibit great structural and immunological similarity and some of which at least may be identical. All of these proteins appear to be secreted by decidual tissue and include placenta specific alpha$_2$-microglobulin (Petrunin et al., 1976) (also called chorionic alpha$_2$-microglobulin (Petrunin et al., 1978)), which has been shown to be antigenically identical to PP14 (Bohn et al., 1982), alpha-uterine protein (Sutcliffe et al., 1980) which was subsequently found to be immunologically identical (Sutcliffe et al., 1982) to progestagen-dependent endometrial protein (Joshi et al., 1980) which in turn is immunologically indistinguishable from PP14 (Julkunen et al., 1986a); and pregnancy associated endometrial alpha$_2$-globulin (Bell et al., 1985) which shows immunological cross-reactivity with PP14 (Bell and Bohn 1986).

Although PP14 is found in endometrial tissue and in the peripheral circulation of non-pregnant women (15-40 ug/ml, Seppala et al., 1985), levels rise rapidly in early pregnancy and as soon as 2-12 days after embryo replacement in IVF (Julkunen et al., 1985). Highest levels are found in the deciduum and peak activities of the

protein appear in the maternal circulation between 6 and 12 weeks of gestation (Julkunen et al., 1985), declining thereafter. The secretion pattern of PP14 resembles that of hCG, however the two proteins are immunologically distinct. The observation that PP14 is extra placental in origin is supported by the fact that PP14 concentrations are significantly higher in amniotic fluid compared with maternal serum (peak values 232 mg/l and 2.2 mg/l respectively). Decidual origin and transport through the membranes into the amniotic fluid explain this kind of distribution of the protein in foetal and maternal compartments (Julkunen et al., 1985).

PP14 has been localised in the cytoplasm of villous syncytiotrophoblast in early human placentae (Inaba et al., 1987). In term placentae the positive staining for PP14 was weakened in the trophoblast cells. The umbilical epithelium has been shown to be cytoplasmically positive for PP14 and there has been clear positive staining for PP14 demonstrated in the cytoplasm of foetal polymorphonuclear neutrophils (Inaba et al., 1987). The decidual large cells were also positive for PP14. In cynomolgus monkey placentae, similar immunostaining results have been obtained. The observation that PP14 was clearly localised in certain decidual cells suggests it to be produced by the decidua. It still remains to be confirmed as there is the possibility that the decidual cells may be invading trophoblastic cells (Inaba et al., 1987).

Using radioimmunoassay a cyclic variation in the circulatory levels of PP14 in serum from non-pregnant females has been observed in ovulatory menstrual cycles which suggests an association with endocrine events (Julkunen et al., 1986b). The levels of PP14 in

serum begin to rise at the time when serum progesterone is at a peak and continue to rise after the decline of progesterone levels. This observation does not preclude the possibility that PP14 is progesterone controlled as the delay may represent the time taken for PP14 to enter the bloodstream from its localised site of secretion. Non-pregnant human secretory endometrium is capable of synthesising PP14 *in vitro* with the PP14 content of the endometrium being highest in the late secretory phase which explains its appearance in the absence of conception. No similar increase takes place in anovulatory cycles indicating that the synthesis of PP14 is related to the action of progesterone (Julkunen *et al.*, 1986a). These results are compatible with the observation that immunochemical and immunohistochemical methods have shown the occurrence of placental protein 14-like material in the human fallopian tube, with the tubal PP14 content being higher in the secretory rather than the proliferative phase (Julkunen *et al.*, 1986d).

PP14 has also been found in the luteinised granulosa cells of preovulatory follicles, corpus luteum and endometrium after the third postovulatory day (Seppala *et al.*, 1985) and in 59 of 122 follicular fluids examined (7.9 - 122 ug/ml). PP14 has also been detected in extracts of cervical mucus (Pockley *et al.*, 1988a, 1988b). Results have also shown that low serum PP14 levels in the late luteal phase are typical of anovulatory cycles and measurement of serum PP14 levels has a potential to become a novel means to identify ovulatory cycles retrospectively for the first days of the next menstrual cycle (Julkunen *et al.*, 1986c).

Table 1.3 details the PP14 levels in a variety of sites, both in pregnant and non-pregnant individuals:

TABLE 1.3 PP14 levels at various sites (Julkunen et al., 1985)

Non-pregnant serum	15-40ug/l
Peak in normal pregnancy serum (6-12 weeks)	2200ug/l
(plateauing after 24 weeks to)	200ug/l
Amniotic fluid (peak 12-20 weeks)	232mg/l
Cord blood (undetectable or)	15-22ug/l
Early pregnancy decidua	41-160mg/g protein
Late pregnancy decidua	60-2700ug/g protein
Early pregnancy placenta	0.25-15mg/g protein
Late pregnancy placenta	3-430ug/g protein

It has been suggested that the elevation of serum PP14 levels may not be a reflection of implantation, but rather a decidual reaction (Julkunen et al., 1985). High levels of PP14 have been detected by RIA in seminal plasma where it may comprise up to 2.5% of the total protein, with the reference range being 19-515 mg/l (Bolton et al., 1986a). PP14 isolated from human plasma and seminal plasma are immunologically indistinguishable, however the isoelectric point of seminal plasma derived PP14 has been reported to be slightly higher than that of maternal serum PP14 which may be explained by a difference in the glycosylation (Julkunen et al., 1984). The observation that PP14 levels are similar in both vasectomised and non-vasectomised men suggests that the protein is not derived from the testes or epididymis even though it could be detected in saline extracts of both these tissues (Bolton et al., 1986a).

PP14 has been recently characterised to be a dimeric 60 kDa glycoprotein with homologous 28 kDa subunits (Westwood et al., 1988), is highly glycosylated (30%) and highly hydrophobic (O.M.R. Westwood, personal communication). The first 24 amino acids of the N terminal were found to be;

Met Asp Ile Pro Gln Thr Lys Gln Asp Leu Glu Leu

Pro Lys Leu Ala Gly Thr Glu His Glu Met Ala Met

The first 17 amino acids showed identity with the sequence data determined by Huhtala et al (1986), the differences observed further along the polypeptide chain possibly being explained by genetic polymorphism. Significant homology exists between beta-lactoglobulins of a number of species and PP14 (Pervais and Brew 1985; Godovac-Zimmerman et al., 1985). Beta-lactoglobulins (BLG) are found in the milk of many animals and possibly man. The protein exists as a dimer and many species appear to possess genetic variants. A possible binding site for retinol in BLG has been identified suggesting a possible role for BLG in vitamin A transport (Papiz et al., 1986).

Pregnancy associated $alpha_2$-globulin ($alpha_2$-PEG) has been shown to be immunologically and biochemically related to PP14 (Bell and Bohn 1986). The 15 amino acid N terminal sequence reported by Huhtala et al. (1986) is identical to the N terminal sequence of $alpha_2$-PEG apparently confirming the work of Bell and co-workers.

$Alpha_2$-PEG appears to be synthesised by the decidua spongiosa of the decidua parietalis (Bell 1985) and has been detected at the highest levels in amniotic fluid with peak levels being observed at

20-25 weeks (Bell and Bohn 1986). From previous studies on alpha$_2$-PEG it has been deduced that although the major route of secretion is into the amniotic fluid (Bell et al., 1986), the protein may also be bound by populations of trophoblast in the placenta resulting in a localisation to the placental trophoblast.

Alpha$_2$-PEG is also the major secretory protein of the mid-late secretory endometrium during the luteal phase of the menstrual cycle and has been suggested as representing the human analogue of rabbit uteroglobulin (Bell et al., 1986). It has been clearly demonstrated that alpha$_2$-PEG is principally associated with glandular epithelium and its secretions and is modulated during the menstrual cycle (Waites et al., 1988). Alpha$_2$-PEG secretion was observed to be initiated well after the histologically defined secretory phase had begun, suggesting a dependance on an extended progesterone exposure or a link with predecidualisation of the stroma rather than a direct steroid hormonal control (Waites et al., 1988).

The observed sequence homology between alpha$_2$-PEG and equine beta-lactoglobulin is intriguing since the latter species exhibits epitheliochorial placentation. In the horse, beta-lactoglobulins have been demonstrated to bind vitamin A and have been suggested to be involved in its transport (Pervais and Brew 1985). Such a role for alpha$_2$-PEG as a vitamin A transporter to the implanting embryo and developing placenta has still to be elucidated. Binding studies attempting to show that PP14 is a retinol binding protein are unconvincing (O.M.R. Westwood, personal communication) and it is unlikely to be of physiological significance.

Due to its tissue and temporal distribution, interest has arisen into a possible immunosuppressive role for PP14 in human reproduction, and preliminary data suggest that both crude decidual extract and purified PP14 exhibit immunosuppressive activity in the allogeneic mixed lymphocyte reaction (MLR) (Bolton et al., 1986b).

1.42 Steroids as Immunosuppressive Molecules in Pregnancy

Progesterone is widely distributed in nature. Its synthesis from cholesterol requires only two enzymatic steps, and many studies have shown it to be essential for the maintenance of pregnancy in a variety of mammalian species. Progesterone production appears to be related to placental structure with higher levels being produced in haemochorial placentae with the levels in humans being by far the highest (1-5 ug/ml) (Stites and Siiteri 1983).

The potential role of high local concentrations of progesterone as a natural anti-inflammatory and immunosuppressive agent was proposed by Stites and Siiteri (1983), and in vitro studies have shown progesterone to block T cell activation in concentrations of 5-20 ug/ml (Mori et al., 1977; Clemens et al., 1979). Progesterone suppresses the recruitment of cells from G_0 to G_1 and S phases of the cell cycle (Mendelsohn et al., 1972), however at concentrations above 20 ug/ml progesterone has significant cytotoxicity. It has been established that cortisol and progesterone exert a dose-dependant suppression of ^{3}H-thymidine incorporation with either mitogenic or allogeneic stimulation in the MLR (Clemens et al., 1979). The effect of progesterone is readily reversible and work suggests that progesterone both reduces DNA synthesis and

inhibits thymidine incorporation. The levels at which these effects were observed (20 ug/ml) are comparable to the possible localised levels at the foeto-maternal interface, but much higher than those levels in the maternal circulation.

It has also been shown that the mechanism by which progesterone and cortisol inhibit T cells differ. Progesterone is primarily directed towards T cells and not the accessory cell function of monocytes (Stites and Siiteri 1983) whereas glucocorticoids seem to have effects on both monocyte and T cell populations. A local anti-inflammatory activity of progesterone has been observed in vivo as confirmed by the absence of inflammatory cells in the pregnant uterus until a few days before birth, although whether this is mediated directly by progesterone is unknown. The appearance of inflammatory cells at the time of removal of progesterone activity may lead to parturition mediated by prostaglandin production and possibly suggests parturition to be a delayed rejection reaction (Stites and Siiteri 1983).

An important additional feature in the process of immunological recognition and proliferation is the capacity of lymphocytes to form aggregates possibly allowing local exchange of immunological information, via messengers such as lymphokines. It has been demonstrated that progesterone directly interferes with cell-cell contact possibly having immunological consequences at the foeto-maternal interface. Inhibition of lymphocyte aggregation in the presence of progesterone is reversible implying that once maternal lymphocytes leave the placental bed they may regain their immunological capability (Van Vlasselaer et al., 1986).

Oestrogens are also produced by the corpus luteum and placenta, however their function in the immunological aspects of the foeto-maternal relationship is unknown.

1.43 Aims of the Study

The aim of this study was to confirm the previously reported immunosuppressive activities of PP14 using both the allogeneic MLR and mitogenic stimulation assay systems. The level of action of the suppressive activity was also investigated, primarily using mitogenic stimulation. The effect of PP14 on interleukin-2 and gamma interferon production were assessed as was the expression and affinity of the IL-2R and the release of the IL-2R from activated lymphocytes. Flow cytometry was used to investigate the expression of activation antigens on stimulated lymphocytes and analyse cell cycle progression. Potential non-specific effects of PP14 on aspects of lymphocyte responsiveness were also investigated.

CHAPTER TWO

MATERIALS AND METHODS

The reagents, equipment and their suppliers are detailed in Appendix I.

Blood samples used in this study were obtained from normal healthy volunteers in the Department of Biomedical Sciences, Sheffield City Polytechnic and the University Department of Haematology, Royal Hallamshire Hospital, Sheffield. Decidual tissue samples were provided by Mr M.G. Chapman of the Department of Obstetrics and Gynaecology, Guy's Hospital, London and Mr P. Stewart of the Department of Obstetrics and Gynaecology, Northern General Hospital, Sheffield.

Monoclonal antibodies to placental protein 14 were produced and kindly provided by E.A. Mowles of the Department of Biology and Biochemistry, North East London Polytechnic, Romford Road, London. The monoclonal antibody has been shown to have less than 1% cross-reactivity with purified hCG, hPL, SP1, PP5, PP12, PAPP-A, prolactin and the human placental enzymes alkaline phosphatase, malic dehydrogenase, sphingomyelinase, arylamidase and choline acetyl transferase. The binding specificity of the monoclonal antibody was tested by the examination of its binding to radioiodinated proteins and its specificity as the labelled antibody reagent in a two-site immunoradiometric assay.

Purified PP14 for use in the in vitro assays was provided by O.M.R. Westwood of the Department of Obstetrics, Guy's Hospital, London.

Purified PP14 (lot 120/135) and PP14 antiserum (lot 201ZA) for use in the radioimmunoassay was kindly provided by Dr. Hans Bohn of Behringwerke AG, Germany.

2.1 Isolation of Peripheral Blood Mononuclear Cells (PMC)

Peripheral blood mononuclear cells (PMC) were isolated from whole blood by density gradient centrifugation as described by Boyum (1968). Peripheral blood was taken aseptically from normal healthy male volunteers into vacutainers containing lithium heparin as anticoagulant and diluted in 2 volumes of Dulbecco's modification of Eagle's medium (DMEM). Fifteen millilitres of the diluted blood was layered onto 10ml of Lymphopaque separation medium (relative density 1.084) contained in sterile plastic universal containers, and centrifuged at 400 x g (calculated at the interface) for 20 minutes at room temperature. The opaque layer of lymphocytes formed at the Lymphopaque interface was carefully aspirated. The lymphocytes were transferred to clean universal containers and the cells washed in approximately 25ml of DMEM. The cells were sedimented at 800 x g for 10 minutes, the supernatant removed and the lymphocytes resuspended gently in DMEM by repeated aspiration into a sterile 1ml pipette tip attached to an adjustable pipette. The washing procedure was performed a total of three times and the cells were finally suspended in 1ml of DMEM containing 3.7g/l $NaHCO_3$, 200iu/ml penicillin, 200ug/ml streptomycin and 10% foetal calf serum (designated $DMEM^+$), or SF1 serum-free Hybridoma growth medium containing the same antibiotic concentration (designated $SF1^+$). All manipulations were performed under sterile conditions using a laminar flow hood.

The viability of the isolated lymphocytes was assessed by Trypan Blue dye exclusion by adding an aliquot of the cell suspension (25ul) to 25ul of Trypan Blue dye (0.2% w/v Trypan Blue in physiological saline). The plasma membrane of viable cells does not permit the entry of non-electrolyte dye substances and non-viable cells stain blue. Viable cells were counted using an improved Neubauer counting chamber and the lymphocytes diluted to 1 x 10^6 viable cells/ml in $DMEM^+$ or $SF1^+$ growth medium prior to inclusion in the cell culture systems. All lymphoproliferation assays were performed in triplicate in 96 well microtitre tissue culture plates.

2.2 The Two-way Allogeneic Mixed Lymphocyte Reaction (MLR)

PMC were isolated as described from two healthy unrelated volunteers and equal numbers of cells from each donor mixed. Two hundred microlitres of the cell mixture or cells from each individual, 2 x 10^5 cells/well, were incubated in the presence of 50ul of the PP14 preparation containing a known amount of PP14 as measured by radioimmunoassay (Bolton et al., 1983). Control cultures contained 50ul of phosphate buffered saline (PBS) in place of the PP14 preparation. The cultures were restricted to the central 60 wells of the plate to minimise evaporation.

The microtiter plates were incubated at 37°C, 100% humidity and 5% CO_2 for 6 days. Twenty four hours prior to the termination of the cultures luCi of tritiated thymidine (^{3}H-Tdr) was added to each well and the extent of lymphoproliferation assessed by its incorporation into the DNA of the proliferating cells. At the termination of the cultures, the cells were harvested onto glass

fibre filter paper, either manually using 5% trichloroacetic acid (TCA) containing 30mM/l pyrophosphate, or by using a Skatron semi-automated cell harvester.

For the manual termination of the cultures, the contents of each well were transferred into 3ml plastic disposable tubes on ice, and 200ul of trypsinising solution (0.05% trypsin, 0.02% EDTA in physiological saline) added to each well in order to free the adherent cells. The plates were then left to incubate at room temperature for 20 minutes after which the contents of each well were transferred to the appropriate cell suspension on ice.

One millilitre of ice cold 5% TCA was added to each cell suspension which was vortex mixed and left to incubate at room temperature for one hour to precipitate the DNA. The TCA precipitable material was isolated by filtration through glass fibre filter paper using a Millipore sintered glass filter assembly attached to a vacuum line.

Each filter was presoaked in TCA, placed into the filtration assembly and 5ml of ice cold TCA dispensed onto it. The sample was vortex mixed and tipped onto the filter followed by two 1ml washes of the tube with TCA. Vacuum was applied and the filter allowed to drain, 5ml of TCA was then added followed by 5ml of ice cold ethanol. The vacuum was ceased, the filters transferred to scintillation vials and allowed to dry in air for about 30 minutes. The extent of ^{3}H-thymidine incorporation was assessed by liquid scintillation counting in an LKB Rackbeta 1212 for 10 minutes using scintillation cocktail '0' (0.3% PPO, 0.01% POPOP in toluene) or LKB 'HiSafe' scintillation fluid.

For the harvesting of the cells using the semi-automated cell harvester, wash fluid (distilled water) was supplied to the harvester by gravity feed and a suction unit positioned over the microplate so that pairs of tubes projected into the wells to be harvested. The inlet tube which is longer and smaller in diameter, fed the wash fluid into the well whilst the shorter and wider outlet tube simultaneously aspirated the well contents. The rinse principle effectively removes all the cells from the wells including the adherent population. The contents of each well were harvested onto glass fibre filter mats which were allowed to dry. The extent of ^{3}H-Tdr incorporation was assessed by liquid scintillation counting as detailed above.

2.3 The Mitogenic Stimulation Assay (MSA)

PMC from a single donor were isolated as described above and 100ul of the washed cell suspension (1 x 10^{5} cells/well) incubated in the presence of 50ul of the PP14 preparation, 50ul of the mitogen at the concentration indicated in the results section and 50ul of growth medium (either DMEM^{+} or SF1^{+}). Control cultures contained PBS in place of PP14 preparations and background stimulation was assessed by incubating cells with growth medium in place of the mitogen solution.

The plates were incubated at 100% humidity, 37°C and 5% CO_2 for 72 hours. Six hours prior to termination, the cultures were pulsed with 1uCi of ^{3}H-Tdr and on termination were harvested using a Skatron semi-automated cell harvester. The degree of lymphoproliferation was assessed as above.

2.4 Control of Isotope Isolation Procedure

For both of the culture systems and harvesting methods, the contamination due to unincorporated ^{3}H-Tdr was assessed. Immediately prior to the termination of the cultures 1uCi of ^{3}H-Tdr was added to control wells which were then treated as detailed, this procedure also serves as a zero hour control.

2.5 Preparation of Decidual Extracts

Decidual tissue, as confirmed by subsequent histological examination, was obtained following elective pregnancy termination at about 14 weeks of gestation and rinsed rapidly in cold saline containing the protease inhibitor PMSF (0.1 mM phenyl methyl sulphonyl fluoride). Samples were stored at -20 °C until use. The tissue samples were homogenised in PBS containing PMSF and the cell debris removed by centrifugation at 5000 x g for 30 minutes at 4 °C. Prior to incorporation into the cell culture systems, the extracts (10 ml) were dialysed overnight at 4°C against PBS (3 x 1 litre of PBS, pH 7.5), diluted in PBS to the concentrations indicated in the results section and filter sterilised. Aliquots of the decidual extracts were assayed for PP14 and total protein as described later.

2.6 The Effect of PP14 on the Production of Soluble Mediators of the Immune Response from Stimulated Lymphocytes

PMC were isolated as described earlier, washed and resuspended at 10^{6} viable cells/ml in SF1^{+} growth medium. 10^{6} cells were cultured in 24 well culture plates in the presence of 200ul of PP14

preparation and 200ul of mitogen (PHA, 5 ug/ml final concentration) for the times indicated in the results section. Control cultures contained PBS in place of the PP14 preparation and DMEM in place of the mitogen. At the termination of the culture period the culture supernatants were harvested and stored at -20 C prior to assay.

2.6.1 Assay of culture supernatants for Interleukin-2

Cell culture supernatants were assayed for Interleukin-2 as per the manufacturer's recommendations using a Genzyme Intertest 2 Human Interleukin 2 ELISA kit.

The provided immunoplate was coated by incubating with monoclonal anti-IL-2 antibody diluted in coating buffer overnight at 37°C in a humidified incubator. Immediately prior to use the plate was washed four times in PBS/Tween washing buffer and finally patted dry on absorbent paper.

One hundred microlitres of each standard (500, 50, 5, 0.5, 0.05 U/ml), controls and dilutions of the culture supernatants were incubated in duplicate in the covered plate at 37°C for 6 hours. At the end of the incubation period the plate was washed as above and each well incubated with 100ul of a 1:50 dilution of 2nd antibody (polyvalent rabbit anti-IL-2) for 1 hour at 37°C.

At the end of the second incubation, the fluid was aspirated and the plate washed. 100ul of the 3rd antibody (goat anti-rabbit alkaline phosphatase conjugate) was dispensed into each well and the plate incubated for 1 hour at room temperature. The washing procedure was repeated and 100ul of substrate reagent (p-NPP) dispensed into each well. The enzymatic colour was allowed to

develop at room temperature for 15-60 minutes. The resulting colour was read on a plate reader at a wavelength of 405nm and a standard curve of absorbance vs log IL-2 concentration plotted. The levels of IL-2 in the culture supernatants were determined by extrapolation from the standard curve.

2.6.2 Assay of culture supernatants for cell-free Interleukin-2 receptors

Cell culture supernatants were assayed for the released form of the IL-2R protein from activated T cells as per the manufacturer's specifications using a T Cell Sciences CELLFREE interleukin-2 receptor enzyme immunoassay test kit.

The provided immunoplate was coated by incubating with monoclonal anti-IL-2R antibody diluted in coating buffer at 4°C for 72 hours. The coating solution was removed from all wells and each well incubated with 300ul of blocking buffer for 2 hours at 37°C. The antibody coated plates containing blocking buffer were stored covered at 4°C until use. Immediately prior to use the blocking buffer was discarded and each well washed three times with washing buffer and patted dry on absorbent paper.

One hundred microlitres of the provided sample diluent buffer was added to each well. Fifty microlitres of each standard (0, 100, 400, 1600 units/ml), controls and dilutions of the culture supernatants were incubated in duplicate in the covered plate at 37°C for 2 hours. At the end of the incubation period the plates were washed 3 times and incubated with 100ul/well of horseradish peroxidase (HRP) conjugated anti-IL-2R antibody for 2 hours at 37°C.

After the second incubation, the plate was washed 3 times and each well incubated with 100ul of substrate solution (OPD) for 30 minutes at room temperature. The reaction was stopped by the addition of 50ul of 2N H_2SO_4 and the resultant absorbance of the wells read at a wavelength of 492nm. A standard curve of absorbance versus the IL-2R concentrations (u/ml) of the standards was plotted and the levels of IL-2R in the culture supernatants determined by extrapolation from the standard curve.

2.6.3 Assay of culture supernatants for Interferon-gamma (IFN-gamma).

Cell culture supernatants were assayed for gamma interferon as per the manufacturer's specifications using a SUCROSEP interferon gamma immunoradiometric assay kit.

Two hundred microlitres of standard IFN (1024, 256, 64, 16, 4, 1, 0 U/ml) or unknown sample were incubated with 50 ul of ^{125}I-anti-interferon gamma monoclonal antibody in 75mm x 13 mm disposable tubes. After 2 hours incubation at room temperature, 50 ul of sheep anti IFN-gamma antibody covalently coupled to solid phase particles was added. The tubes were covered and agitated on an orbital shaker at 300-350 rpm for 2 hours at room temperature.

One millilitre of the provided pre wash buffer was added to all tubes and 2 ml of Sucrosep reagent layered below the incubate in each tube using a Sucrodispenser. The solid phase was allowed to settle for 15 minutes and the tube contents aspirated leaving approximately 0.3 ml in the bottom of each tube. The separation procedure was repeated and the pellet counted using an LKB Multigamma. A standard

curve of log counts vs log standard concentration was plotted and the levels of interferon gamma in the culture supernatants determined by extrapolation from the standard curve.

2.7 Effect of PP14 on Interleukin-2-Induced Lymphoproliferation.

To investigate the possibility that PP14 may interfere with the binding of IL-2 to its cell surface receptor once expressed, the effect of PP14 on IL-2-induced lymphoproliferation was assessed.

Peripheral blood mononuclear cells were isolated as described, washed and resuspended at 10^6 viable cells/ml in $SF1^+$ growth medium. 10^6 cells were cultured in the presence of 200ul of PHA (5ug/ml final concentration) for 6 days at 37°C and 5% CO_2 in 24 well culture plates. The resulting 'PHA blasts' produced little IL-2 as determined in the above assay, but most (70%) expressed IL-2 receptors (Tac antigen) as determined by flow cytometry (see below). The cells were washed once in PBS and the adherent cells harvested by incubating with 0.5 ml of 2% EDTA at 37°C for 15 minutes. The harvested cells were washed twice in DMEM growth medium and resuspended at 10^6 viable cells/ml in $SF1^+$ growth medium.

The proliferative response of PHA blasts.

a) To PHA.

100ul of the 'PHA blast' cell suspension or control cells (cells incubated as above, but in the absence of PHA) were cultured in the presence of 50ul of PHA

(2.5 ug/ml final concentration, DMEM as control), 50ul of PP14 preparation (PBS as control) and 50ul of $SF1^+$ medium.

b) To IL-2.

100ul of the 'PHA blast' cell suspension or control cells were incubated in the presence of 50ul of recombinant IL-2 (rIL-2 ala 125, Amersham International; final concentration 0.25 U/ml in DMEM growth medium, DMEM as control), 50ul of PP14 preparation (PBS as control) and 50ul of $SF1^+$ growth medium.

All cultures were incubated for 72 hours at 37°C, 5% CO_2 in 96 well microtitre plates and ^{3}H-Tdr uptake into the proliferating cells assessed following a 6 hour pulse as detailed above (section 2.2).

2.8 Cell Surface Marker Analysis of Stimulated Lymphocytes by Flow Cytometry

Flow cytometry was used to investigate the effects of crude decidual extract and immunoadsorbed decidual extract on the levels of surface expression of a number of antigens associated with lymphocyte activation.

2.8.1 Mitogenically stimulated lymphocytes

Peripheral blood mononuclear cells were isolated as described, washed and resuspended at 10^6 cells/ml in $SF1^+$. 10^6 cells were incubated in 24 well culture plates in the presence of 200ul of the PP14 preparation and 200ul of PHA at a final concentration of 5ug/ml for the times indicated in the results section.

Constitutive (background) expression of the cell surface antigens was assessed by incubating the cells in the absence of both PP14 preparation and mitogen. Control expression of the cell surface antigens on stimulated lymphocytes was assessed by incubating the cells with PHA in the absence of the PP14 preparation.

2.8.2 Allogeneically stimulated lymphocytes

The cell surface marker analysis of allogeneically stimulated lymphocytes was assessed using an MLR culture system. Peripheral blood mononuclear cells from two unrelated individuals were isolated as described, washed and resuspended at 10^6 cells/ml in $SF1^+$ growth medium. Equal volumes of the two cell preparations were mixed together and 10^6 cells incubated in the presence of 200ul of the PP14 preparation (PBS as control) in 24 well culture plates. Control (constitutive) expression of antigens was assessed by incubating single cell types.

2.8.3 The staining of cell surface markers using monoclonal antibodies

All plates were incubated at 37°C, 100% humidity and 5% CO_2 for the times indicated in the results section. On termination of

the cultures the cells were washed twice with PBS and the cells harvested by incubating each well with 0.5ml of 2% EDTA at 37°C for 15 minutes. The cells were transferred to 10ml plastic conical centrifuge tubes followed by 2 x 1ml washes of PBS. The volume was made up to 10ml with PBS containing 1% foetal calf serum (PBS/FCS) and the tubes centrifuged at 1000 x g for 10 minutes. The cells were resuspended, counted and 10^6 cells transferred into plastic 10mm x 64mm disposable polystyrene tubes and washed twice in PBS/FCS.

After the second wash in PBS/FCS, the supernatant was discarded and the cells resuspended in the residual volume. 100ul of an appropriate dilution (1/40) of monoclonal antibody was added to the cell suspension and incubated at 4°C for 20 minutes. After incubation with the first layer antibody, the cells were washed twice in PBS/FCS, the supernatants decanted and the cells resuspended in the residual volume. One hundred microlitres of an appropriate dilution (1/40) of FITC goat anti-mouse antibody was added to each cell suspension and the cells incubated in the dark at 4°C for 20 minutes. Non specific binding of fluorescein isothiocyanate-conjugated goat anti-mouse IgG antibody (FITC control) was assessed by incubating cells with PBS/FCS in place of the first layer antibody.

After the incubation the cells were washed twice in PBS/FCS. When possible, the cells were resuspended in 300ul of PBS/FCS and immediately analysed by flow cytometry on a Beckton Dickinson FACS 420 flow cytometer at an excitation wavelength of 488 nm. Both the percentage of cells expressing antigen and the antigen density as determined by the mean channel of fluorescence were measured. If

immediate analysis was not performed, the cells were resuspended in 1% paraformaldehyde and stored at 4°C in the dark overnight until analysis.

The monoclonal antibodies used in this study were directed towards:-

Interleukin-2 receptor (Tac, CD25)

MHC class II (HLA-DR)

Human Transferrin receptor (OKT9)

T helper cell (OKT4, Leu 3a, CD4)

T suppressor/cytotoxic (OKT8, Leu 2a, CD8)

E rosette receptor (OKT 11, T11)

Cells were also stained for the presence of cell-surface associated PP14 and interleukin-2 using the relevant monoclonal antibodies.

2.9 Effect of PP14 on the Distribution of Cells in the Cell Cycle

Subsequent to analysis for antigen expression, cells were stained with the nuclear dye propidium iodide and analysed for DNA content to determine the distribution of the cells within the cell cycle. Cells were cultured, harvested and washed as detailed above. One hundred microlitres of Triton X100 (0.2% in PBS) was added to the resuspended cell pellet to solubilise the cell membranes and the cells left at room temperature for 15 minutes. Propidium iodide (20 ug/ml in PBS) was added and the cells mixed and analysed after a minimum of 15 minutes. In practice the cells were incubated with the dye for as long as possible to allow the interaction of the stain with the DNA to reach equilibrium.

The cells were excited at a wavelength of 488 nm and the resulting fluorescence measured through red filters at a wavelength of 620 nm. The intensity of the resulting fluorescence is proportional to the nuclear DNA content and is indicative of passage of the cells through the cell cycle.

2.10 The Effect of PP14 on the Expression of High Affinity IL-2R

Peripheral blood mononuclear cells were isolated as described, washed and resuspended at 10^6 viable cells/ml in $SF1^+$ growth medium. 10^6 cells were cultured in the presence of the PP14 preparation (200ul) and 200ul of PHA (5 ug/ml final concentration) in 24 well culture plates for the times indicated in the results section. At the termination of the culture period the cells were washed once with PBS and incubated with 2% EDTA for 15 minutes at 37 °C to remove the adherent cells. The harvested cells were washed twice in DMEM growth medium, counted and resuspended at 5 x 10^6 cells/ml in RPMI 1640 medium supplemented with 10% FCS (RPMI/FCS). The binding of IL-2 to the cells was assessed by incubating radiolabelled ^{125}I-IL-2 (4.7 pM - 0.3 pM) with 5 x 10^5 cells in a total volume of 200ul of RPMI/FCS in 12mm x 75mm disposable polystyrene tubes. The tubes were incubated at 37 °C for 20 minutes and the cell suspension overlayed onto a 200ul mixture of 80% silicone oil 550 and 20% paraffin oil 0-119 in 1.5ml Eppendorf microfuge tubes. The tubes were centrifuged at 8500 x g for 90 seconds and the aqueous supernatant containing unbound ^{125}I-IL-2 aspirated. The cell bound and free activity was determined by counting on an LKB Multigamma. The number of binding sites/cell and

the affinity of the receptors for IL-2 were derived by Scatchard analysis of equilibrium binding data. All determinations were corrected for non-specific binding as assessed by incubating cells with a 150 molar excess of unlabelled IL-2.

2.11 Effect of PP14 on the Expression of Functional IL-2R.

It is generally assumed that the response of T lymphocytes to exogenous IL-2 is dependent on the number and affinity of the IL-2R borne by the cells. It is possible to determine the sensitivity of the cells to IL-2 by incubating dilutions of IL-2 with decreasing numbers of responding cells and plotting the results as the fractional response versus the concentration of added ligand (Ashwell et al., 1986). At some point, as the number of responding cells is increased, the normalised dose-response curves will shift so that more ligand will be required to achieve a given fractional response. The transition point is defined as that number of responding cells beyond which shifts in the dose-response curves are discernable and is a reflection of the receptor number and affinity.

Peripheral blood mononuclear cells were isolated, cultured and harvested as detailed in section 2.10. The harvested cells were resuspended at 1 x 10^6 cells/ml in $SF1^+$ growth medium and assessed for their ability to proliferate in response to exogenous IL-2. One hundred microlitres of an appropriate dilution of the cell suspension (10^6, $5x10^5$, $2.5x10^5$, $1.25x10^5$ and $6.25x10^4$ cells/ml) were incubated with 50ul of IL-2 at the concentrations indicated in the results section, and 100ul of $SF1^+$ growth medium

for 72 hours in 96 well microtitre plates. The degree of proliferation was assessed following a 6 hour pulse with ^{3}H-Tdr.

2.12 The Radioimmunoassay of Placental Protein 14

Prior to inclusion in the assays, all samples were assayed for PP14 by radioimmunoassay (Bolton et al., 1983). PP14 was radioiodinated using the oxidising agent chloramine T. Five microlitres (0.5 mCi) of $Na^{125}I$ was added to a 12mm x 75mm disposable polystyrene tube (LP4) and was buffered by the addition of 10ul of sodium phosphate buffer (0.25M, pH 7.5 + 0.1% sodium azide as preservative). Five micrograms of the purified protein in 10ul of phosphate buffer (0.05M, pH 7.5 + 0.1% azide) was added, and the reaction started by the addition of 5 ug (10ul) of chloramine T in the same buffer. After 30 seconds the reaction was stopped by the addition of 100ul of cysteine solution (17 ug) in phosphate buffer and the volume made up to 1ml with a solution of KI (2 mg/ml) in assay diluent buffer (0.05M phosphate buffer, 0.1% azide and 2% horse serum). The labelled protein was separated from the unreacted iodide by desalting on a 10ml Sephadex G25 column equilibrated with assay diluent buffer (Bolton 1985).

The labelled PP14 was further purified by lectin affinity chromatography. The protein was applied to a 1ml column of Con-A-Sepharose which was then washed with five bed volumes of assay diluent buffer and the retained material (PP14) eluted with 0.2 mol/l mannose in the same buffer.

For the assay, 100ul of PP14 standards diluted in assay diluent buffer, or an appropriate dilution of the preparations, were

incubated in LP4 tubes overnight at room temperature with 100 ul of 1 ng/ml PP14 tracer containing a 1/133 dilution of non-immune rabbit serum (to bulk up the final precipitate) and 100ul of rabbit anti-PP14 antiserum at a dilution to bind approximately 40 - 45% of the available tracer. The tracer bound to the antibody was then separated from that unbound using a polyethylene glycol (PEG) assisted double antibody system, by adding 0.5ml of a 1/160 dilution of donkey anti-rabbit antiserum in 7% PEG in assay diluent buffer.

The tubes were incubated at room temperature for 30 minutes and the bound fraction sedimented by centrifugation at 2500 x g for 20 minutes. The supernatants were aspirated and the radioactivity in the bound fraction determined by counting in an LKB Multigamma. A standard curve of PP14 concentration versus bound tracer was plotted. The PP14 content of the unknown preparations was extrapolated from the standard curve as a mean of duplicate measurements. All data analysis was performed using an LKB Riacalc DM software package.

2.13 The Interaction of Radiolabelled PP14 with Phytohaemagglutinin

The possibility that PP14 could exert suppressive activity on PHA-induced lymphoproliferation by binding to the mitogen itself was investigated by assessing the binding of radiolabelled PP14 to a solid phase linked PHA system. PP14 was iodinated as above and PHA linked to cyanogen-bromide activated Sepharose 4B (CNBr-S4B) as described below.

2.13.1 The coupling of PHA to CNBr-S4B

One gram of CNBr-S4B was swollen for 15 minutes in 1mM HCl to give a final gel volume of 3.5ml. The swollen gel was washed by repeated gentle centrifugation with a total volume of 200ml of 1mM HCl. HCl was used to preserve the activity of the reactive groups which hydrolyse at high pH.

Five milligrams of PHA was dissolved in coupling buffer ($NaHCO_3$ buffer, 0.1M pH 8.3). One millilitre of the gel was washed in 2.5ml of coupling buffer and rapidly transferred to the solution of PHA. This stage was completed immediately as the reactive groups on the gel rapidly hydrolyse at the coupling buffer pH. The protein/gel suspension mixture was mixed on an end-over-end mixer overnight at 4°C.

2.13.2 Blocking of the remaining active groups

After overnight mixing, the gel was transferred to 0.2M glycine (pH 8.0) as blocking agent and incubated for 2 hours at room temperature. The blocking agent introduces a small number of charged groups into the gel, the effect of these charges is overcome by the use of 0.5M NaCl in the buffers. Excess uncoupled ligand was removed by washing the gel alternately with high and low pH buffers four times (coupling buffer pH 8.3 and 0.1M acetate buffer pH 4.0). The washing cycle ensures that no free ligand remains ionically bound to the immobilised ligand as no desorption occurs of covalently bound protein. After washing, the gel was stored until use at 4°C in an equal volume of PBS.

As a control for the experiment, the procedure was performed as above using both bovine serum albumin (BSA) as a ligand and without a ligand to act as a non-specific binding control. At each stage of the procedure all supernatants were retained and assayed for protein content by the method of Ohnishi and Barr (1978) to determine the coupling efficiency of the procedure.

2.13.3 Binding of PP14 to the solid phase linked PHA

One hundred microlitres of the PHA solid phase (or control solid phase) was incubated with 100ul of ^{125}I-PP14 (5 ng/ml in assay diluent buffer) in LP4 tubes on an orbital shaker for 2 hours at room temperature. After the incubation period, the solid phase was washed twice by gentle centrifugation with assay diluent buffer containing Tween 20 (1000 x g, 1min) and the activity bound to the solid phases determined using an LKB Multigamma. ^{125}I-carbonic anhydrase III (^{125}I-CA III) was used as a further non-specific binding control.

2.14 Immunoadsorption of PP14 using a Monoclonal anti-PP14 Antibody Solid Phase System

To act as controls for the cultures detailed in the results section, PP14 was specifically removed from decidual tissue extracts by treatment with a monoclonal anti-PP14 antibody solid phase system.

Monoclonal antibodies to PP14 were produced as described (Bolton et al., 1987) and linked to cyanogen bromide activated Sepharose 4B as detailed above.

One millilitre of decidual extract was reacted with 1ml of the monoclonal anti-PP14 solid phase immunoadsorbent by end-over-end mixing for 3 hours at room temperature. After the reaction, the gel was gently centrifuged and the supernatant removed using a pasteur pipette. After use, the solid phase was regenerated by treatment with 3M KI followed by repeated and extensive washing with PBS.

The immunoadsorbed samples were filter sterilised, diluted and incorporated into the in vitro culture systems as detailed in the results section. Samples were also retained for PP14 determinations by radioimmunoassay.

2.15 Determination of Protein

Protein was determined quantitatively by the method of Ohnishi and Barr (1978) which involves a combination of the method of Lowry et al., (1951) and the Biuret method. The method produces a more stable colour complex and enables large sample numbers to be processed with increased reproducibility.

Eight hundred microlitres of BS7 reagent (Appendix II) was added to 200ul of the sample, the solutions mixed thoroughly and allowed to stand at room temperature for 10 minutes. The colour was developed by adding 25ul of Folin and Ciocalteau phenol reagent and the tubes mixed and left to develop for 20 minutes. The samples were read at 600nm against a distilled water blank.

A calibration curve was produced using bovine serum albumin (BSA, fraction V) dissolved in sodium acetate buffer (0.1M; pH 4.8) as a standard and the protein content of the unknowns extrapolated from the curve.

2.16 Two-dimensional Crossed Immunoelectrophoresis (2D-CIE) of PP14 Preparations

Two-dimensional crossed immunoelectrophoresis was used in addition to the use of radioimmunoassay for confirming the specific removal of PP14 from decidual tissue extracts.

2.16.1 Development of antisera to decidual tissue extract proteins

Decidual tissue extracts were prepared as described previously and emulsified with two volumes of Freund's complete adjuvant either using a mechanical emulsifier or by repeated passage through a fine bore tube between two disposable syringes. The stability of the emulsion was assessed by dropping a sample into distilled water. One millilitre of the stable emulsion was injected subcutaneously at four sites along the neck and back of a New Zealand white rabbit. After 3 - 4 weeks a booster immunisation was given followed by further boosters at 2 - 3 week intervals. About 10 days after each booster, 20 - 30ml of blood was collected from a marginal ear vein into a plastic universal and the blood allowed to clot at room temperature for 2 hours. The clot was released from the sides of the universal using a spatula and the sample stored overnight at 4°C to allow the clot to retract.

The universal was centrifuged at 2000 x g for 15 minutes and the serum collected and stored in aliquots at -20°C until use. The antisera produced were screened for anti-PP14 immunoreactivity by assessing the binding of radiolabelled PP14 in an RIA.

2.16.2 2D-CIE of PP14 preparations

One percent agarose gels in barbitone buffer pH 8.6 (Appendix II) were prepared by steaming and allowing the gel to cool to 56°C. The agarose gels were cast onto 8cm x 8cm glass plates and placed onto the cooling plate of an LKB Multiphor electrophoresis bath and the samples applied. This procedure avoided the diffusion of the samples into the gel prior to electrophoresis. The PP14 preparations were electrophoresed at a constant current of 20mA at 4°C in 0.1M barbitone buffer pH 8.6 for approximately 1.5 hours. Each plate was used to run three samples with one sample being mixed with bromophenol blue in glycerol to act as a tracker dye. After the first electrophoretic dimension, rectangular gel strips which contained each of the separated samples were cut from the agarose gel (2.5cm x 8cm) and transferred to the cathodic end of a separate clean glass plate. Seven millilitres of 1% agarose gel containing 200ul of antiserum was cast onto the plate. The samples were electrophoresed at 20mA constant current into the antibody containing gel at 4°C for 16 - 20 hours.

After electrophoresis the gel was wetted with distilled water and covered with one wet and five dry filter papers. A glass plate and a weight (1 kg) were placed on top of the filter papers. The pressing was repeated twice at three-minute intervals. The gel was washed in 0.1 M sodium chloride (2 x 15 min) and the pressing repeated twice more.

The plate was placed in staining solution (Appendix II) for approximately 10 minutes and, after rinsing with distilled water, was

placed in destaining solution for approximately 2 hours. The gel was dried to a fine film using hot air from a hair dryer and the precipitin lines visualised using back illumination from a light box.

CHAPTER THREE.

RESULTS AND DISCUSSION.

3.1 The Radioimmunoassay of PP14

A typical G25 elution profile for radioiodinated PP14 is shown in Fig 3.1. The incorporation of ^{125}I into purified PP14 was typically 60 - 70% of the available isotope with purification of the labelled protein on Con-A-Sepharose giving two peaks of activity (Fig 3.2). The specific activity of the labelled tracer was typically 78 uCi/ug and its binding to excess antiserum was increased from typically 36% before Con-A-Sepharose purification to typically 65% after. These results are comparable to those reported by Bolton et al. (1983).

A typical standard curve and error profile is shown in Fig 3.3. The error profile was derived from duplicate standard curves run before and after each batch of samples and from replicate determinations of all the unknowns in the assay. The working range of each assay was defined as the region of the standard curve which exhibited a CV of less than 10%. The intra-assay precision of the assay was assessed from the mean of 20 determinations and ranged from 5 - 10% Inter-assay variation ranged from 10 - 15%.

3.2 The Standard Curve for Protein

A typical standard curve and error profile for protein determinations is shown in Fig 3.4. The working range was derived and defined as for the PP14 reference curve detailed above.

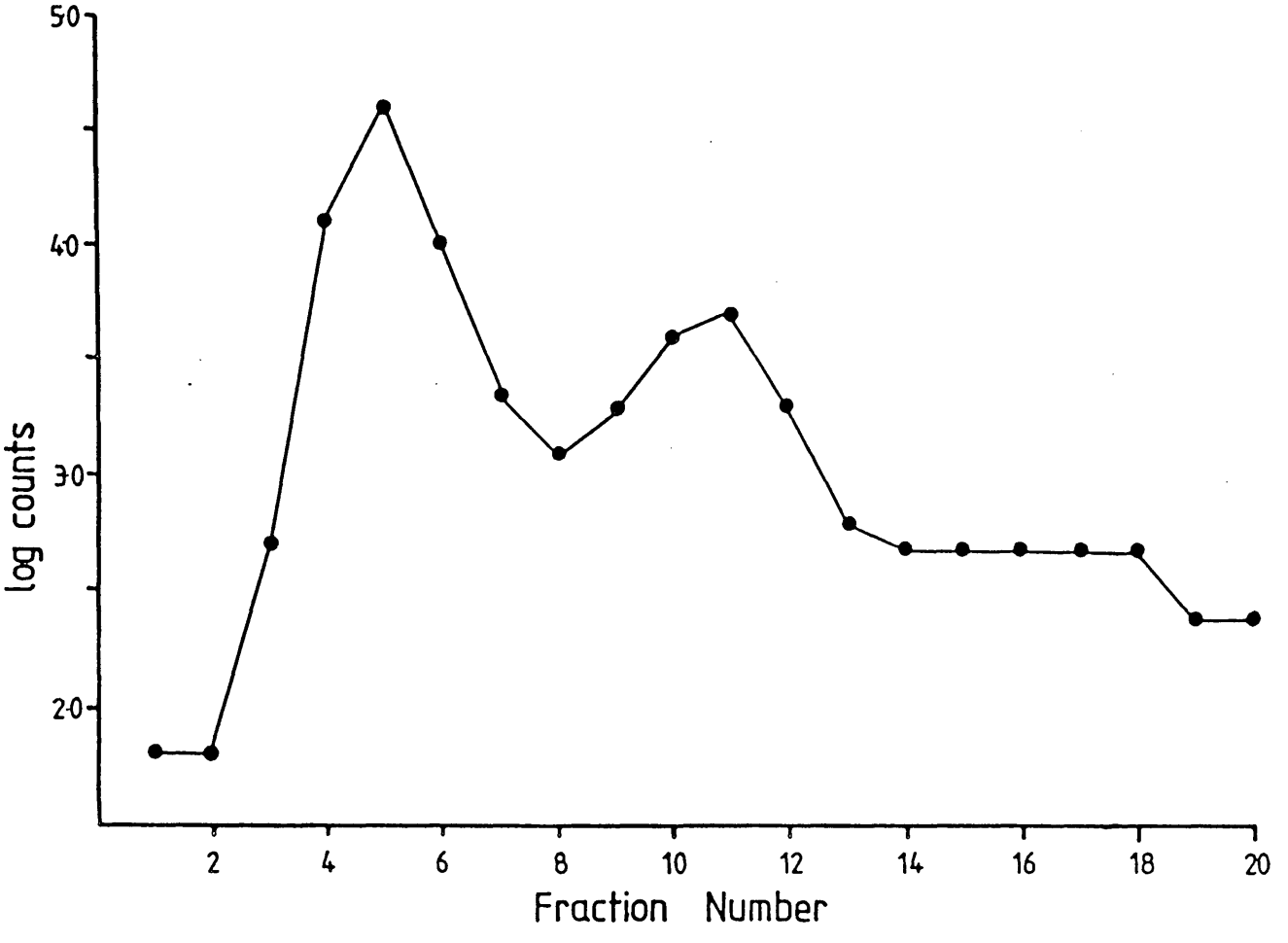


Figure 3.1 A typical G25 elution profile for radioiodinated PP14

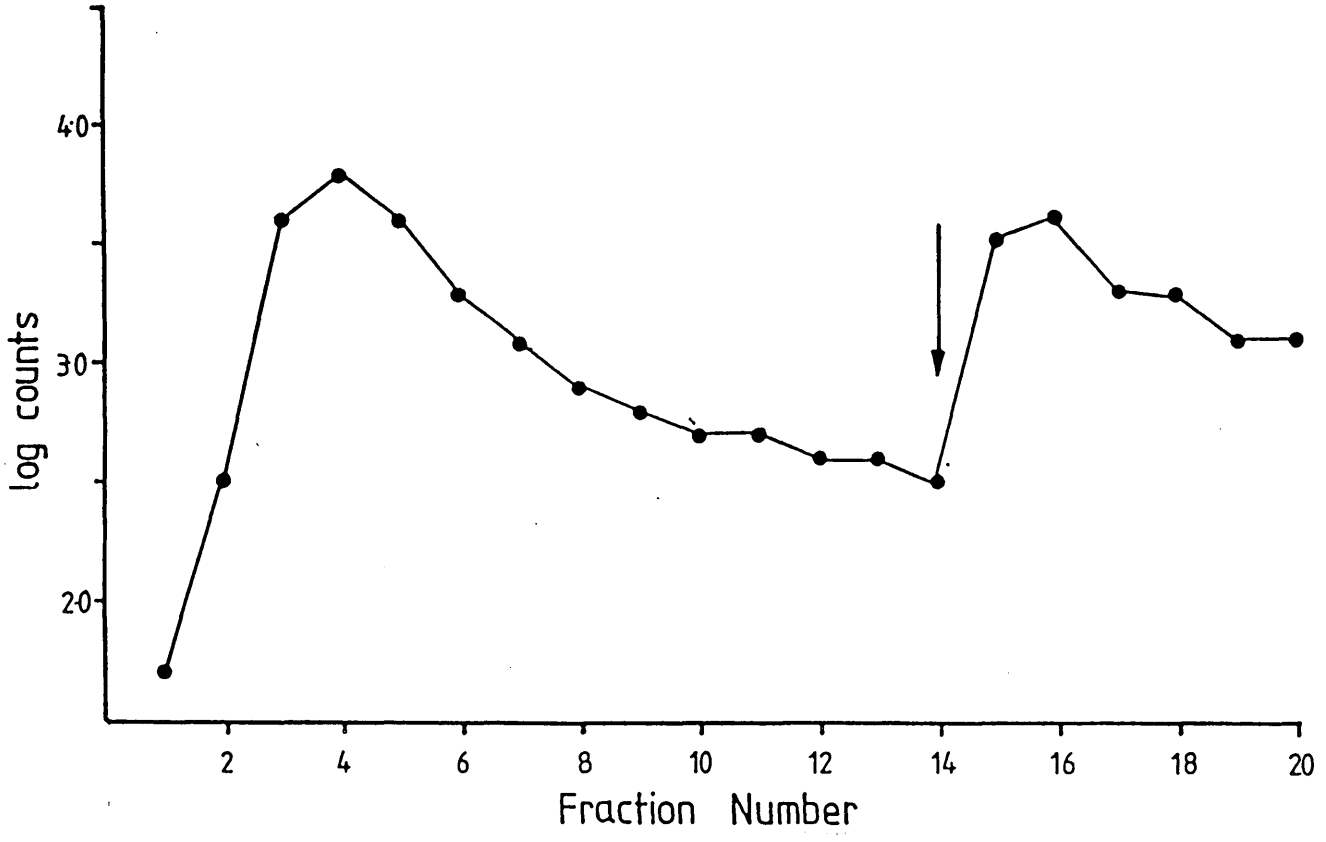


Figure 3.2 Elution profile for the purification of radioiodinated PP14 on Con-A-Sepharose. The arrow indicates the start of the elution of the retained material with 0.2 mol/l mannose in assay diluent buffer.

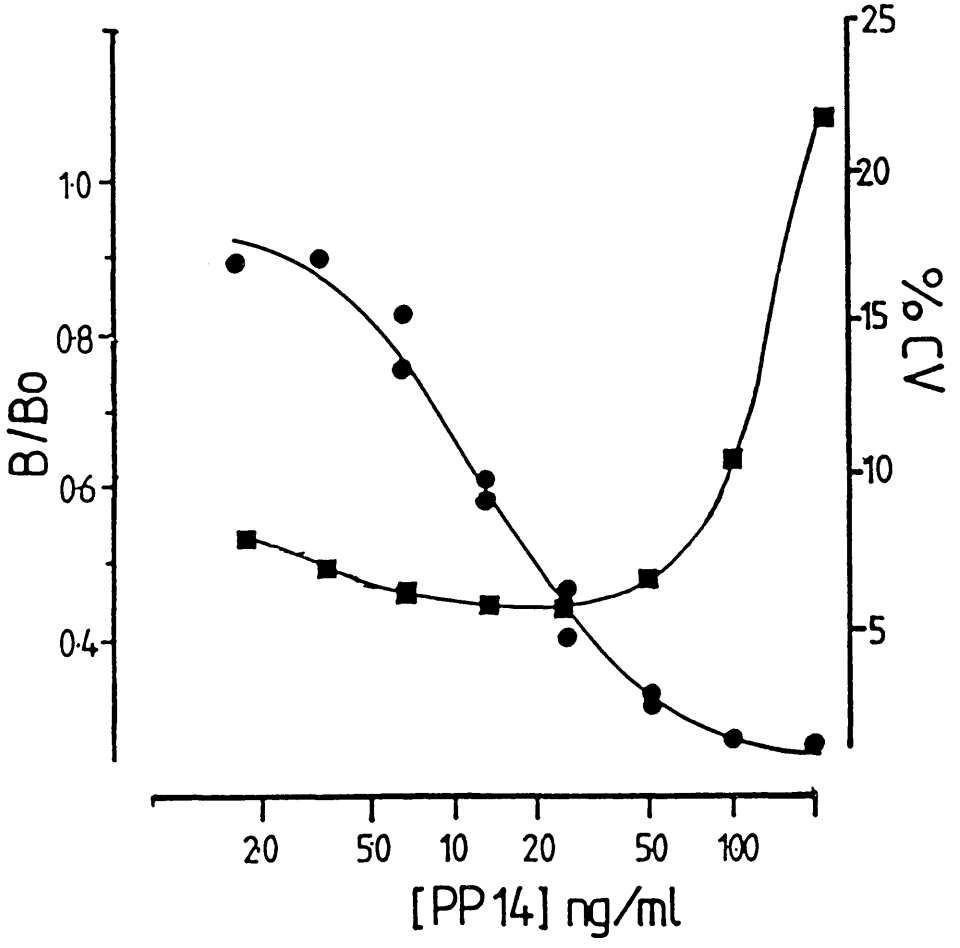


Figure 3.3 A typical standard curve (●) and error profile (■) for the radioimmunoassay of PP14.

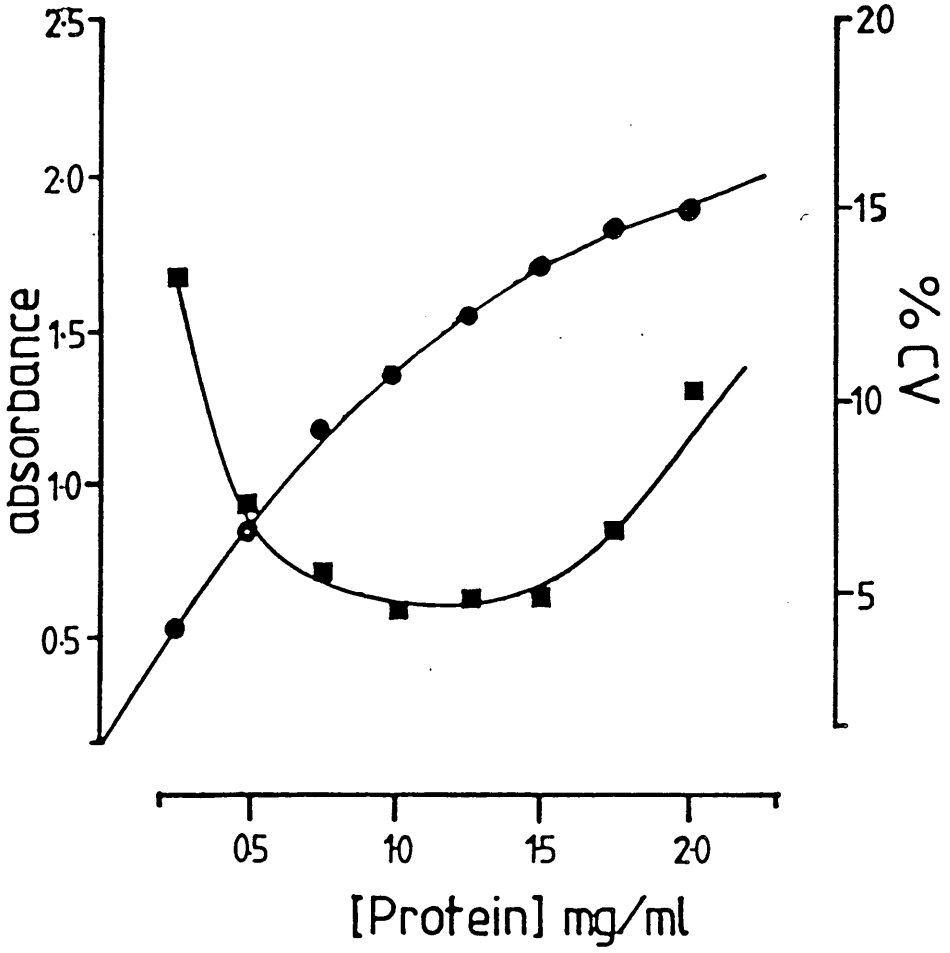


Figure 3.4 A typical standard curve (●) and error profile (■) for the protein assay.

3.3 PP14 and Protein Content of Decidual Extracts

The data for nine individual decidual tissue extracts assayed for both protein and PP14 content are shown in Table 3.1. PP14 comprised some 7.1% of the total soluble protein content of the extracts which confirmed previous data published by Julkunen et al. (1985).

Unless stated, pooled extracts from a number of tissue samples were used throughout this study.

3.4 Immunoadsorption of Decidual Tissue Extracts

The treatment of decidual tissue extracts with the anti-PP14 immunoadsorbent specifically reduced that protein peak which was immunologically indistinguishable from PP14 as assessed by two-dimensional crossed immunoelectrophoresis (Plate 3.1). The reduction of PP14 content was confirmed by assaying the samples for PP14 by radioimmunoassay.

SECTION A

THE EFFECT OF PP14 ON THE RESPONSE OF LYMPHOCYTES TO ALLOGENEIC STIMULATION

3.5 The Effect of PP14 on the Proliferative Response of Lymphocytes to Allogeneic Stimulation

Both purified PP14 and PP14-containing crude decidual tissue extracts exhibited a dose dependent inhibition of ^{3}H-Tdr uptake into allogeneically stimulated lymphocytes (Fig 3.5). The inhibitory

Decidual Extract	[PP14] ug/ml	[Protein] mg/ml
1	1.92	0.043
2	1.28	0.037
3	2.10	0.047
4	1.05	0.023
5	0.26	0.049
6	0.13	0.024
7	6.65	0.010
8	3.33	0.005
9	1.10	0.016
	1.98 $\pm$ 2.00	0.028 $\pm$ 0.16

Table 3.1 The PP14 and total protein content of 9 individual decidual extracts.

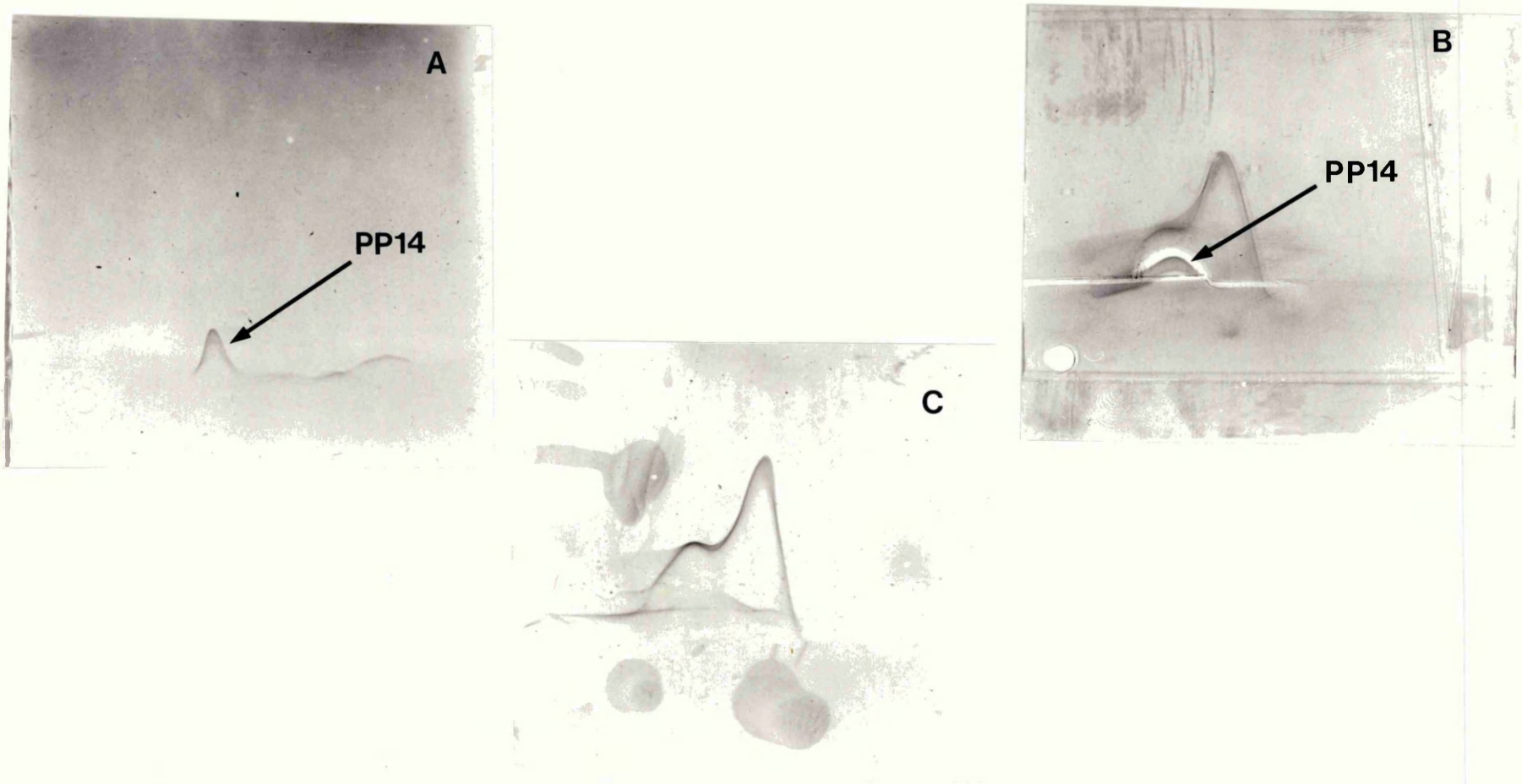


Plate 3.1 Two-dimensional crossed immunoelectrophoresis of purified PP14 (A, lot number 120/135, Behringwerke AG, Germany), unadsorbed decidual extract (B) and immunoadsorbed decidual extract (C). The PP14 peak observed in B has been accentuated for clarity. Immunoadsorption of the extract removed the peak immunologically indistinguishable from purified PP14.

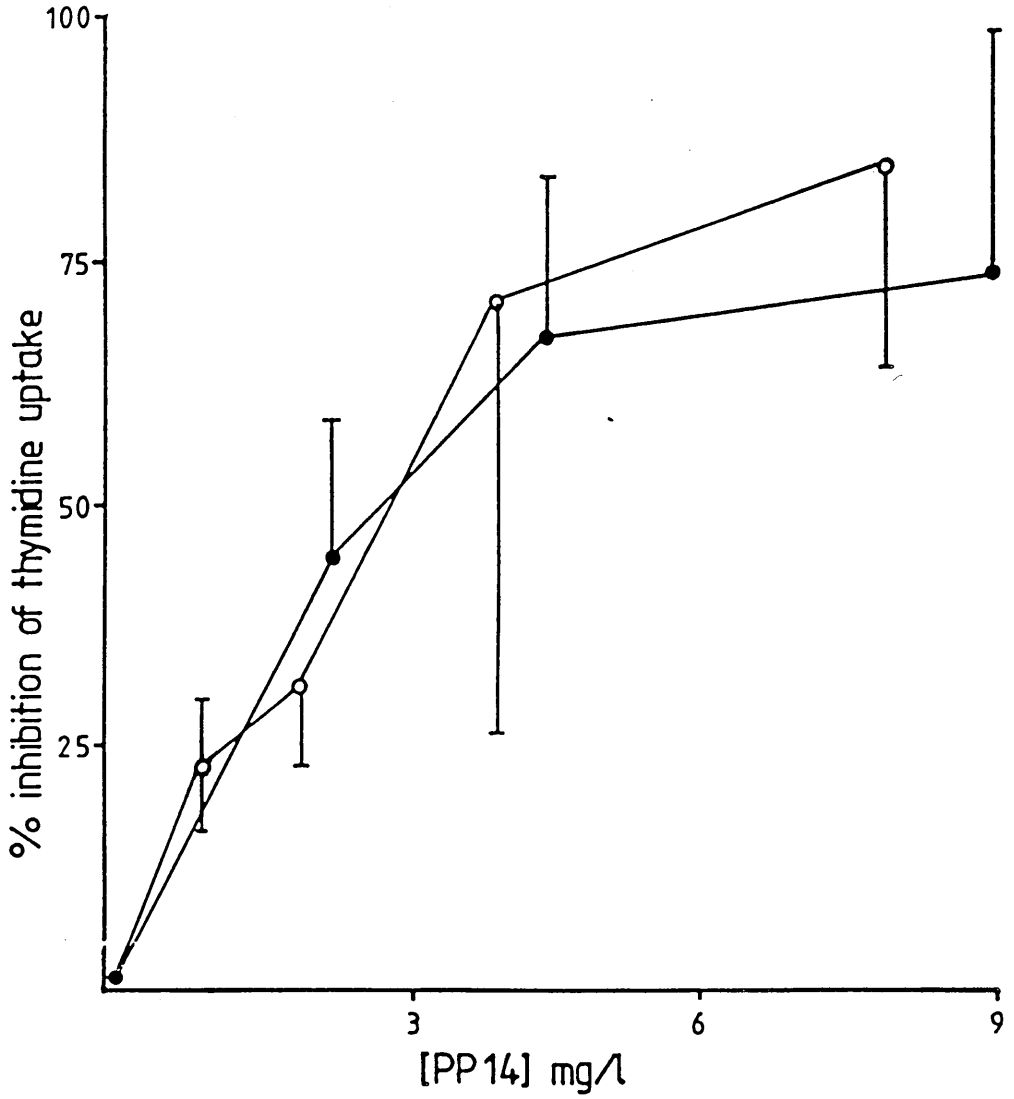


Figure 3.5 The effects of crude decidual extracts (●) and purified PP14 (O), related to their PP14 content, on the uptake of ^{3}H-Tdr by mixed lymphocyte cultures. Results are expressed as means ± sd, n = 10.

activity of the tissue extracts related closely to both their PP14 content as measured by RIA and that observed by purified PP14. There was no significant difference in the inhibitory activity of decidual extracts when the cells were cultured in foetal calf supplemented medium or serum-free medium (Table 3.2). Treatment of the decidual extract preparations with the monoclonal anti-PP14 immunoadsorbent resulted in a reduction in the inhibitory effects on ^{3}H-Tdr incorporation (Fig 3.6). The reduction in the suppressive activity paralleled the reduction in PP14 content as measured by RIA. The data presented in Fig 3.6 suggest that all of the immunosuppressive activity of the decidual tissue extracts could be removed by treatment with the monoclonal anti-PP14 immunoadsorbent.

3.6 The Effect of Neuraminidase Treatment on the Inhibitory Activity of Purified PP14

In order to assess whether the high carbohydrate content of PP14 was necessary for it to exert its suppressive activity, a treatment system consisting of neuraminidase linked to a solid phase was prepared. Purified PP14 was incubated with the solid phase by end-over-end mixing for 3 hours at room temperature. Subsequent to this treatment PP14 lost a significant element of its suppressive activity (Table 3.3), suggesting that sialo residues may play a part in the modulatory activity of the protein. These data need to be confirmed by further experimental work as it is possible that the apparent inactivation of PP14 may be an artefactual effect of impurities such as proteases in the commercial neuraminidase preparations.

[PP14] ug/ml	Inhibition of ^{3}H-Tdr uptake (%).
4.50 + FCS	69 ± 8.0
4.50 - FCS	67 ± 7.0

Table 3.2 The effect of foetal calf serum supplementation on the suppression of the mixed lymphocyte reaction by crude decidual extracts. Results are expressed as means ± sd, n = 9.

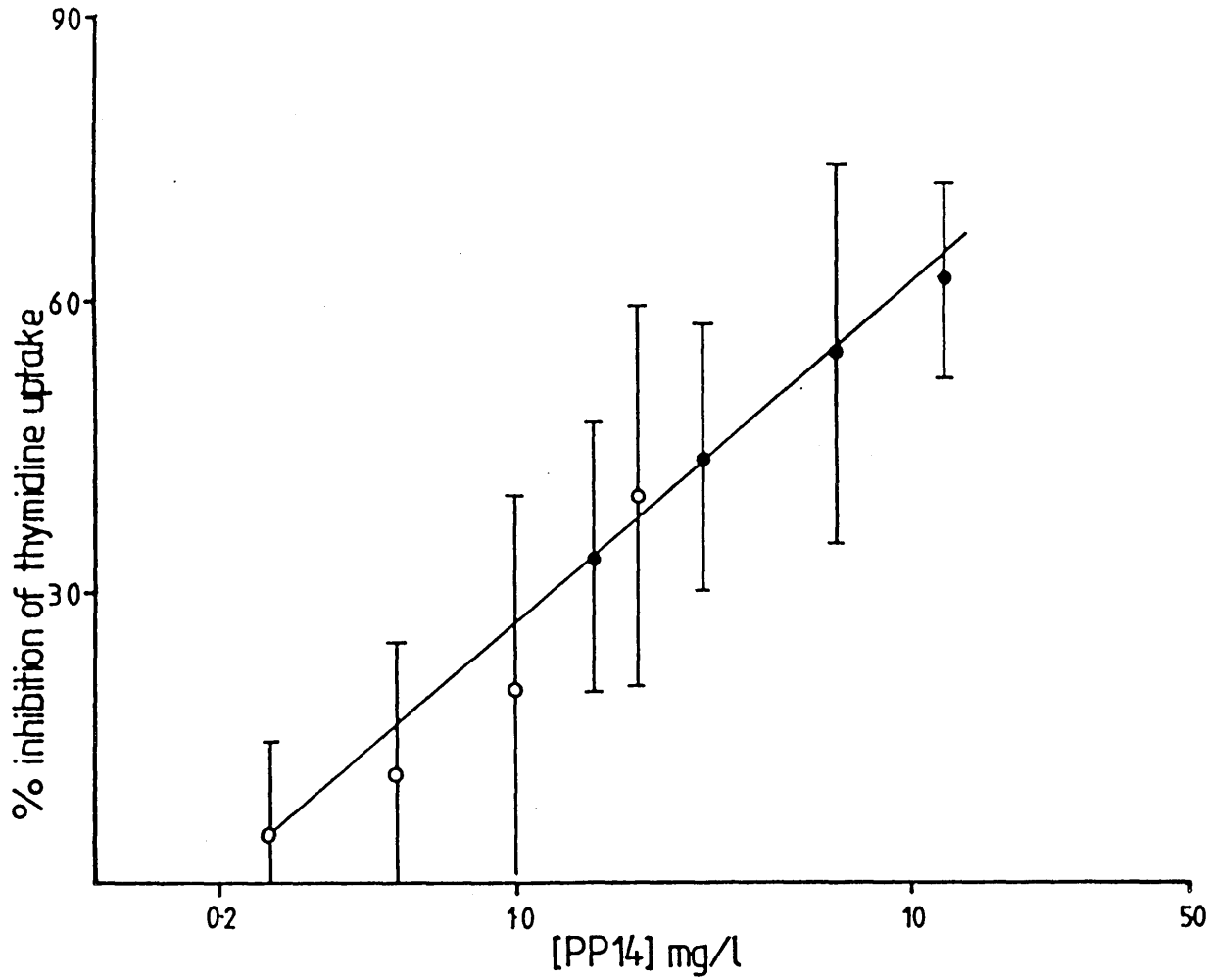


Figure 3.6 The effect of treating dilutions of pooled crude decidual extract with a monoclonal anti-PP14 antibody on its ability to inhibit ^{3}H-Tdr uptake by mixed lymphocyte cultures. Results are expressed as means ± sd. (●) unadsorbed; (O) immunoadsorbed.

	Inhibition of ^{3}H-Tdr uptake (%).	
[PP14] ug/ml	+ treatment	- treatment
8.0	24 ± 16	51 ± 13 **
4.0	20 ± 18	32 ± 13 *
2.0	15 ± 15	32 ± 13 **
1.0	14 ± 14	21 ± 15 ns

** P<0.01; * P<0.05; ns not significant.
(paired Student t test)

Table 3.3 The effect of treatment of purified PP14 with a neuraminidase solid phase on the suppression of the MLR. Results are means ± sd, n = 3.

Several oligo- and monosaccharides have been studied for their capacity to modulate lymphocyte proliferation in human allogeneic and autologous MLR. A defined subset of sugars showed a marked inhibitory activity on lymphocyte proliferative responses (Licastro et al., 1987). The inhibitory activity appeared to be effective at an early stage of the response, as addition of the sugars after 96 hours abrogated the inhibitory activity. The data suggested that carbohydrate determinants were involved in the proliferative response of lymphocytes in the MLR and may play a role in cell-cell recognition during such a response. The possibility that sugars may react with soluble mediators or growth factors produced in the MLR was excluded with the observation that oligosaccharides did not inhibit the response of human lymphocytes to PHA (Muchmore et al., 1980). The latter observation would appear to eliminate the possibility that the activity of PP14 is mediated through the carbohydrate moiety, for the mitogenic stimulation at least.

3.7 The Effect of 'Conditioned Medium' on the Inhibitory Effect of PP14

The incorporation of purified PP14 into the MLR resulted in an inhibition of ^{3}H-Tdr uptake into the stimulated cells. The inhibitory activity was partially reversed by the incorporation of previously generated MLR supernatant ('conditioned medium') into the assay (Table 3.4). The inhibitory effects of crude decidual extracts having a range of PP4 concentrations (2.0 - 10.0 ug/ml) was also partially reversed by the addition of conditioned medium (Table 3.4). These results support a possible level of control at the IL-1 or IL-2 level of the response.

	Inhibition of ^{3}H-Tdr uptake (%).	
	- CM	+ CM
Purified PP14	42 ± 12	30 ± 12 *
Decidual Extract	58 ± 30	28 ± 25 **

* P<0.10, ** P<0.01
(paired Student t test)

Table 3.4 The effect of conditioned medium on the suppression of the MLR by purified PP14 and crude decidual extracts (PP14 concentrations 2.0 - 10.0 ug/ml). Results are expressed as means ± sd, n = 5. For clarity of presentation, results are expressed as the means of the inhibitions observed at all PP14 concentrations investigated. Statistical analysis was performed by paired Student t test using the data obtained at each individual PP14 concentration.

3.8 The Effect of PP14 on Cell Surface Marker Expression

Low levels of Tac antigen expression were detected on unstimulated lymphocytes (5.0% cells staining; Table 3.5). The Tac antigen expressed was of low intensity, having a mean channel of fluorescence of 20. Incubation with allogeneic cells for 6 days increased both the number of Tac^+ cells and Tac antigen density. Incorporation of both immunoadsorbed and unadsorbed (PP14 concentrations 2.0 - 8.0 ug/ml) decidual extracts had no significant effect on the number of Tac^+ cells or antigen density as compared to the control (Table 3.5).

Allogeneic stimulation of lymphocytes also increased the number of TFR^+ cells from 6.0% to 23% with a concomitant increase in antigen density (Table 3.6). Incubation of the stimulated cells with decidual extract preparations had no significant effect either on the number of TFR^+ cells nor on the antigen density as compared to the control (Table 3.6).

The HLA-DR antigen was detectable on unstimulated cells at low levels (8.0%; Table 3.7). The antigen density on unstimulated cells appeared to be highly variable. Allogeneic stimulation gave rise to an increase in $HLA\text{-}DR^+$ cell number. However such expression was not significantly affected by incubation of the cells in the presence of decidual extract preparations (Table 3.7).

The data presented in Tables 3.5, 3.6 and 3.7 indicate that allogeneic stimulation causes an increase in surface expression of

Tac Antigen Expression

	% Cells	Mean Channel
Unstimulated	5 ± 4.0	20 ± 21
Stimulated control	30 ± 7.0	173 ± 64
I/A DE	28 ± 3.0	154 ± 41
U/A DE	25 ± 6.0	144 ± 72

Table 3.5 The effect of immunoadsorbed decidual extract (I/A DE, PP14 concentration 0 - 0.10 ug/ml) and unadsorbed decidual extract (U/A DE, PP14 concentration 2.0 - 8.0 ug/ml) on the expression of Tac antigen on allogeneically stimulated lymphocytes. Results are means ± sd, n = 5. The mean channel of fluorescence is a measure of the intensity of staining and hence the antigen density. Statistical analysis of data obtained at individual PP14 concentrations (paired Student t test) indicated no significant difference of antigen expression in the presence of PP14. For clarity of presentation, data are expressed as the means of the results obtained at all PP14 concentrations investigated.

Transferrin Receptor (TFR) Expression

	% Cells	Mean Channel
Unstimulated	6 ± 2.0	54 ± 74
Stimulated control	23 ± 7.0	197 ± 130
I/A DE	20 ± 3.0	93 ± 37
U/A DE	19 ± 10	81 ± 22

Table 3.6 The effect of immunoadsorbed decidual extract (I/A DE, PP14 concentration 0 - 0.10 ug/ml) and unadsorbed decidual extract (U/A DE, PP14 concentration 2.0 - 8.0 ug/ml) on the expression of transferrin receptor (TFR) on allogeneically stimulated lymphocytes. Results are means ± sd, n = 5. The mean channel of fluorescence is a measure of the intensity of staining and hence the antigen density. Statistical analysis of data obtained at individual PP14 concentrations (paired Student t test) indicated no significant difference of antigen expression in the presence of PP14. For clarity of presentation, data are expressed as the means of the results obtained at all PP14 concentrations investigated.

HLA-DR Antigen Expression

	% Cells	Mean Channel
Unstimulated	8 ± 3.0	92 ± 106
Stimulated control	33 ± 7.0	114 ± 20
I/A DE	28 ± 18	65 ± 12
U/A DE	22 ± 13	108 ± 51

Table 3.7 The effect of immunoadsorbed decidual extract (I/A DE, PP14 concentration 0 - 0.10 ug/ml) and unadsorbed decidual extract (U/A DE, PP14 concentration 2.0 - 8.0 ug/ml) on the expression of the HLA-DR antigen on allogeneically stimulated lymphocytes. Results are means ± sd, n = 5. The mean channel of fluorescence is a measure of the intensity of staining and hence the antigen density. Statistical analysis of data obtained at individual PP14 concentrations (paired Student t test) indicated no significant difference of antigen expression in the presence of PP14. For clarity of presentation, data are expressed as the means of the results obtained at all PP14 concentrations investigated.

Tac, TFR and HLA-DR antigens. Such stimulation did not give rise to the same degree of expression as did PHA stimulation, however as with PHA stimulation, PP14 did not appear to affect such expression. These results make unlikely the possibility that PP14 may modulate proliferative activity by either modulating activation antigen expression or by the masking of such antigens.

SECTION B.

THE EFFECT OF PP14 ON LYMPHOCYTE RESPONSIVENESS TO PHA

3.9 The Response of Peripheral Blood Lymphocytes to PHA

On culturing lymphocytes with varying concentrations of PHA (40 - 1.25 ug/ml) for 72 hours, maximum incorporation of ^{3}H-Tdr was observed at a final PHA concentration of 2.5 ug/ml (Fig 3.7). When cultured with 2.5 ug/ml of PHA, the maximum incorporation of ^{3}H-Tdr into the proliferating cells occured after 72 hours (Fig 3.8). Culturing the lymphocytes in the presence of $SF1^{+}$ medium containing 10% foetal calf serum increased the incorporation of ^{3}H-Tdr into unstimulated cells from 877 $\pm$ 110 cpm to 1876 $\pm$ 243 cpm. There was also a decrease in the stimulation index (stimulated cpm/unstimulated cpm) of the cultures (344 in $SF1^{+}$ medium; 154 in $SF1^{+}$ medium supplemented with foetal calf serum). Whilst medium supplementation with autologous plasma had no effect on the above parameters (data not shown), serum-free medium was routinely used throughout the study. Typically the background stimulation of lymphocytes was less than 2% of the uptake into PHA stimulated cells.

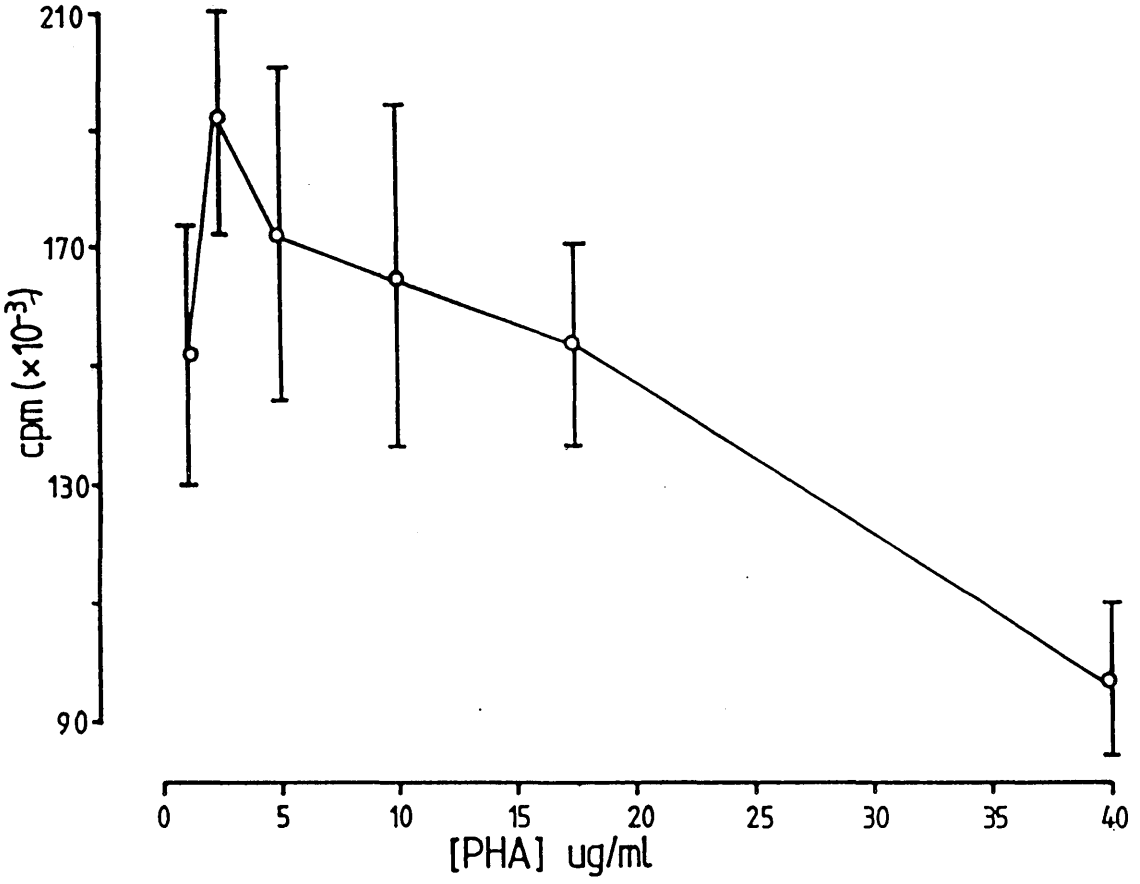


Figure 3.7 The effect of PHA concentration on the incorporation of ^{3}H-Tdr into stimulated lymphocytes. Results are expressed as means ± sd, n = 3.

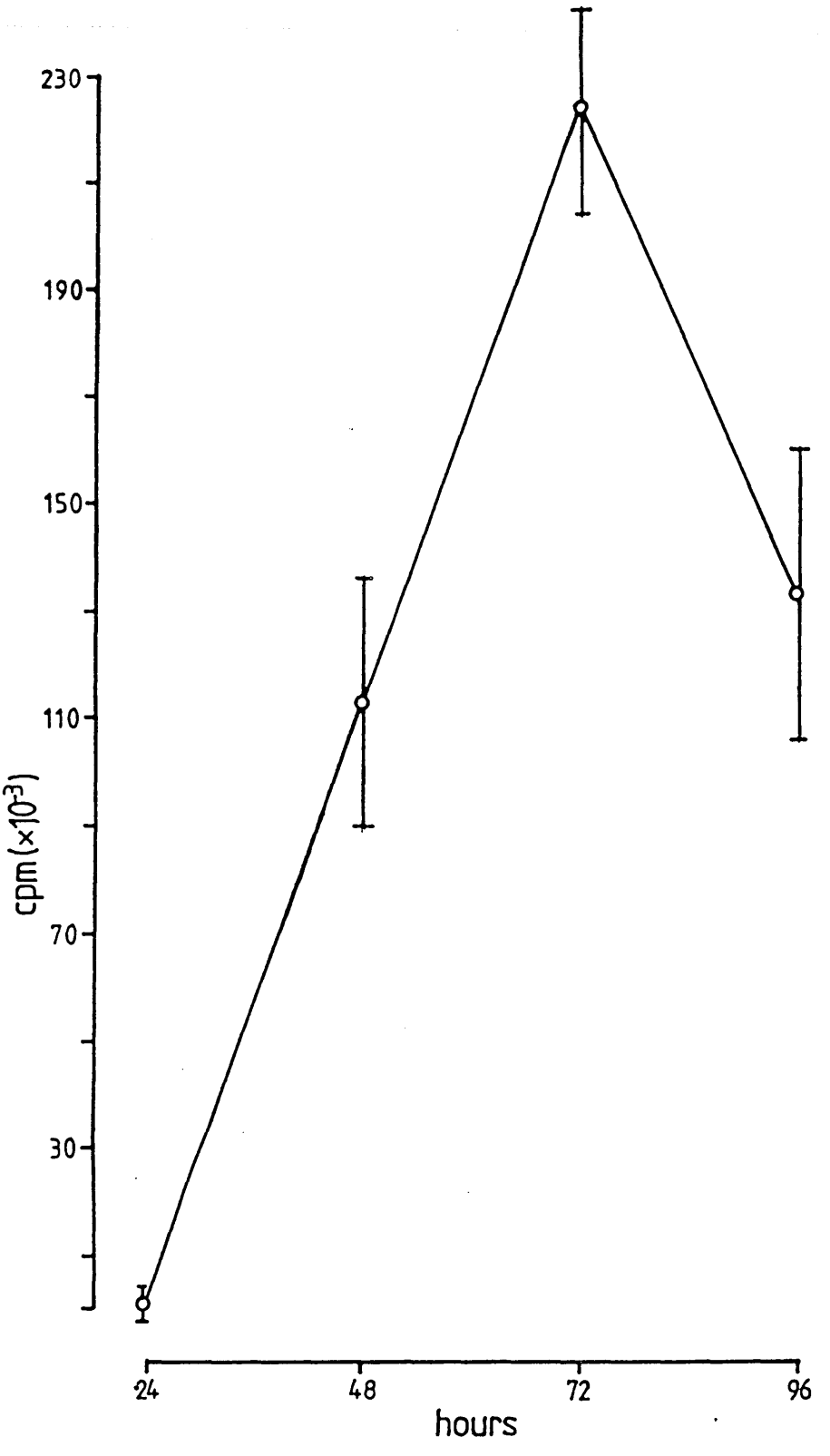


Figure 3.8 The effect of the culture period on the incorporation of ^{3}H-Tdr into PHA stimulated lymphocytes. Results are expressed as means ± sd, n = 3.

Optimisation was repeated for each preparation of PHA obtained, however the parameters remained constant throughout the culture period.

3.10 The Effect of PP14 on PHA Induced Lymphoproliferation

Both purified PP14 and PP14-containing crude decidual extracts exhibited a dose dependent inhibition of ^{3}H-Tdr uptake into PHA stimulated lymphocytes (Fig 3.9). These data and those obtained using the MLR above confirm previous observations on the immunomodulatory activity of PP14 on allogeneically stimulated lymphocytes (Bolton et al., 1986b). On the incorporation of 8 individual extracts into the assay, there was a significant correlation ($r=0.96$, $P<0.001$) between the log of the PP14 concentration and the suppressive activity, but not with the soluble protein concentration (Table 3.8). Culturing the cells in the presence of either foetal calf serum or autologous plasma had no effect on the suppressive activity of the extracts (Table 3.9).

The treatment of the extracts with the monoclonal anti-PP14 immunoadsorbent significantly reduced the inhibitory activity (Fig 3.10). The reduction in the suppressive activity paralleled the reduction in the PP14 content as measured by radioimmunoassay and confirmed the specificity of the activity. There was no significant suppression of the mitogenic response at PP14 concentrations less than 1.3 mg/l (Fig 3.10).

3.11 Time Dependency of the Inhibitory Activity

Decidual extracts were added at timed intervals after the initiation of the cultures in order to investigate the level of the

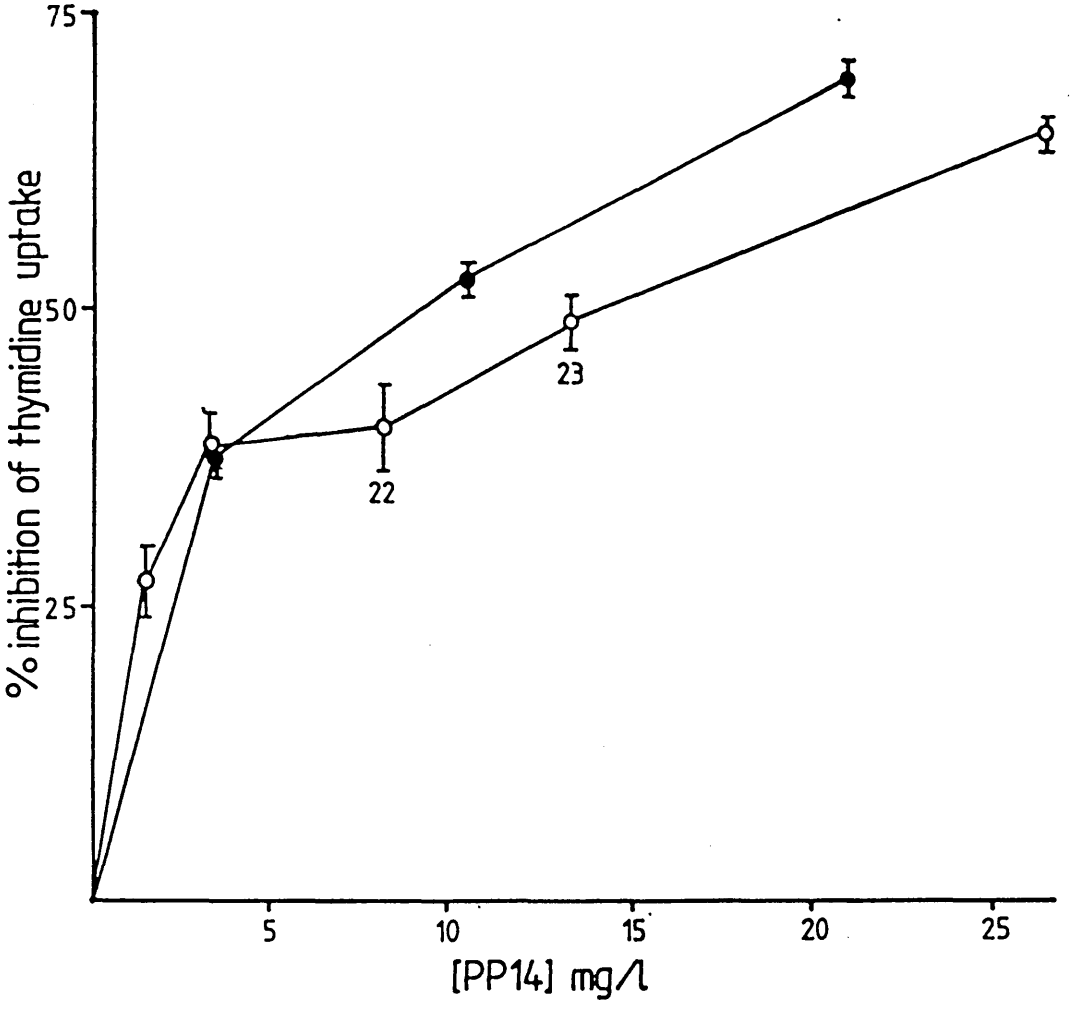


Figure 3.9 The effect of purified PP14 (●, n = 3) and pooled crude decidual extract (O, n = 20, unless stated), related to their PP14 content on the incorporation of ^{3}H-Tdr into PHA stimulated lymphocytes. Results are expressed as means ± SEM. ^{3}H-Tdr incorporation into control cultures = 115223 ± 5414 cpm, ^{3}H-Tdr incorporation into unstimulated lymphocytes = 1201 ± 134 cpm.

log [PP14] ug/ml	suppression of ^{3}H-Tdr uptake (%).	log [soluble protein] mg/ml
0.2844	50.7 ± 0.6	-1.3617
0.1703	47.9 ± 3.1	-1.4365
0.3222	51.7 ± 2.9	-1.3298
0.0212	45.4 ± 3.4	-1.6308
-0.5918	24.6 ± 1.0	-1.3125
-0.8928	1.30 ± 1.2	-1.6135
0.8228	64.4 ± 4.9	-2.0000
0.5218	49.0 ± 8.4	-2.3010
	r = 0.96*	r = -0.26^{ns}

* $P<0.001$

Table 3.8 The correlation between log PP14 concentration and log soluble protein concentration and the suppression of ^{3}H-Tdr uptake into PHA stimulated lymphocytes. Results are expressed as means ± sd, n = 3.

	Suppression of ^{3}H-Thymidine incorporation (%)		
	SF1	Autologous plasma	10% FCS
DE A	50.7 ± 0.6	68.8 ± 0.3	64.3 ± 0.8
DE B	47.9 ± 3.1	28.2 ± 0.7	36.2 ± 1.6
DE C	51.7 ± 2.9	34.8 ± 0.7	41.6 ± 1.3
DE D	45.4 ± 3.4	10.4 ± 0.9	32.8 ± 3.8
DE E	24.6 ± 1.0	40.9 ± 0.6	55.8 ± 1.0
DE F	1.3 ± 1.2	18.8 ± 0.8	35.2 ± 1.5
	36.9 ± 20.1	33.7 ± 20.0	44.3 ± 12.8

Table 3.9. The effect of medium supplementation (autologous plasma or 10% FCS) on the suppression of ^{3}H-Tdr uptake by 6 individual decidual extracts (PP14 concentrations 0.1 ug/ml - 5.0 ug/ml). Results are expressed as means ± sd, n = 3.

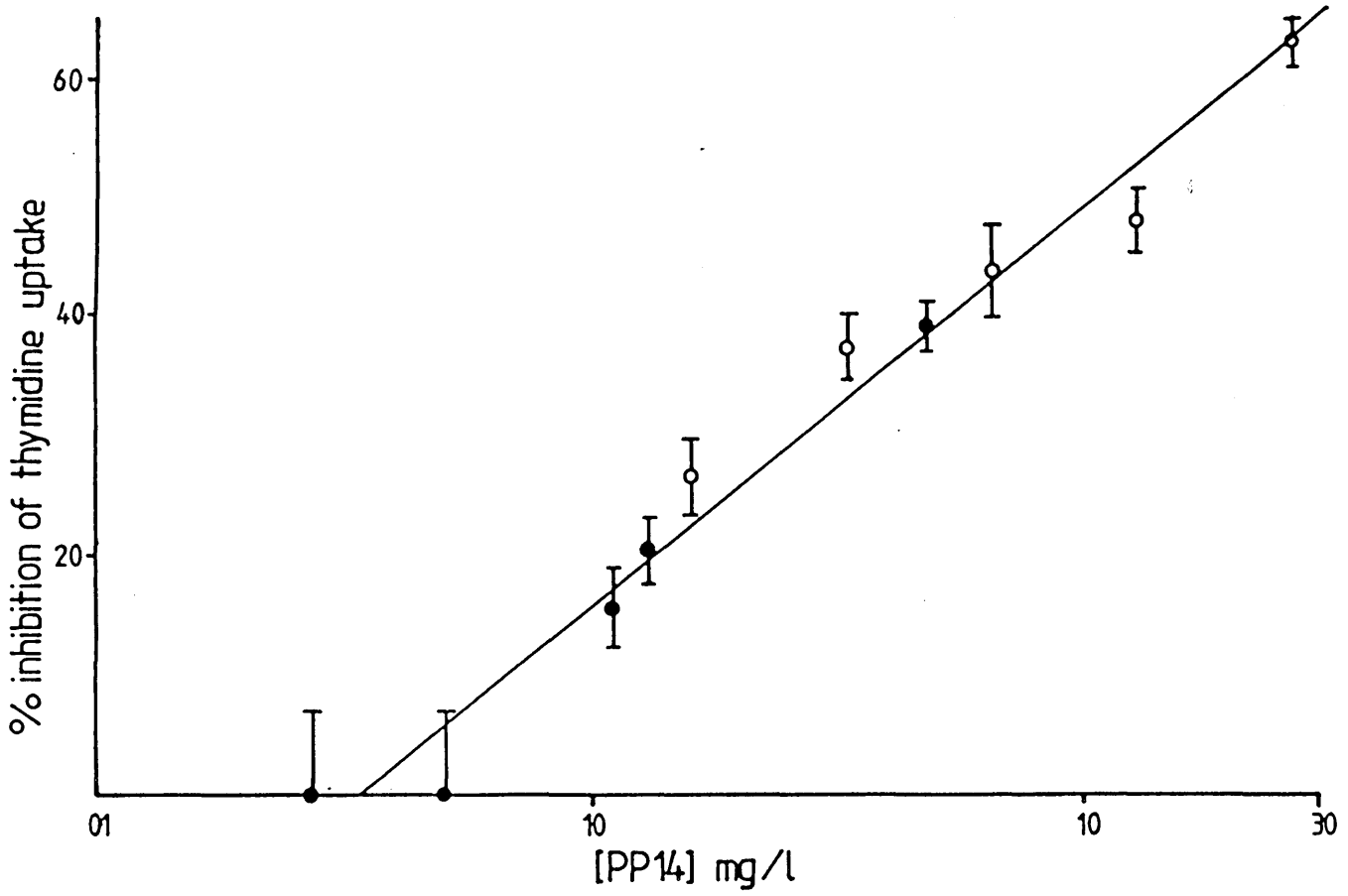


Figure 3.10 The effect of treating dilutions of pooled crude decidual extract with a monoclonal anti-PP14 antibody immunoadsorbent on its ability to inhibit ^{3}H-Tdr uptake by PHA stimulated lymphocytes. Data were related to PP14 content. Results are expressed as means ± SEM. ^{3}H-Tdr incorporation into control cultures = 115939 ± 4256, ^{3}H-Tdr incorporation into unstimulated cells = 1791 ± 285 cpm.
(O) unadsorbed; (●) immunoadsorbed.

suppressive activity. The activity of the extracts was independent of the time of addition up to 36 hours (Fig 3.11), with no significant inhibitory activity being observed if the tissue extracts were added after 48 hours of culture. These results suggest PP14 to exert its effect on a level of the immune response occurring early in stimulation. Addition after 48 hours of culture may allow sufficiently high levels of high affinity IL-2R to be developed to allow a normal proliferative response or alternatively allow normal levels of IL-1, IL-1R or the IL-1 inducing lymphokine suggested by Mizel (1987) to be attained. Addition of decidual extract at the time of pulsing the cells (66 hours) had no effect on ^{3}H-Tdr incorporation. This discounts the possibility that PP14 merely inhibits the uptake of ^{3}H-Tdr.

When lymphocyte cultures were incubated with PHA for varying time periods (1, 3, 5 and 7 days) in the presence of immunoadsorbed and unadsorbed decidual extracts, maximum suppression of ^{3}H-Tdr uptake was observed on day 3 (Fig 3.12), although significant suppression ($P<0.001$) could be observed after only 1 day of culture. Subsequent to day 3, inhibition of ^{3}H-Tdr uptake was reduced in parallel to the reduction of ^{3}H-Tdr incorporation into the cells of the control cultures containing immunoadsorbed decidual extracts (day 5 115409 $\pm$ 1982 cpm; day 7 64256 $\pm$ 969 cpm).

3.12 The Effect of PP14 on Cell-cell Interaction

There are a number of non-specific mechanisms which may lead to an apparent immunosuppressive activity.

An important feature in the process of immunologic recognition and proliferation is the capacity of lymphocytes to form aggregates.

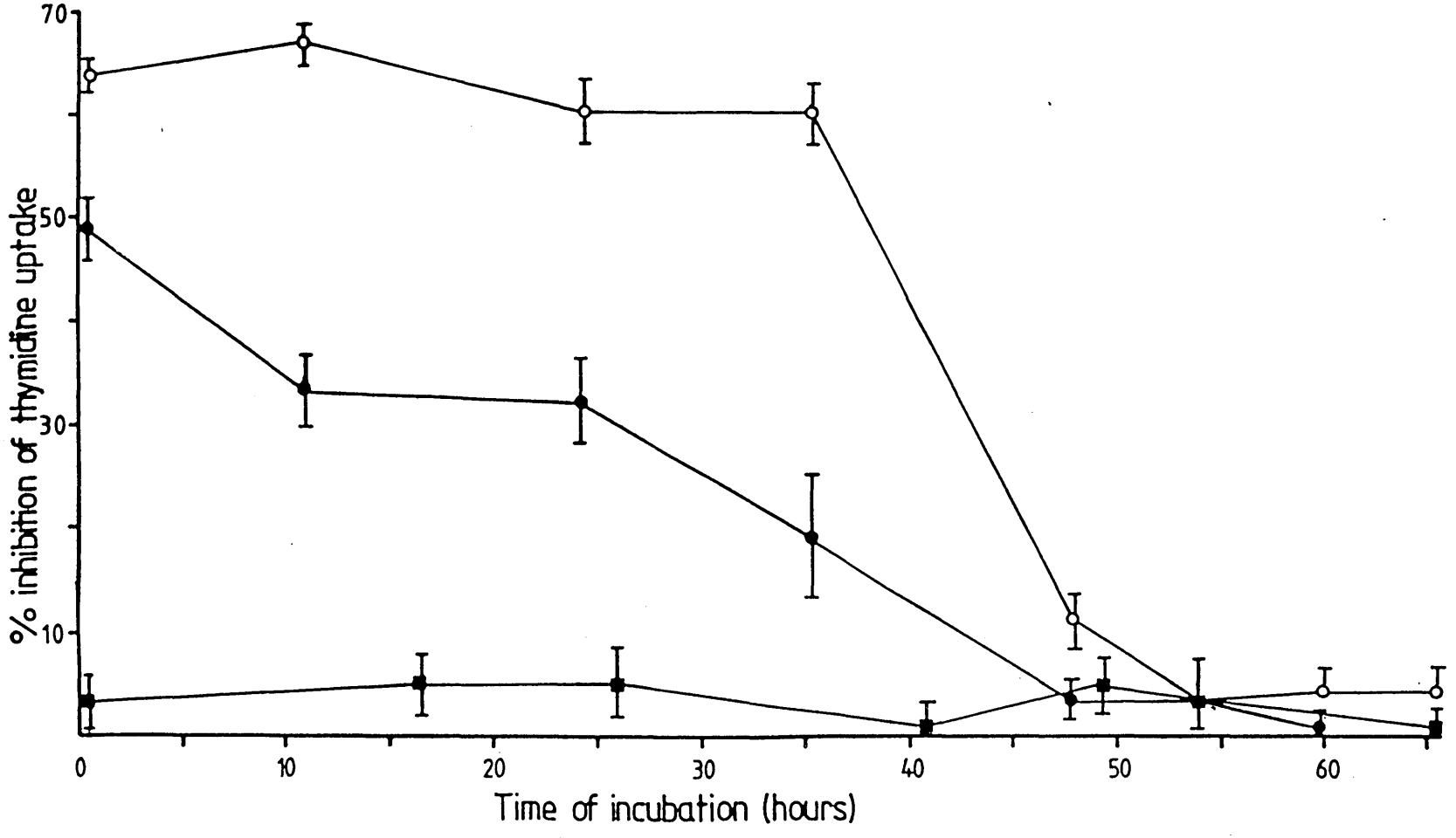


Figure 3.11 The effect of the delayed addition of unadsorbed (O , PP14 concentration 26.7 ug/ml; ●, PP14 concentration 13.4 ug/ml) and immunoadsorbed (■, PP14 concentration 1.0 ug/ml) decidual extracts on the inhibition of ^{3}H-Tdr uptake into PHA stimulated lymphocytes. Results are expressed as means ± SEM, n = 10.

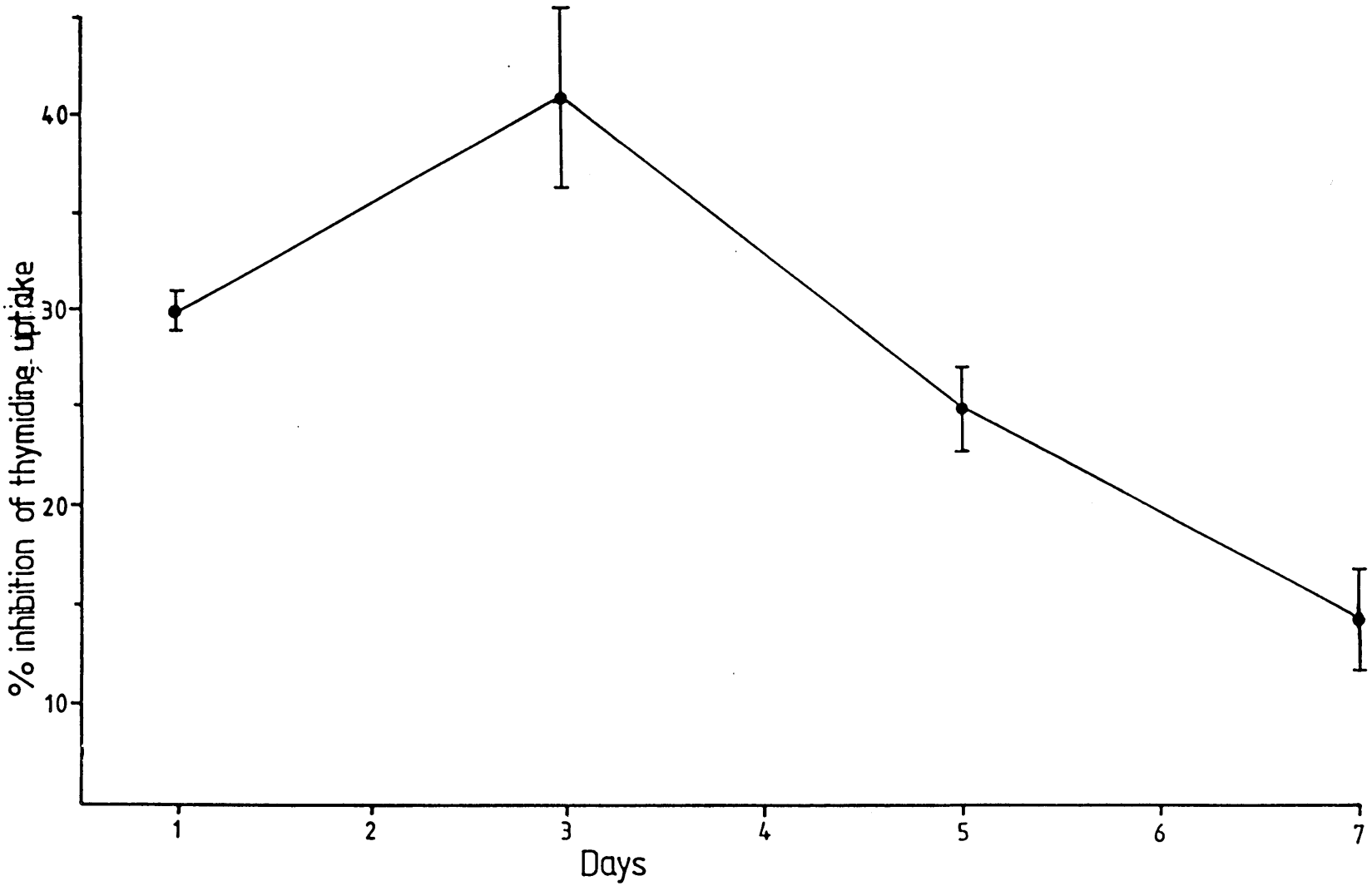


Figure 3.12 The effect of incubation period on the inhibition of ^{3}H-Tdr incorporation into PHA stimulated lymphocytes. Results are expressed as means ± sd, n = 3.

This process may mediate the local exchange of immunological information such as the secretion of lymphokines. An element of the immunomodulatory activity of progesterone appears to be attributable, in part, by an interference with cell-cell contact. Crude decidual extracts which had been shown to exhibit suppressive activity and whose activity was related to the PP14 content did not inhibit lymphocyte aggregation induced on activation with PHA as compared to control cultures. Representative results are shown in Plate 3.2.

3.13 Non-specific Cellular Effects of PP14

The suppression of ^{3}H-Tdr uptake induced by the decidual extracts was not due to a non-specific toxic effect on the cultured cells as after 5 days of culture under these conditions cell viability, as assessed by Trypan Blue dye exclusion, did not significantly decrease as compared to the control (Fig 3.13). The viability of both resting and stimulated lymphocytes in the presence of decidual extracts was 87.5% as compared to 88.5% for the control.

There is the possibility that PP14 may be a non-specific inhibitor of cellular growth and proliferation. This was shown not to be the case. Incubation of the human T leukaemic cell line K562 (Lozzio and Lozzio 1975), the choriocarcinoma cell line SW802 and the myeloid monocytic cell line U937 with decidual extract had no effect on the incorporation of ^{3}H-Tdr into the DNA of the proliferating cells (Table 3.10).

3.14 The Interaction of PP14 with Phytohaemagglutinin

An inhibition of mitogenic responsiveness may be due to an interaction of PP14 with the mitogenic lectin. PP14 is known to bind

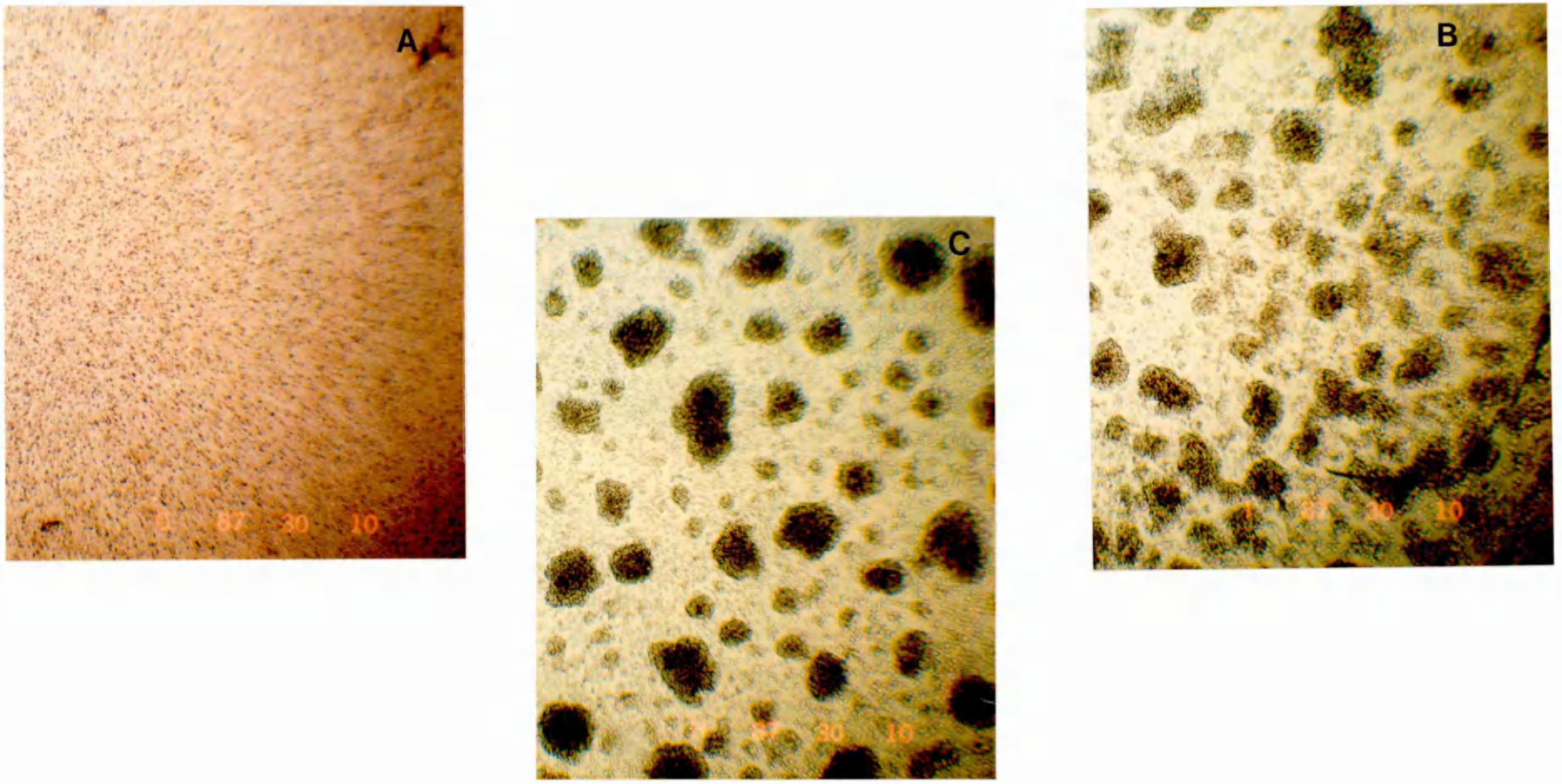


Plate 3.2 Following 3 days of culture, unstimulated lymphocytes showed no aggregation response (A), whereas stimulation of the cells with PHA induced a marked lymphocyte aggregation (B) which was unaffected by the presence of unadsorbed crude decidual extract (C).

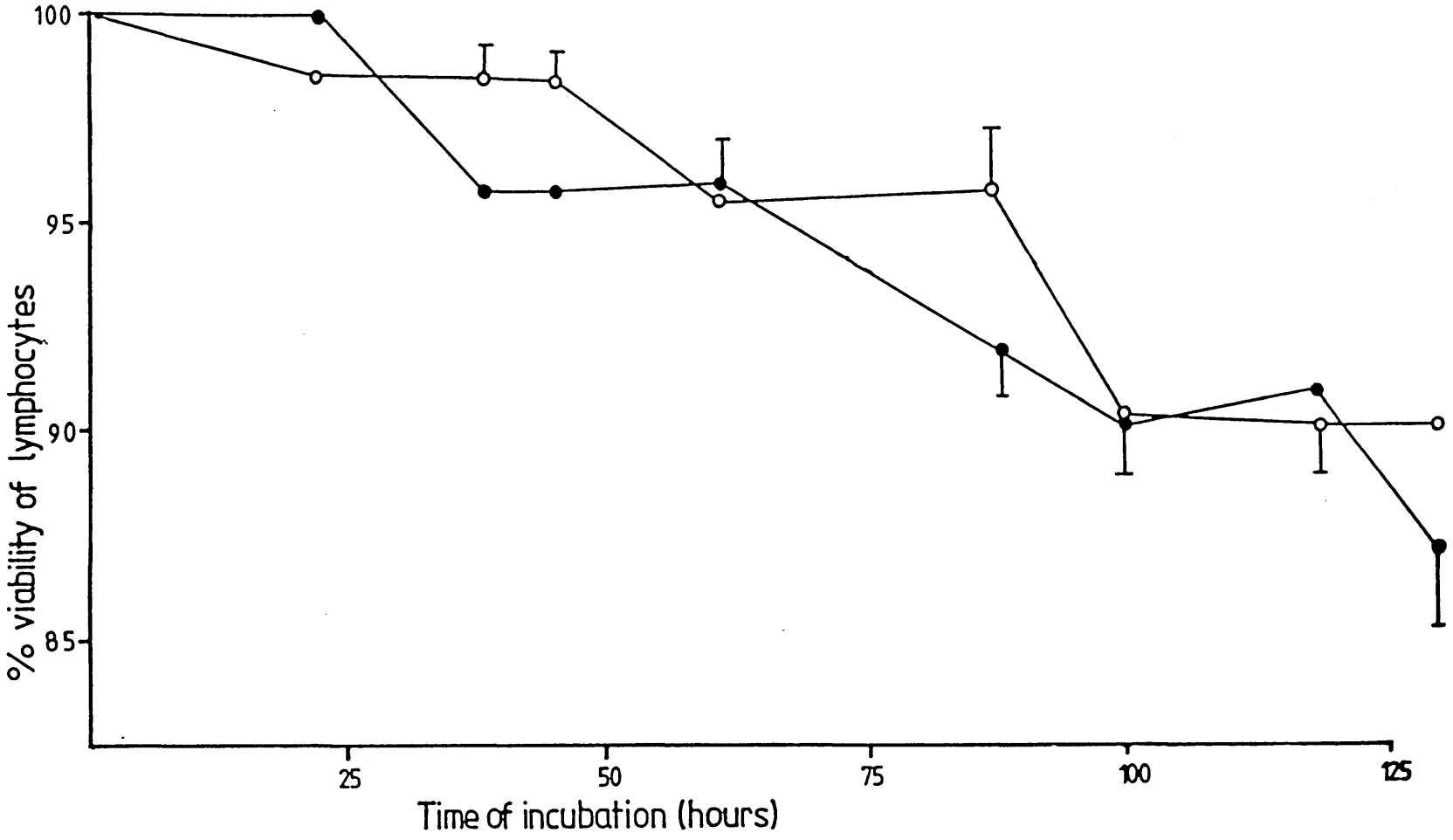


Figure 3.13 The effect of crude decidual extract (O) on cellular viability over the culture period as compared to the control (●). Results are expressed as means ± sd, n = 5.

Incorporation of ^{3}H-Thymidine (cpm).

	SW 802	U 937	K 562
Control	15610 ± 523	18180 ± 1562	31752 ± 550
+ DE	15140 ± 500	21378 ± 2274	29949 ± 4999

Table 3.10 The effect of crude decidual extracts on the incorporation of ^{3}H-Tdr into the DNA of three different cell lines. Results are expressed as means ± sd, n = 5.

to concanavalin A and binding to PHA may be the primary mediator of the modulatory activity.

The binding of radiolabelled PP14 to solid phase linked PHA was higher than its binding to either the BSA linked solid phase or the control solid phase. The observed binding to the PHA solid phase was also higher than the binding of radiolabelled CA III which was used as a non-specific control (Table 3.11). The binding of PP14 to PHA was 5.0% higher than its binding to control solid phases and the binding of radioiodinated CA III, which has no carbohydrate content. Such a low level of interaction would not be expected to account for the inhibitory activity of the protein.

The suppressive activity of decidual tissue extracts was not dependent on the PHA concentration (Fig 3.14). This would appear to eliminate the possibility that PP14 exerts its immunosuppressive activity by the formation of an inactive complex with PHA thereby making it unavailable for cell transformation. If there is binding of PHA by PP14, then it is likely that a depression of lymphocyte reactivity could be overcome by increasing concentrations of PHA. However, as seen in Fig 3.14, the inhibition of lymphocyte reactivity could not be removed by an elevation in PHA concentrations even up to 16 times the optimal.

3.15 The Effect of PP14 at the IL-2 Level of the Immune Response

Incorporation of 6 individual extracts (PP14 concentrations 1.6 - 20 ug/ml) into the mitogenic stimulation assay inhibited the uptake of ^{3}H-Tdr into PHA-stimulated lymphocytes (40 $\pm$ 14% inhibition, $P<0.001$) as compared to the control. The observed inhibition was

Binding of Radiolabelled Protein (%).

	PHA solid phase	BSA solid phase	control solid phase
^{125}I-PP14	6%	1%	1%
^{125}I-CAIII	2%	1%	1%

Table 3.11 The binding of radiolabelled proteins (PP14 and CAIII) to PHA, BSA and control solid phases. Results are expressed as the percentage of the total activity. Total activity for PP14 6215 $\pm$ 128 cpm; total activity for CAIII 11769 $\pm$ 222 cpm.

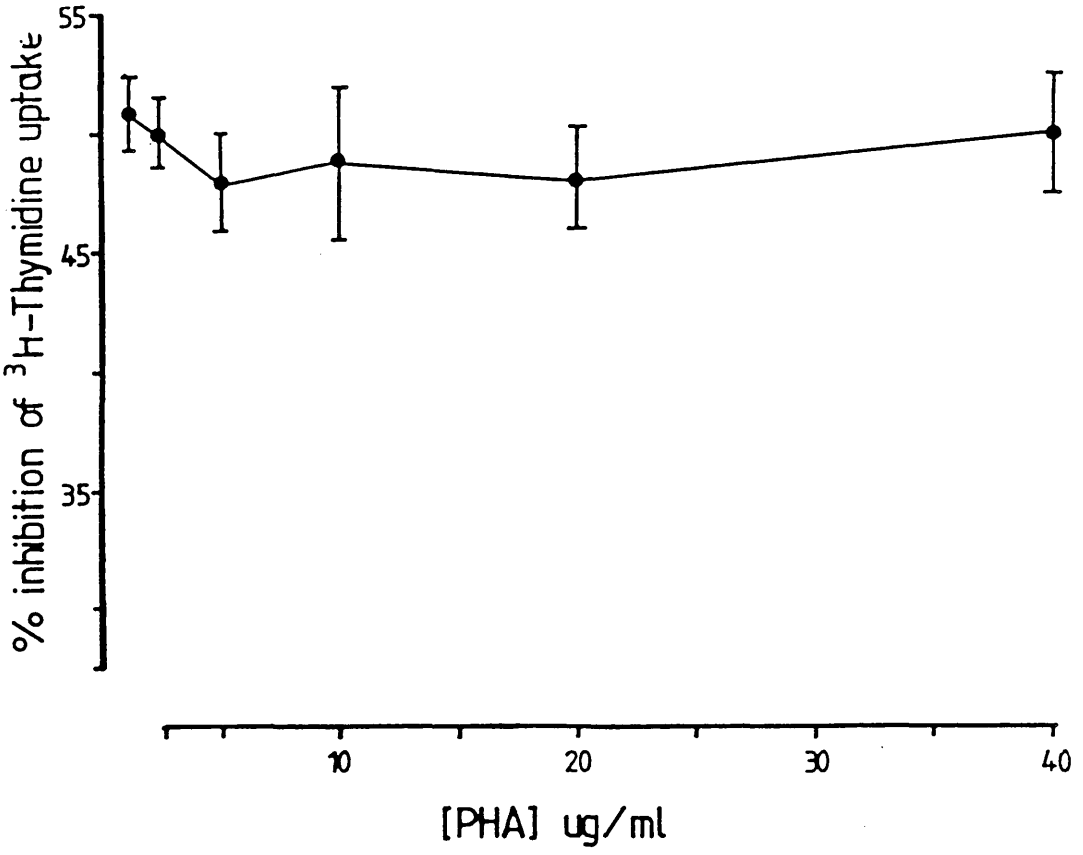


Figure 3.14 The effect of PHA concentration on the inhibition of ^{3}H-Tdr uptake into stimulated lymphocytes by crude decidual extracts (PP14 concentration 9.0 ug/ml). Results are expressed as means $\pm$ sd, n = 3.

significantly reduced ($P<0.002$, paired Student t test) by the addition of 2.5 units/ml recombinant IL-2 (21 $\pm$ 13% inhibition, $P<0.002$; Table 3.12). These data suggest an involvement of PP14 at the IL-2 level of the immune response.

The proliferative response of PHA blasts to recombinant IL-2 (rIL-2, 2.5 U/ml) was minimally suppressed by PP14. However the same concentrations of PP14 inhibited the proliferation of the blasts to PHA (Fig 3.15). These results suggest that PP14 does not mediate its suppressive activity by blocking the binding of IL-2 to the IL-2R.

A typical standard curve for IL-2 determinations is shown in Fig 3.16. The minimum detectable level of IL-2 by this test has been shown to be 0.05 U/ml. Detectable levels of IL-2 (0.25 U/ml) were observed after 18 hours of PHA stimulation (Fig 3.17). The maximum level of IL-2 detectable in the culture supernatants of PHA stimulated lymphocytes occured after around 66 hours of incubation (Fig 3.17). Unadsorbed decidual extract inhibited IL-2 production throughout the culture period investigated (0 to 90 hours) as compared to immunoadsorbed extract as control (Fig 3.18), with the suppressive activity being dose-dependent over the range of PP14 concentrations investigated (Fig 3.19). Maximum inhibition occured after 66 hours which coincided with maximum IL-2 levels (Fig 3.20). Such data support the observations that exogenous IL-2 was able to partially reverse the suppressive activity of decidual tissue extracts. It may be that there is a threshold of IL-2 concentration above which normal proliferation occurs. Addition of the extract

	Inhibition of ^{3}H-Thymidine uptake (%)	
	without IL-2	with IL-2
DE A	31.0 ± 1.0	15.0 ± 1.8
DE B	46.0 ± 1.7	18.0 ± 0.9
DE C	48.0 ± 7.3	36.0 ± 5.7
DE D	20.0 ± 3.2	0
DE E	35.0 ± 9.5	25.0 ± 3.1
DE F	58.0 ± 14.5	33.0 ± 3.6
Mean	40.0 ± 14.0	21.0 ± 13.2*

* $P<0.002$ (paired Student t test)

Table 3.12 The effect of exogenous rIL-2 (2.5 U/ml) on the inhibition of ^{3}H-Tdr uptake into PHA stimulated lymphocytes by 6 individual decidual tissue extracts (PP14 concentrations 1.6 ug/ml - 20.0 ug/ml). Results are expressed as means ± sd.

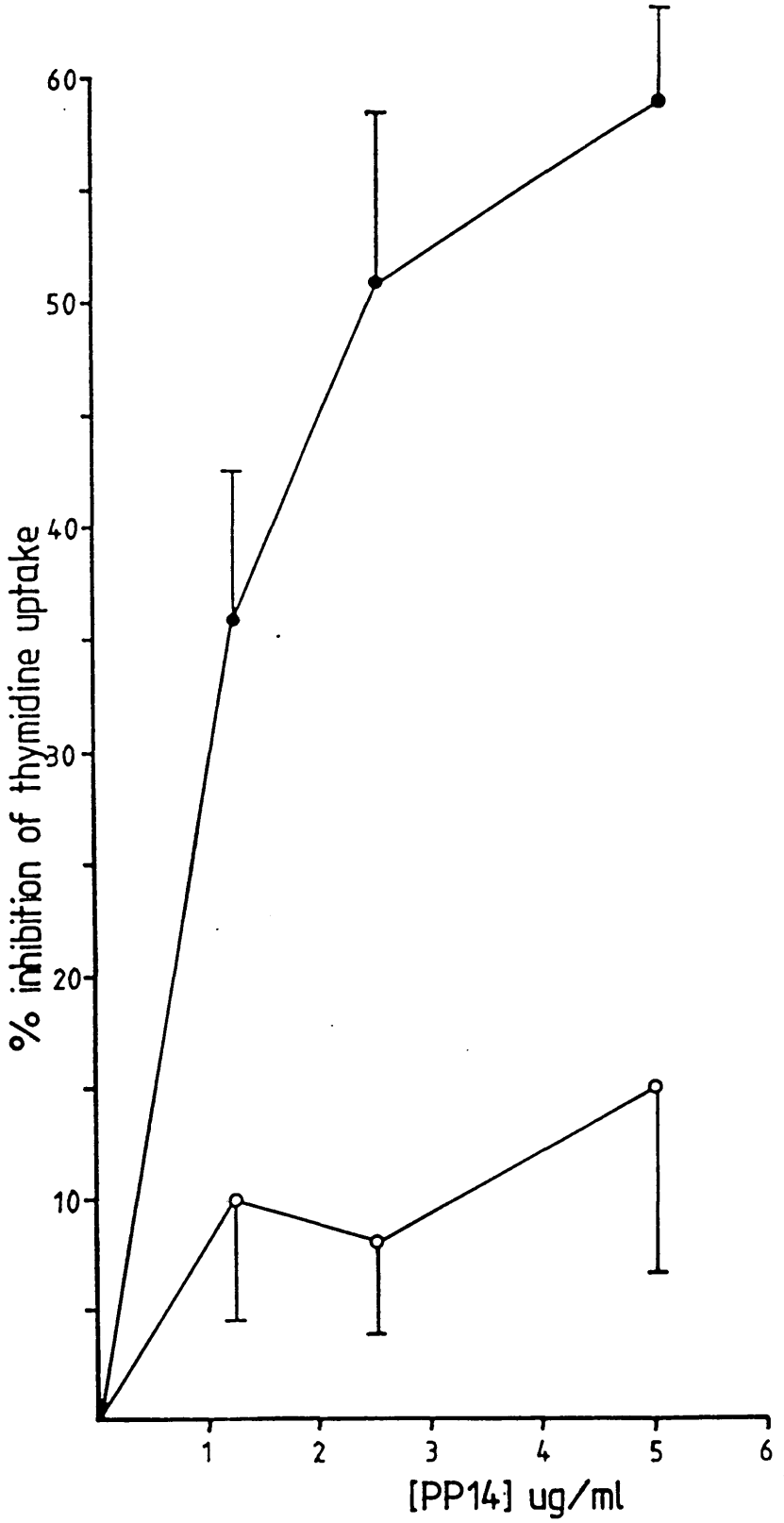


Figure 3.15 The effect of PP14 on the incorporation of ^{3}H-Tdr into 'PHA blasts' stimulated with either 2.5 units/ml of rIL-2 (O) or 2.5 ug/ml PHA (●). Results are expressed as means ± sd. n = 3.

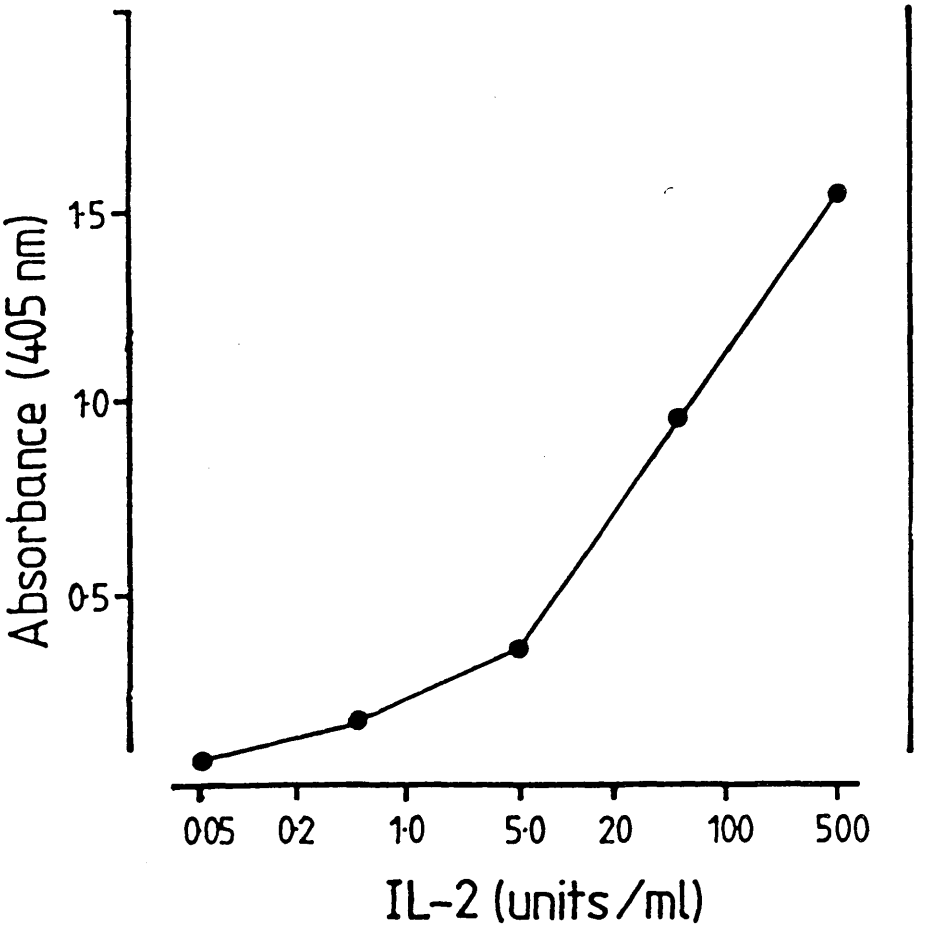


Figure 3.16 A typical standard curve for interleukin-2 determinations.

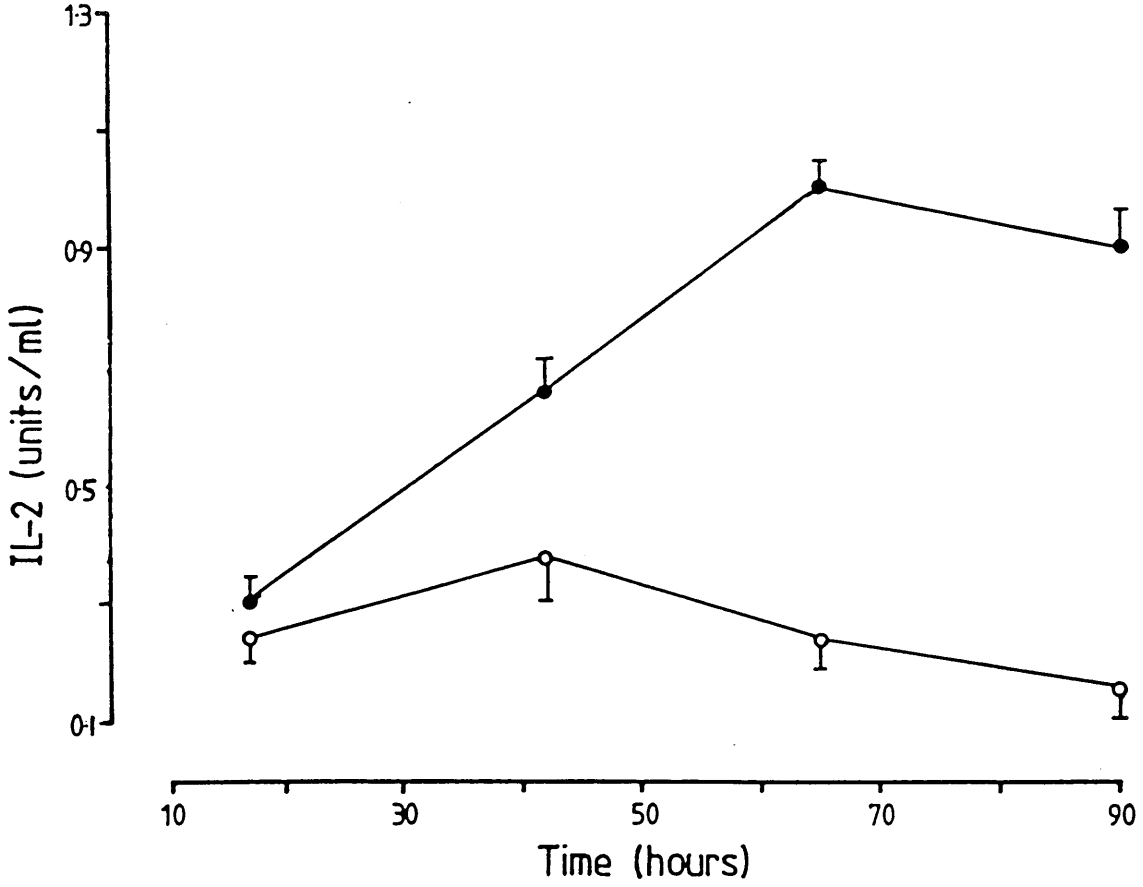


Figure 3.17 The release of IL-2 from unstimulated (O) and PHA stimulated lymphocytes (●). Results are expressed as means ± sd, n = 3.

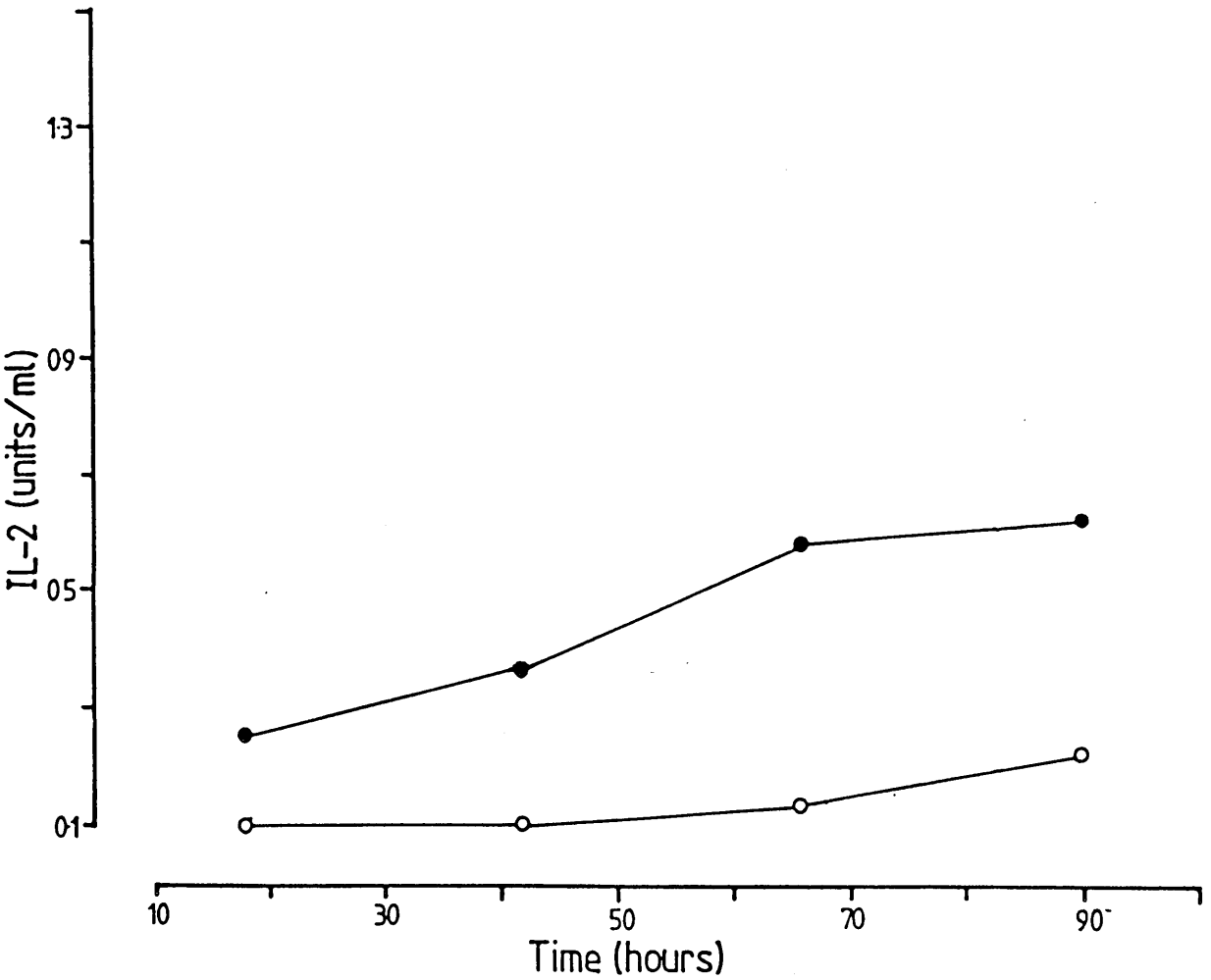


Figure 3.18 The effect of immunoadsorbed (● , PP14 concentration 1.0 ug/ml) and unadsorbed (O , PP14 concentration 8.0 ug/ml) decidual extracts on the release of IL-2 from PHA stimulated lymphocytes. Results are means of duplicate experiments which were concordant in their interpretation.

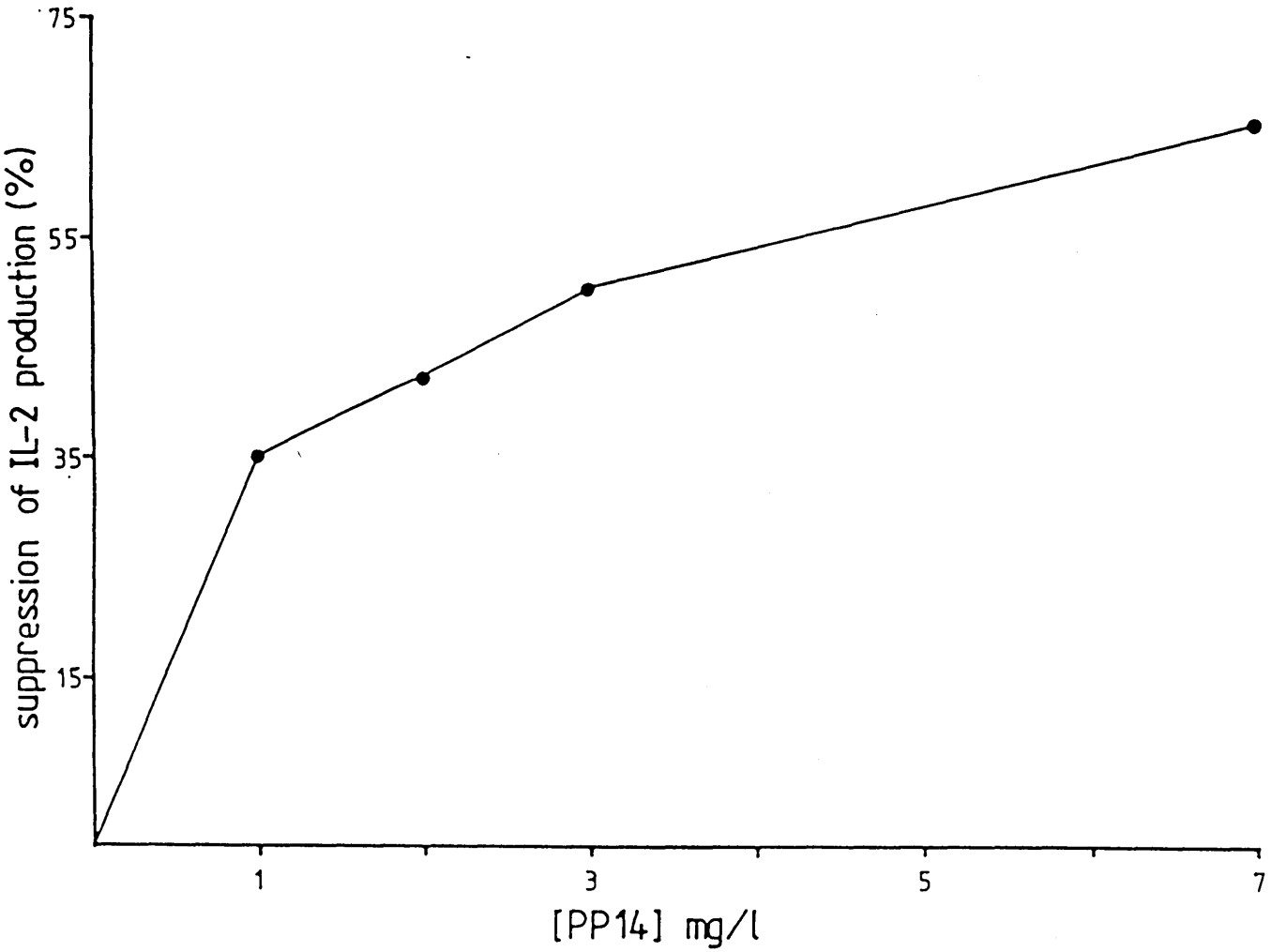


Figure 3.19 The effect of crude decidual extracts, related to the PP14 content, on the release of IL-2 from PHA stimulated lymphocytes. Results are means of duplicate experiments which were concordant in their interpretation.

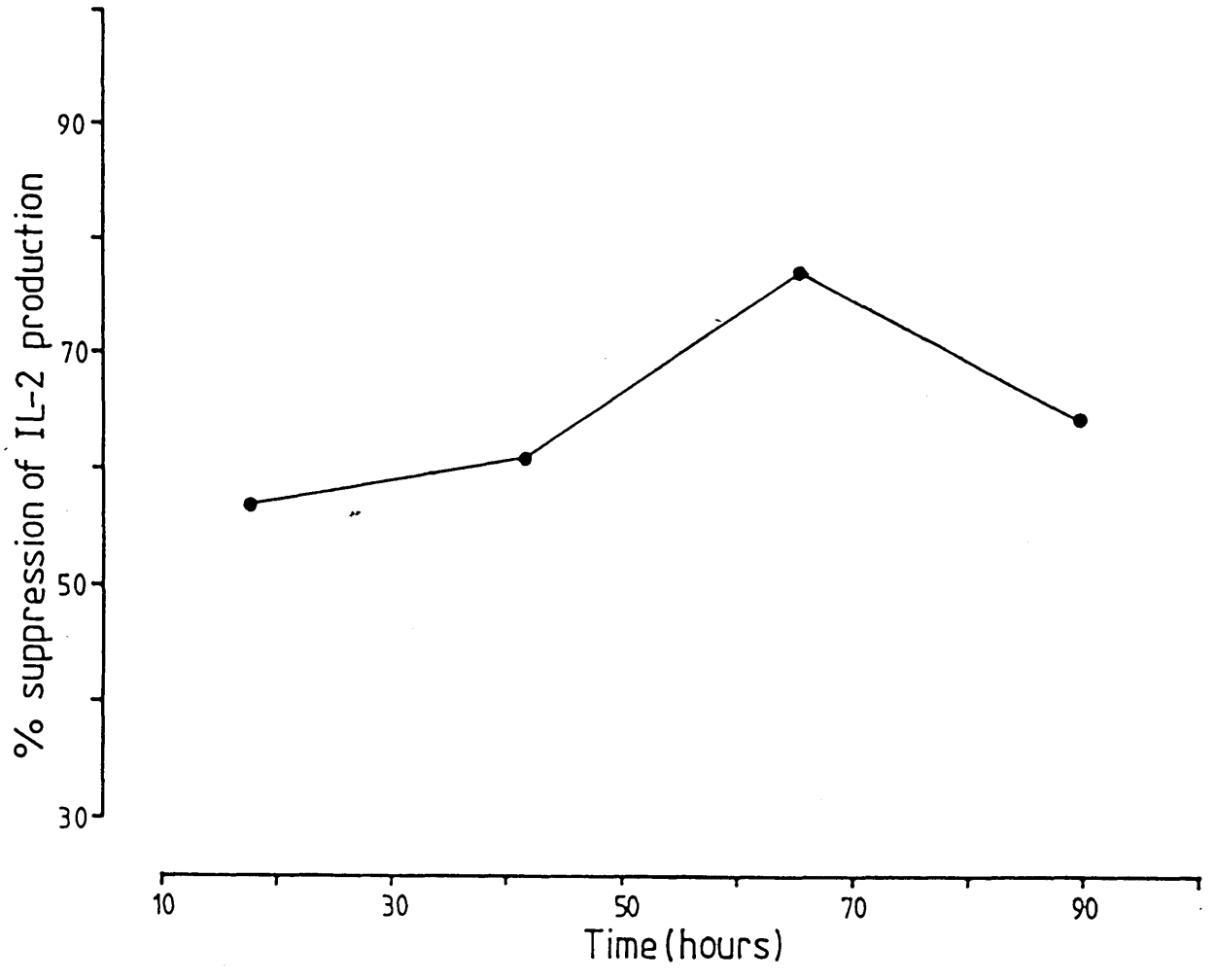


Figure 3.20 Time dependent inhibition of IL-2 release from PHA stimulated lymphocytes by unadsorbed decidual extracts (PP14 concentration 8.0 ug/ml) as compared to the control (immunoadsorbed decidual extract, PP14 concentration 1.0 ug/ml). Results are means of duplicate experiments which were concordant in their interpretation.

after 36 hours may allow such a threshold to be attained and may explain the observed lack of suppression following the delayed addition of decidual tissue extract.

3.16 The Effect of PP14 at the IL-2R Level of the Immune Response

Peak Tac antigen expression on PHA stimulated lymphocytes occured after 72 hours. The peak expression was preceded at 48 hours by the maximum mean channel of fluorescence (peak antigen density) (Fig 3.21). The number of cells expressing Tac antigen declined after 72 hours and reached a plateau towards the end of the culture period investigated (10 days). Tac antigen density decreased after 48 hours and continued to fall for the remainder of the culture period (Fig 3.21). Immunoadsorbed and unadsorbed decidual extract had no effect either on the number of cells expressing the Tac antigen (Fig 3.22) nor on Tac antigen density (Fig 3.23) over the culture period.

The observation of an apparent suppression of IL-2 levels, yet a normal expression of Tac antigen is not consistent with the observations of Diamantstein and Chen (1982), who postulated that the induction of IL-2R required two signals, one from the activating factor (antigen or mitogen) and a second from IL-2 itself. It may be that the suppression of IL-2 levels may not be sufficient to elicit control on IL-2R expression. Different signalling requirements exist for the induction of the IL-2R and IL-2 genes (Isakov and Altman 1985), with the genes controlling IL-2 and IL-2R expression having different intracellular [Ca^{2+}] requirements for their maximal expression (Chopra et al., 1987). In addition, protein kinase C

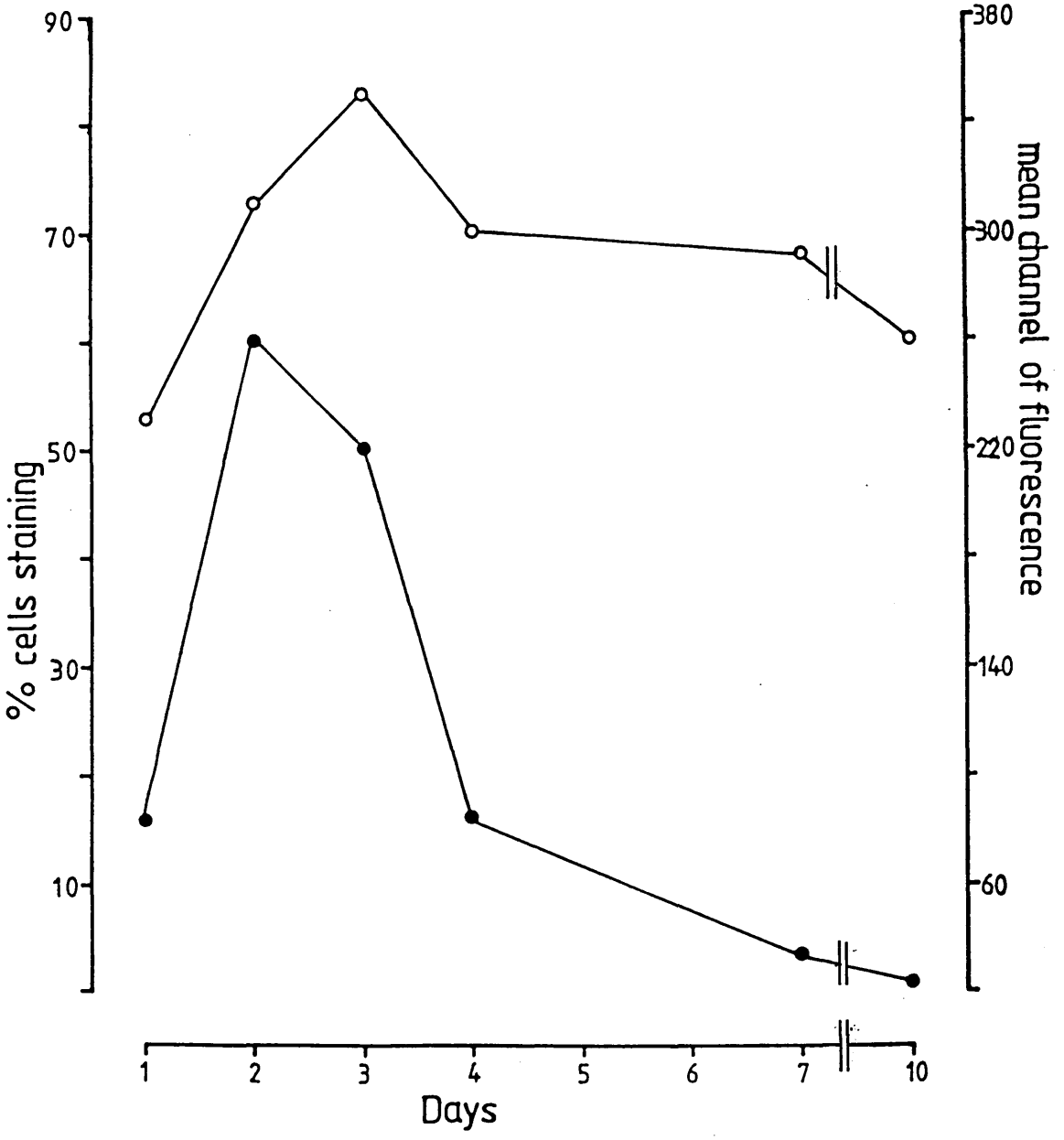


Figure 3.21 The kinetics of Tac antigen expression (O) and antigen density (●) on PHA stimulated lymphocytes. Data are summarised from control data presented in Figs 3.22 and 3.23.

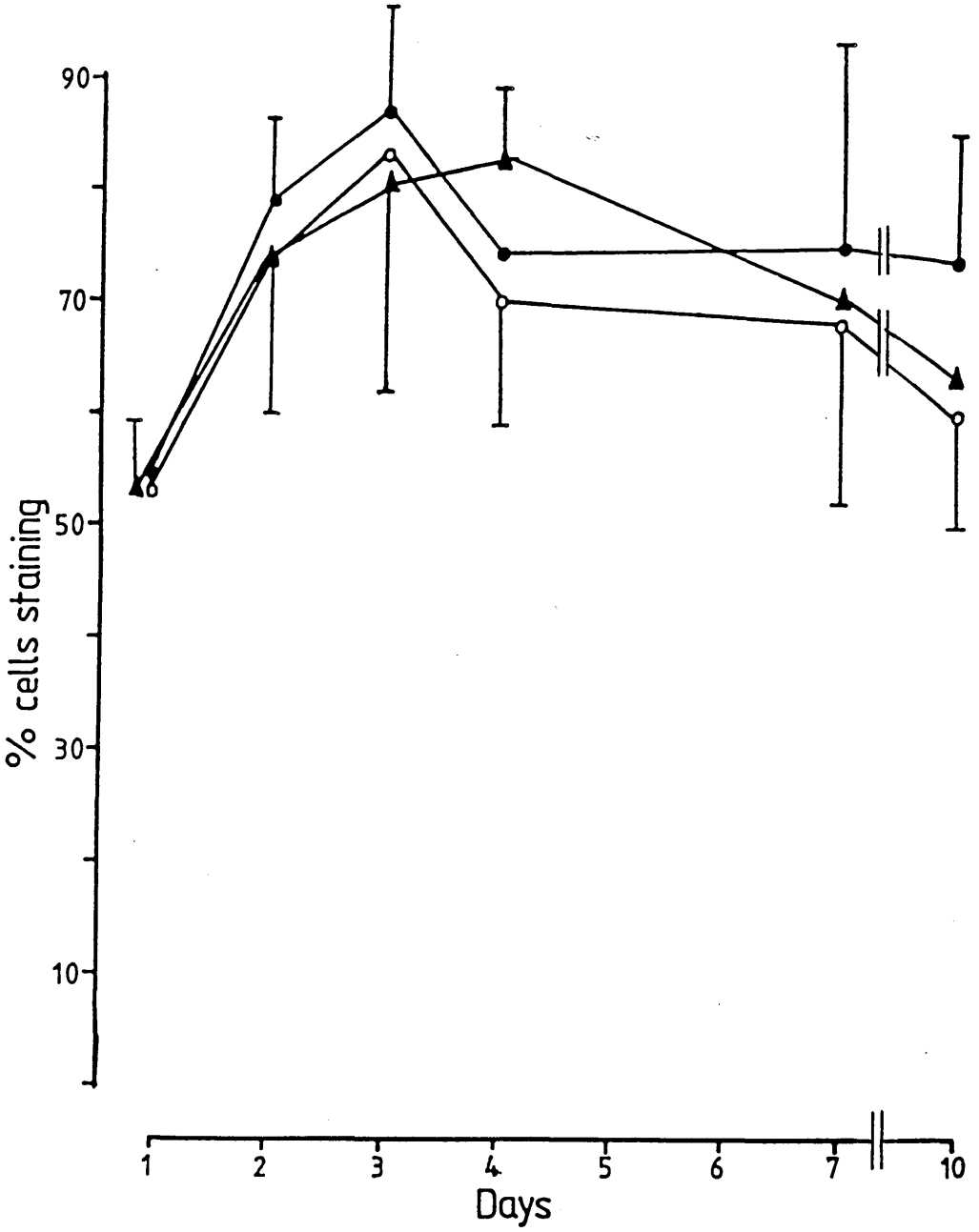


Figure 3.22 The effect of unadsorbed (▲, PP14 concentration 2.0 - 8.0 ug/ml) and immunoadsorbed (●, PP14 concentration 0 - 0.1 ug/ml) decidual extract on the expression of the Tac antigen on PHA stimulated lymphocytes as compared to the control (O). Results are expressed as means ± sd, n=5.

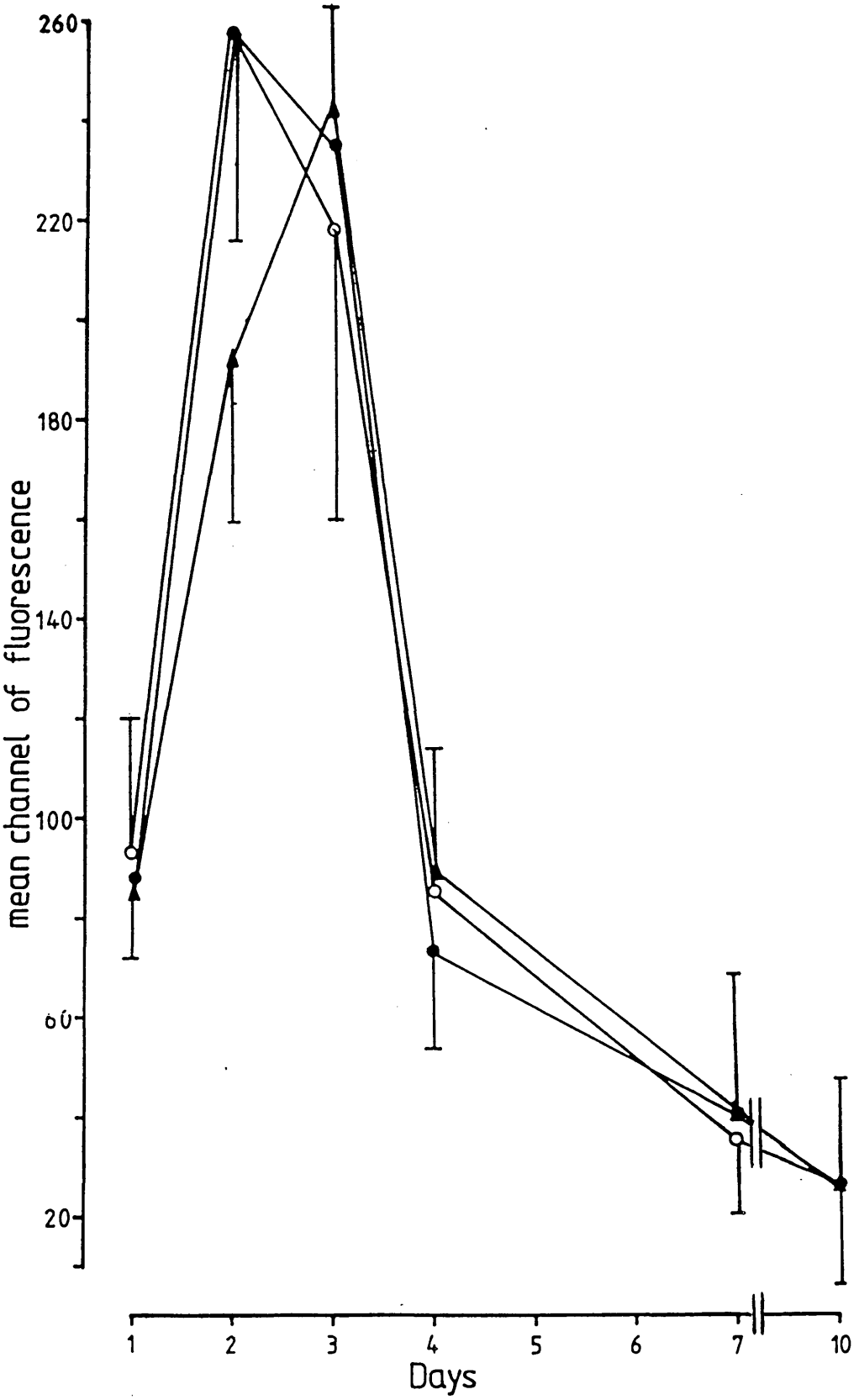


Figure 3.23 The effect of unadsorbed (▲, PP14 concentration 2.0 - 8.0 ug/ml) and immunoadsorbed (●, PP14 concentration 0 - 0.1 ug/ml) decidual extract on Tac antigen density on PHA stimulated lymphocytes as compared to the control (O). Results are expressed as means ± sd, n = 5.

activation is a sufficient signal for IL-2R expression, but not for IL-2 secretion. Such observations may explain the apparent paradoxical results obtained here.

A typical standard curve for soluble interleukin-2 receptor (sIL-2R) is shown in Fig 3.24. The detection limit of the interleukin-2 receptor test kit is approximately 50 U/ml IL-2R. Maximum levels of sIL-2R from PHA stimulated lymphocytes were observed after around 40 hours of culture, with the levels remaining constant for the remainder of the culture period. Peak sIL-2R levels preceded peak IL-2 levels by around 24 hours (Fig 3.25). Soluble IL-2R were not detectable in the culture supernatants of unstimulated cells at any time during the culture period.

There was a small, but significant ($P<0.001$, Student paired t test) inhibition of sIL-2R levels by unadsorbed decidual extract as compared to immunoadsorbed extract over the whole culture period, with the observed inhibition of sIL-2R levels decreasing towards the end of the culture period (Fig 3.26).

Whether this observation reflects the general decrease in proliferative activity caused by PP14 or whether it is a more specific marker of an effect at the IL-2R level of the response is uncertain. Certainly binding of IL-2 to the 75 kDa protein of the IL-2R on stimulated lymphocytes has been reported to mediate rapid internalisation of membrane bound IL-2 and the possible release of IL-2R into the culture supernatant (Robb and Greene 1987). Reduced sIL-2R levels may indicate a control at this level.

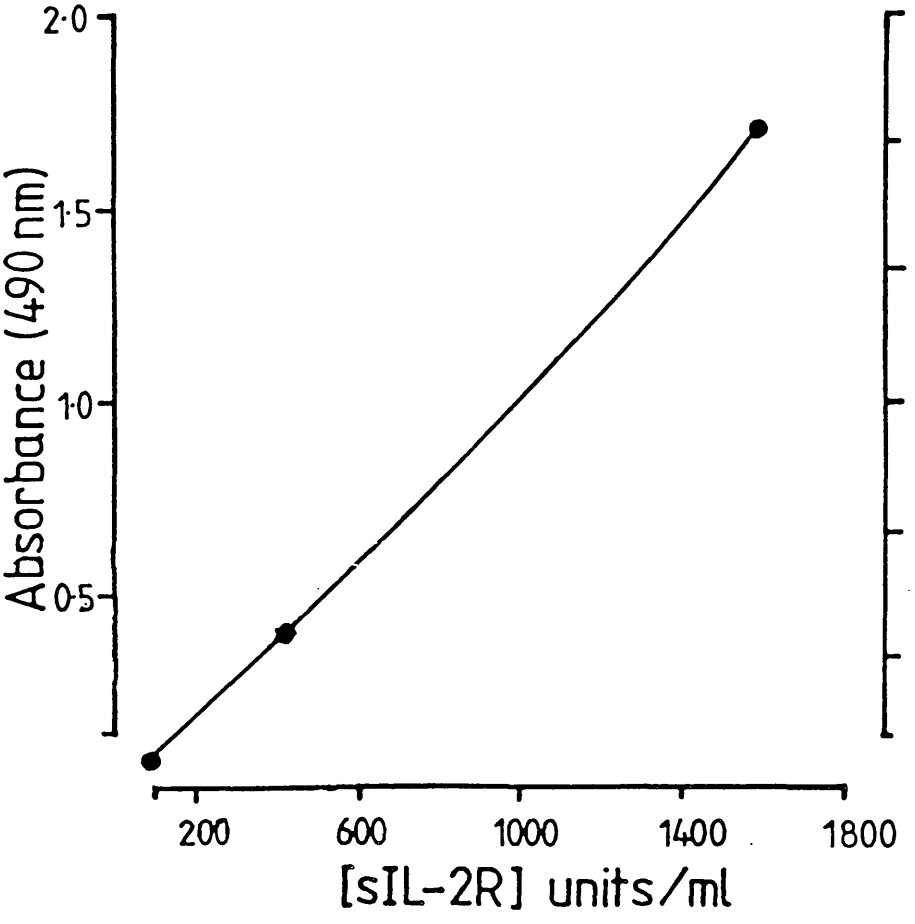


Figure 3.24 A typical standard curve for the determination of soluble IL-2 receptor (sIL-2R) levels.

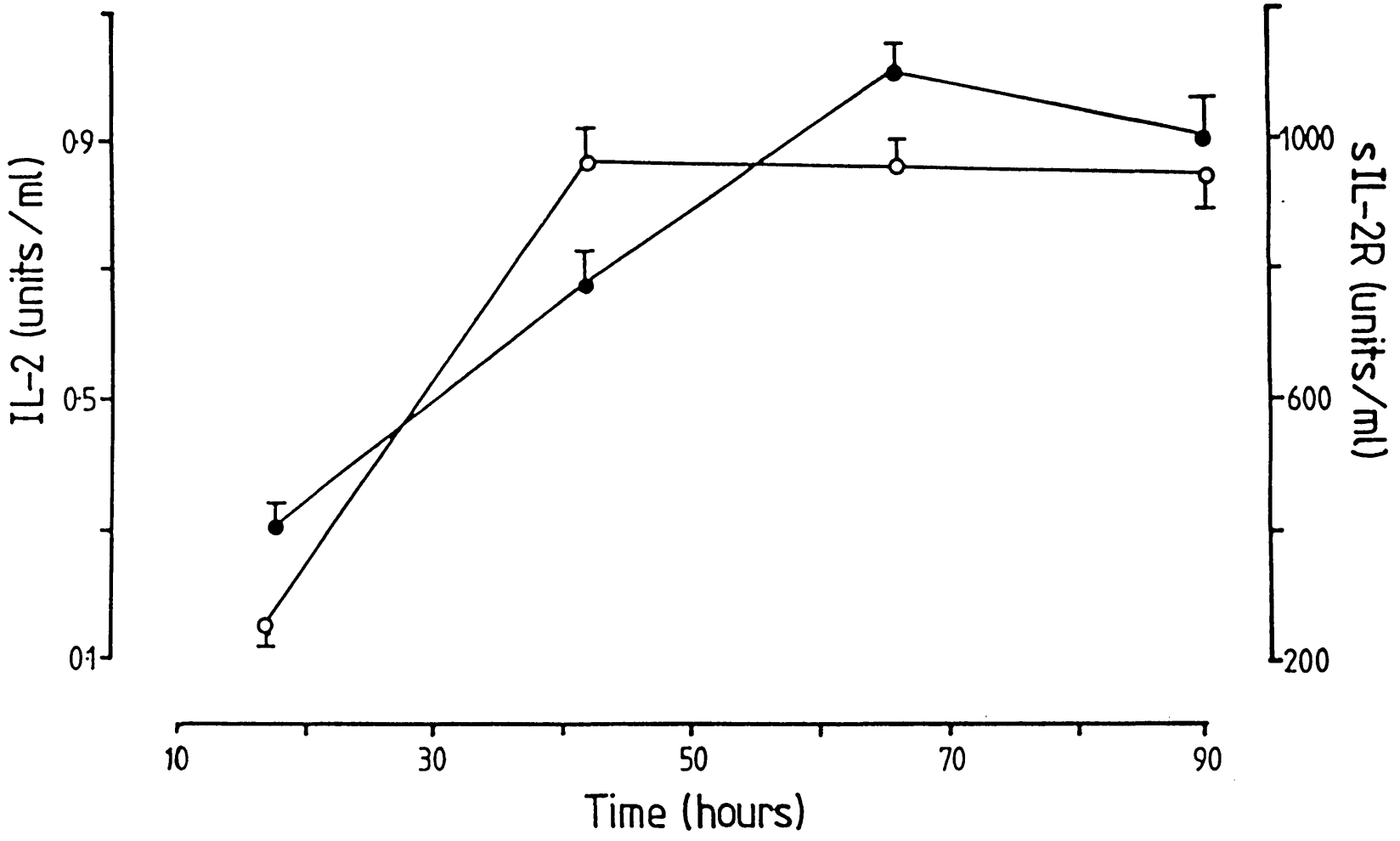


Figure 3.25 The kinetic analysis of the production of IL-2 (●) and the release of soluble IL-2R (O) from PHA stimulated lymphocytes. Results are expressed as means ± sd, n = 3.

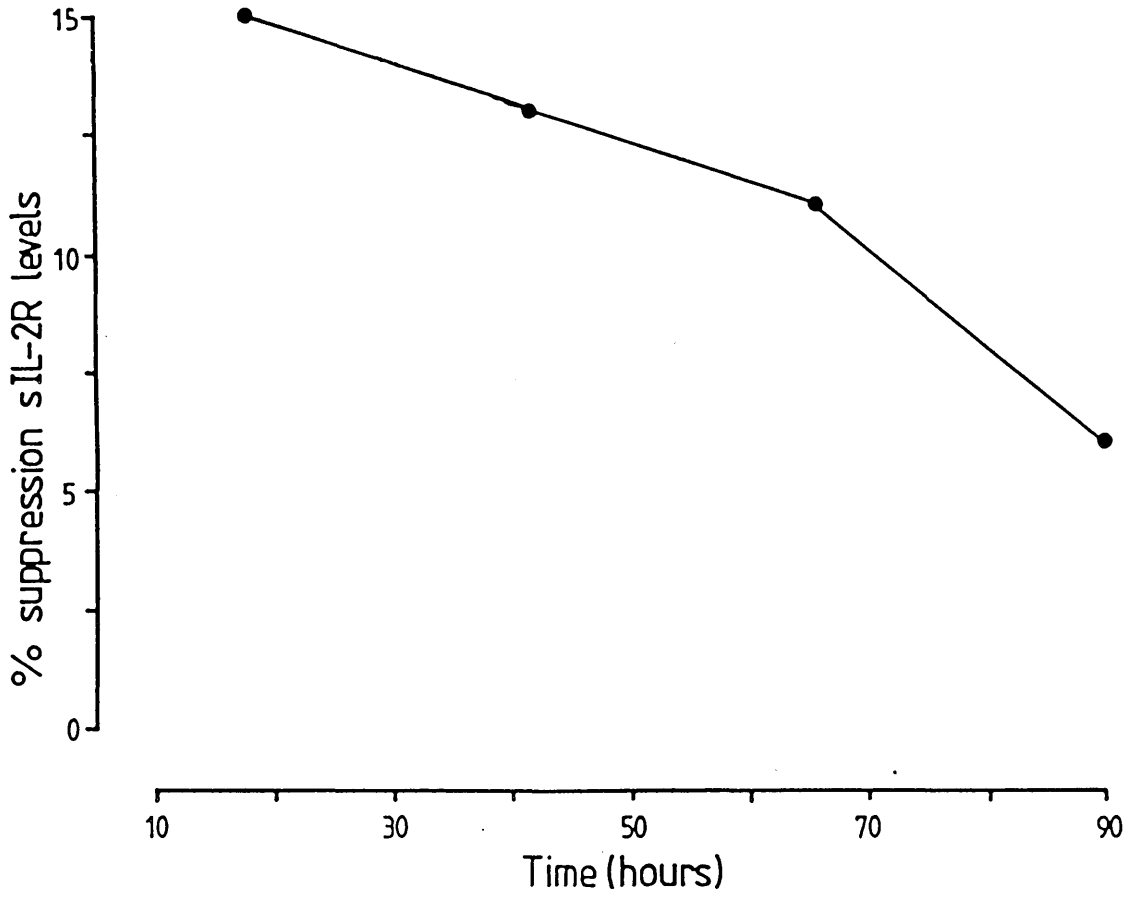


Figure 3.26 The suppression of soluble IL-2R levels by unadsorbed decidual extracts (PP14 concentration 8.0 ug/ml) as compared to the control (immunoadsorbed decidual extract, PP14 concentration 1.0 ug/ml). Results are means of duplicate experiments which were concordant in their interpretation.

3.17 Cell-surface Associated IL-2 and the Effect of PP14

PHA stimulated lymphocytes incubated in the presence/absence of decidual extract for 72 hours were analysed by flow cytometry for cell-surface associated IL-2 using anti-IL-2 monoclonal antibody. The presence of cell surface IL-2 possibly reflects the degree of internalisation of IL-2 once bound to its surface receptor. A low number (7.0%) of unstimulated cells were observed to be IL-2^+. Stimulation of cells by PHA increased the number of IL-2^+ cells to 13% (Table 3.13). Incubation of the stimulated cells with unadsorbed decidual extract (PP14 concentration 5.0 ug/ml) increased the number of IL-2^+ cells (32.0%, $P<0.001$, paired Student t test) as compared to both the stimulated control cells and cells incubated with immunoadsorbed decidual extract (20%; PP14 concentration 0.10 ug/ml, Table 3.13). There was no difference in cell surface associated IL-2 density observed between unstimulated, stimulated and decidual extract containing cultures (Table 3.13).

The increase in cell surface associated IL-2 in the presence of PP14 may confirm the data obtained on the assay of cell culture supernatants for sIL-2R. The lower number of IL-2^+ cells observed in stimulated control cultures as compared to PP14 containing cultures may reflect differing degrees of internalisation of IL-2 once bound to its cell surface receptor. The internalisation of IL-2 once bound to the Tac antigen of the IL-2 receptor is negligible (Robb and Smith 1987). These data suggest that PP14 may alter the relative numbers of the IL-2R 75 kDa and Tac antigen chains and influence the IL-2R affinity on PHA stimulated lymphocytes.

	Cell-Surface Associated IL-2	
	% Cells	Mean Channel
Unstimulated	7 ± 3.0	63 ± 12
Stimulated control	13 ± 3.0	62 ± 27
I/A DE	20 ± 13	74 ± 35
U/A DE	32 ± 13*	70 ± 42

* $P<0.001$ (paired Student t test)

Table 3.13 The effect of immunoadsorbed decidual extract (I/A DE, PP14 concentration 0.1 ug/ml), and unadsorbed decidual extract (U/A DE, PP14 concentrations 5.0 ug/ml) on the expression of cell-surface associated IL-2 on PHA stimulated lymphocytes. Results are means ± sd, n = 5.

3.18 The Effect of PP14 on High Affinity IL-2R Expression

Avidity is defined as the energy of binding of an antibody for its antigen and is denoted by K. K is numerically equal to the energy of binding of antigen for its antibody. The term avidity refers to the properties of an antibody, and affinity to those of the antigen. An antibody-antigen reaction is reversible and obeys the Law of Mass Action:-

$$Ag + Ab \rightleftharpoons AgAb$$

at equilibrium

$$K = \frac{k_1}{k_2} = \frac{[AgAb]}{[Ag][Ab]}$$

The avidity of an antiserum may be measured in a number of ways, but is usually determined by means of a Scatchard plot (Scatchard 1949). For the Scatchard plot, the above equation was rearranged:-

$$B = n - \frac{B}{F}.K^{-1}$$

where B = concentration of bound ligand or bound antibody

F = concentration of free ligand

n = total concentration of binding sites present

If the ratio of bound to free ligand is plotted against the concentration of bound ligand, the slope is -K which has the dimensions of litres/mole and reflects the affinity of the binding of the ligand to its receptor (Scatchard 1949). The intercept on the

abscissa gives the total number of binding sites present. The Scatchard plot obtained may consist of a single straight line indicating that the cells are expressing entirely high affinity receptors for IL-2. The Scatchard plot may be composed of two straight lines which intersect at an angle. The one with the steeper slope relates to the presence of higher avidity binding sites, whilst the one with the shallower slope represents binding sites of apparently lower avidity.

Scatchard analysis is able to determine the affinity of the cell surface receptors for the ligand, and also the concentration of the binding sites (receptors) present. Scatchard analysis of the equilibrium binding data for lymphocytes stimulated in the presence of unadsorbed and immunoadsorbed decidual extracts and control cultures are shown in Fig 3.27. The specific radioactivity of the ^{125}I-IL-2 (600 Ci/mMol) was used to convert the slopes of the lines into values of the dissociation constant (Kd). For all three cultures the Scatchard plots were composed of two straight lines indicating a mixed population of affinities of receptors, the data is summarised in Table 3.14. Incubation of lymphocytes in the presence of unadsorbed decidual tissue extract caused a reduction in the affinity of the higher affinity population of receptors from 1.4 x 10^{-14} M for the control (Fig 3.27 A) to 3.2 x 10^{-14} M for cultures containing unadsorbed decidual extract (Fig 3.27 C). Stimulated lymphocytes incubated with immunoadsorbed extract displayed IL-2 receptors with an affinity comparable to that of the control cultures (1.4 x 10^{-14} M, Fig 3.27 B). Conversion of the intercept of the abscissa into the total number of binding sites

	Dissociation constant (Kd) (x 10^{-14} M)		Binding sites (x 10^{-3} pM)
	Higher affinity receptors	Lower affinity receptors	Higher affinity receptors
Unstim	4.0	42.0	1.3
Stim control	1.4	28.0	5.4
I/A DE	1.4	22.0	8.0
U/A DE	3.2	6.0	10.0

Table 3.14 The effect of immunoadsorbed decidual extract (I/A DE, PP14 concentration 1.0 ug/ml) and unadsorbed decidual extract (U/A DE, PP14 concentration 18.0 ug/ml) on the affinity and the total concentration of binding sites present on PHA stimulated lymphocytes. Representative results from 3 analyses.

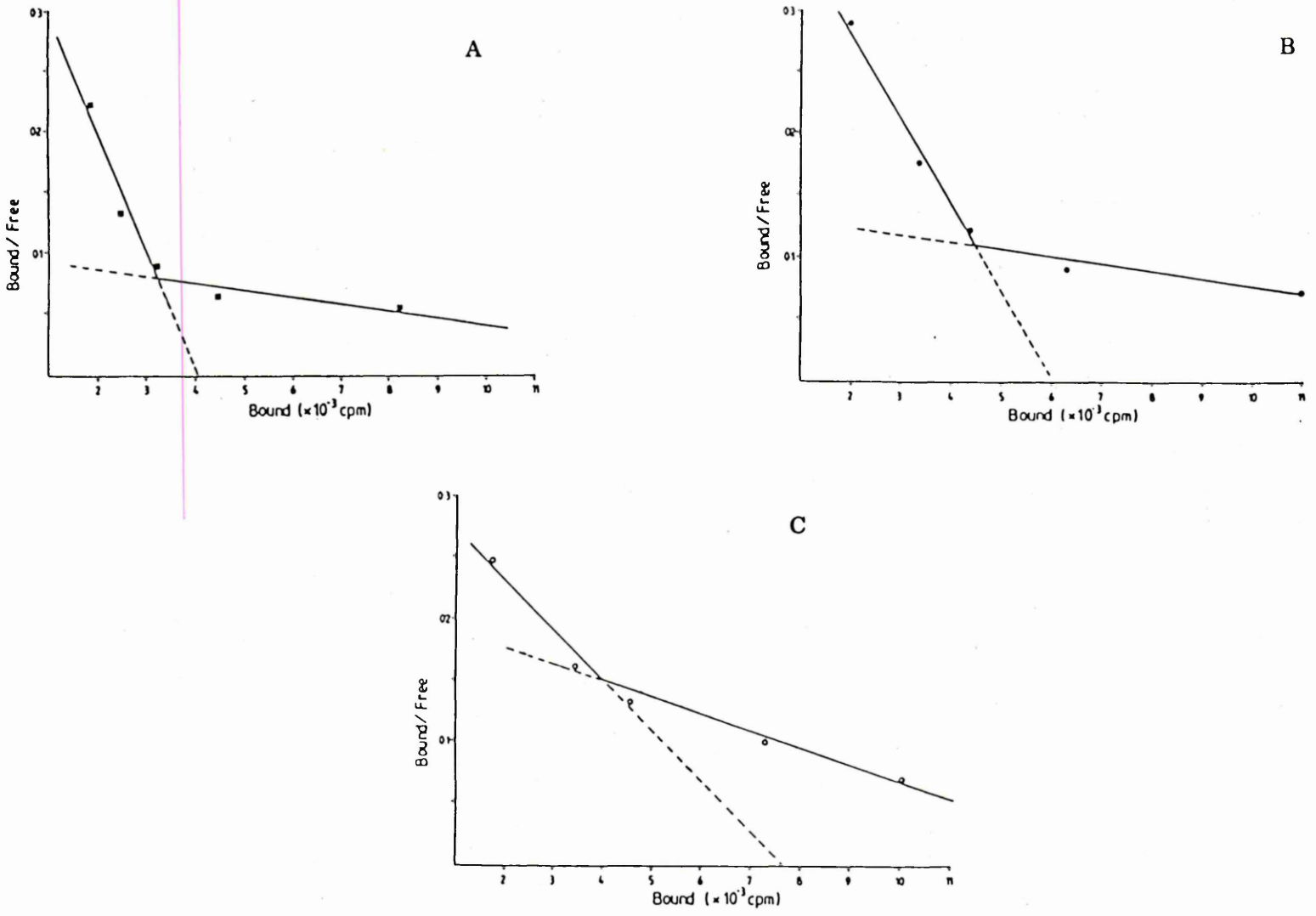


Figure 3.27 Representative data for Scatchard analysis of equilibrium binding data for lymphocytes pre-stimulated with PHA alone (A) and in the presence of immunoadsorbed (B, PP14 concentration 1.0 ug/ml) and unadsorbed (C, PP14 concentration 18.0 ug/ml) decidual extract, n = 3.

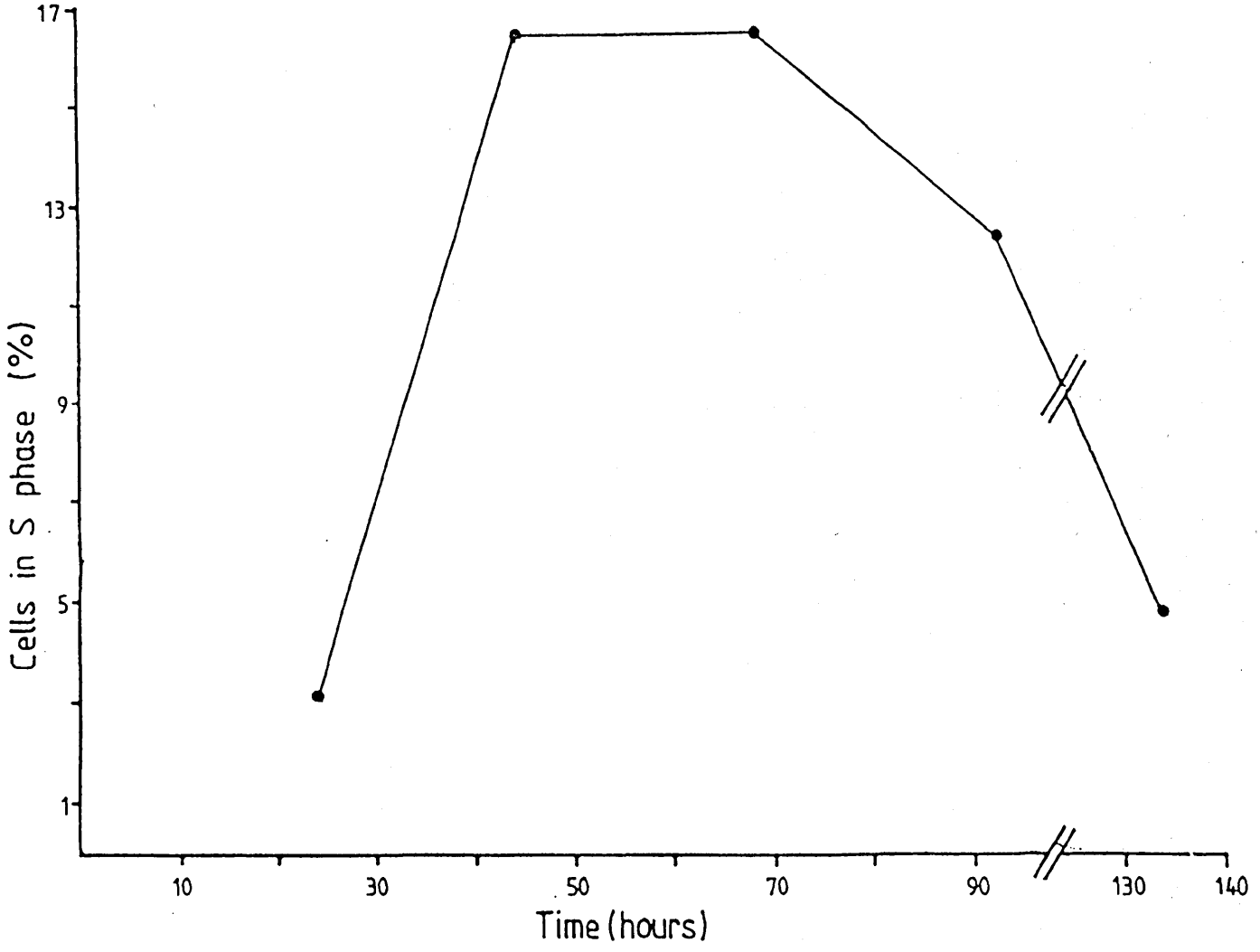


Figure 3.39 Kinetic analysis of the proportion of lymphocytes in the S phase of the cell cycle following stimulation with PHA. The percentage of cells in S phase was obtained by computer fit analyses of the DNA distribution curves.

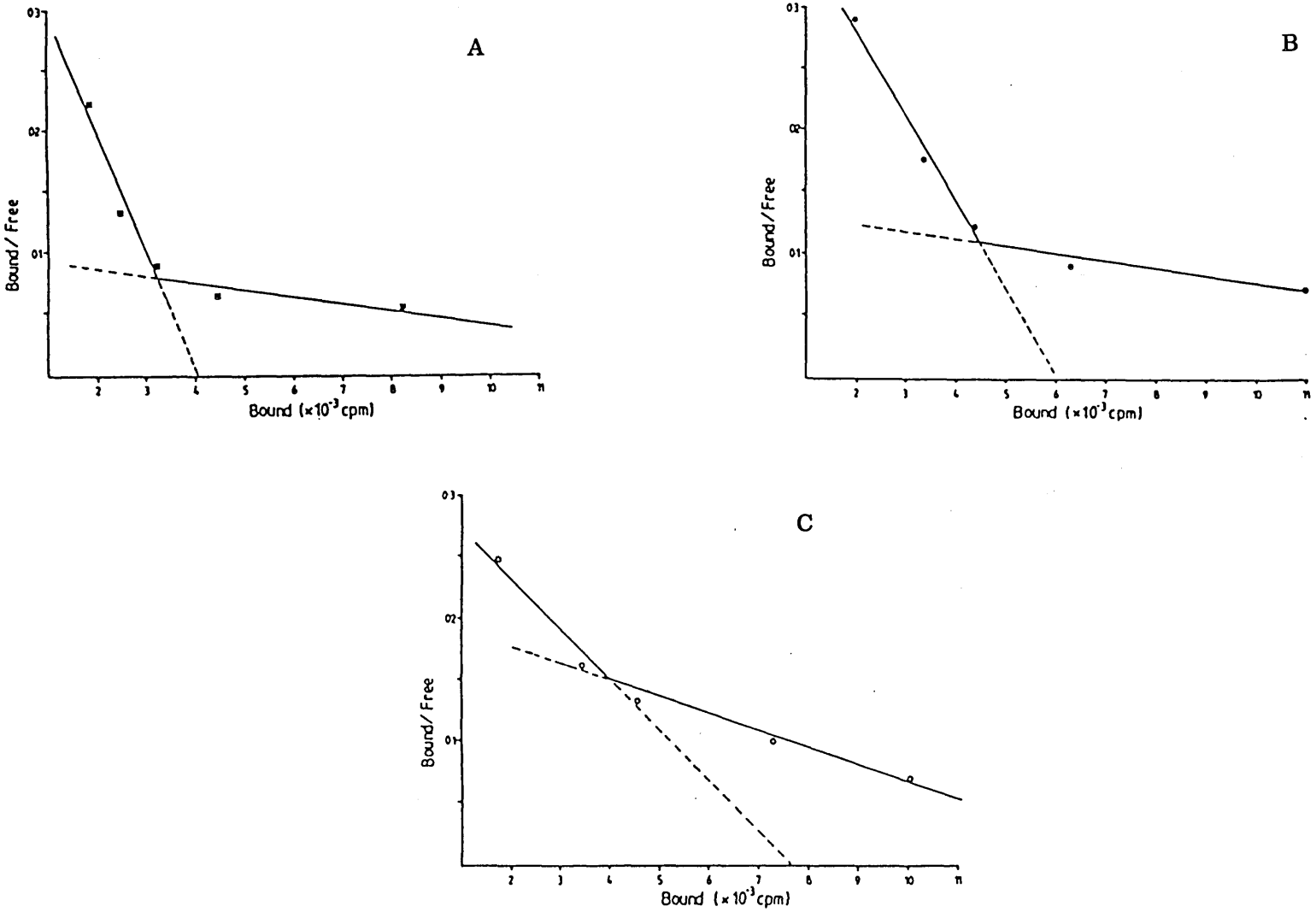


Figure 3.27 Representative data for Scatchard analysis of equilibrium binding data for lymphocytes pre-stimulated with PHA alone (A) and in the presence of immunoadsorbed (B, PP14 concentration 1.0 ug/ml) and unadsorbed (C, PP14 concentration 18.0 ug/ml) decidual extract, n = 3.

present indicated that there were a greater number of the higher affinity receptors on cells incubated with unadsorbed extract (10.0 x 10^{-3} pM, Fig 3.27 C) as compared to the control (5.4 x 10^{-3} pM, Fig 4.27 A) or cultures containing immunoadsorbed extract (8.0 x 10^{-3} pM, Fig 3.27 B). The affinity of the lower affinity receptors was greater on the cells incubated in the presence of unadsorbed decidual extract (6 x 10^{-14} M) as compared to the control (28 x 10^{-14} M) and cultures containing immunoadsorbed extract (22 x 10^{-14} M). An accurate determination of the number of the lower affinity receptors was not possible, however they formed the majority of the surface receptors detectable. Unstimulated cells also exhibited a dual population of receptors having affinities of 4.0 x 10^{-14} M and 42 x 10^{-14} M. The higher affinity receptor was expressed at 1.3 x 10^{-3} pM (Table 3.14).

Such observations indicate that PP14 may elicit a suppressive activity by a modulation of the affinity, and hence biological activity, of the IL-2R.

The affinities of the IL-2R reported here are somewhat higher than those reported by Teshigawara et al., (1987) who reported that the cell line designated YT exhibited high affinity IL-2R binding with a Kd of around 10^{-11} M. Robb et al., (1985) have reported the high affinity IL-2R on human PHA blasts to have a Kd of 4.1 x 10^{-12} M and IL-2R on the human cell line HUT 102B2 to have a Kd of 4.3 x 10^{-12} M.

The reasons for the differing Kd values obtained are unknown. One explanation could be the differing experimental conditions utilised. Alternatively, the difference may be due to the different

preparations of IL-2 used in the studies. Robb et al., (1985) used radioiodinated immunoaffinity purified JURKAT cell line derived IL-2 for the binding studies, whereas Teshigawara et al., (1987) used radioiodinated homogeneous recombinant IL-2. The ^{125}I-IL-2 used in this study was obtained as the ala-125 analogue from Amersham International. This analogue contains an extra methionine residue at the N-terminus of the natural sequence which has no reported effect on the biological activity of the molecule (specification sheet). In addition the cysteine at position 125 has been substituted for by alanine which has resulted in a five-fold increase in the specific activity and a significant improvement in the stability of the molecule. This observation is supported by data suggesting that the region of the IL-2 molecule close to positions 58 and 125 may be important for the functional activity of IL-2 as the deletion of cysteine 125 or of amino acids in the vicinity removes nearly all of the biological activity (Liang et al., 1986). It may be that the substitution of cysteine 125 for alanine alters the binding characteristics of the IL-2 to the IL-2 receptor.

3.19 The Effect of PP14 on the Expression of Functional IL-2 Receptors

The presence of functional IL-2R (i.e receptors capable of eliciting a proliferative response to IL-2) on PHA stimulated lymphocytes was studied by producing IL-2 dose-response curves. Lymphocytes were stimulated in the presence or absence of PP14 for 72 hours and their sensitivity to subsequent stimulation by IL-2 assessed by culturing the cells for a further 72 hours in the presence of different concentrations of IL-2.

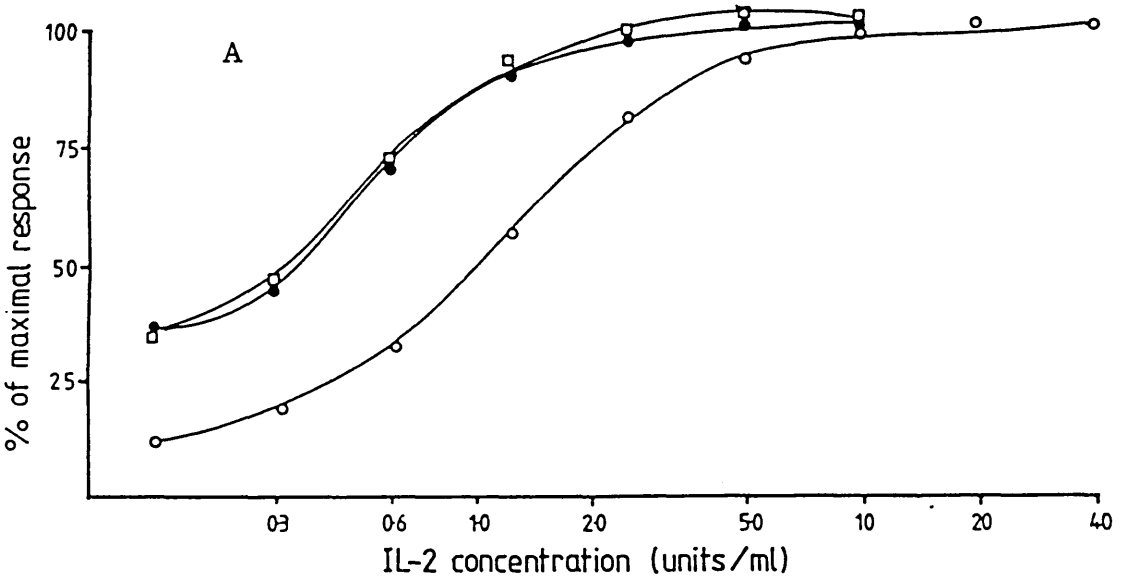
The dose-response curves for unstimulated, stimulated and test cultures were generated with different numbers of responding cells as detailed earlier (section 2.11). Representative results obtained in three independent analyses are displayed in Fig 3.28.

The cell numbers at which the dose-response curves ceased to shift (transition points: unstimulated cells 5 x 10^4 cells/well [Fig 3.28A]; stimulated control 1.25 x 10^4 cells/well [Fig 3.28B]; culture containing immunoadsorbed extract 1.25 x 10^4 cells/well [Fig 3.28D]; culture containing unadsorbed extract 2.4 x 10^4 cells/well [Fig 3.28C]) reflect the fact that all the activated cells expressed more high affinity receptors than did unstimulated cells. Cells incubated in the presence of unadsorbed decidual extract expressed a lower responsiveness as compared to the control with immunoadsorption of the extract removing such inhibitory activity.

The apparent difference in sensitivity to IL-2 between cells from control cultures and cells from unadsorbed decidual extract-containing cultures may reflect different affinities of the IL-2R present on the stimulated cells. These results confirm the earlier data obtained following Scatchard analysis and again suggest that PP14 may exert its immunoregulatory activity by affecting the affinity of the IL-2R present on stimulated lymphocytes.

3.20 The Effect of PP14 on gamma Interferon (gamma IFN) Production from PHA Stimulated Lymphocytes

A typical standard curve and error profile for gamma IFN determinations is shown in Fig 3.29. The minimum detection limit of the assay was reported in the manufacturer's specifications as less



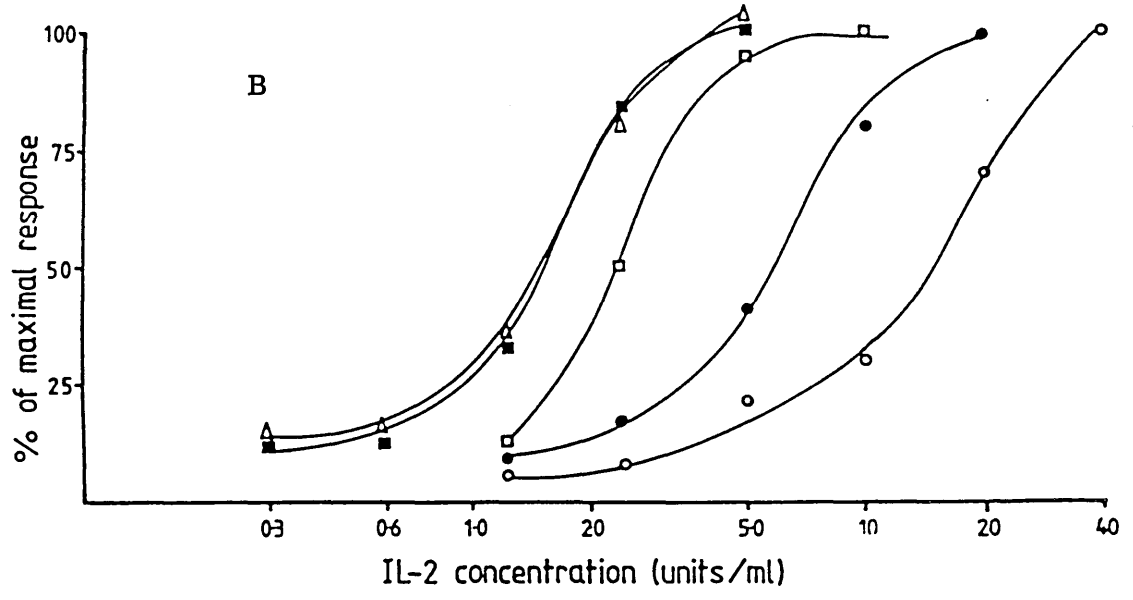
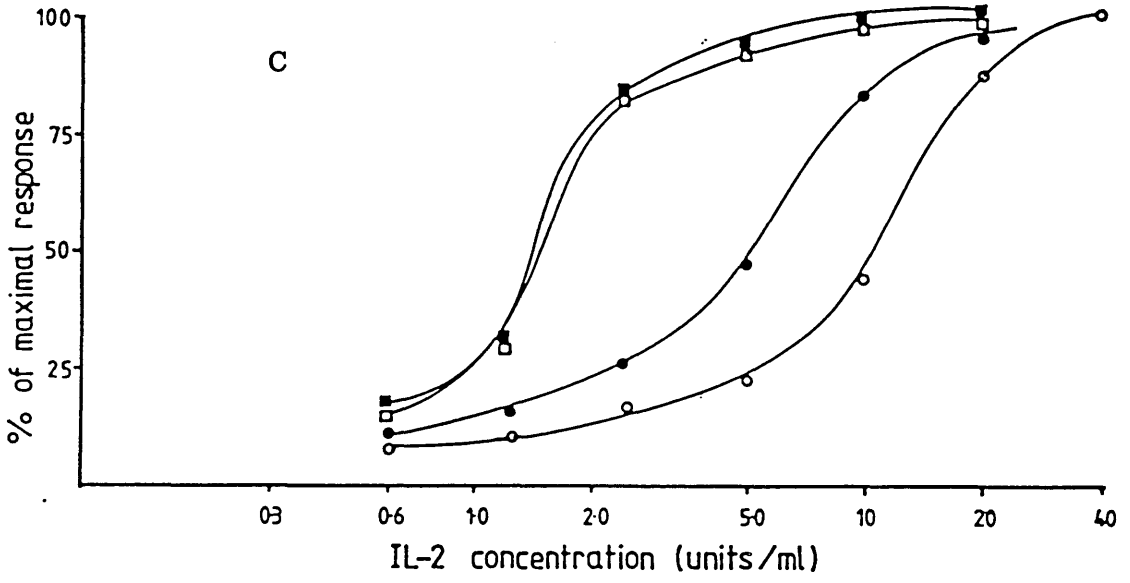


Figure 3.28 A,B Representative data for IL-2 dose-response curves for unstimulated (A) and PHA pre-stimulated (B) lymphocytes. The number of cells per well was 1 x 10^5 (O), 5 x 10^4 (●), 2.5 x 10^4 (□), 1.25 x 10^4 (■) and 0.625 x 10^4 (Δ), n = 3.



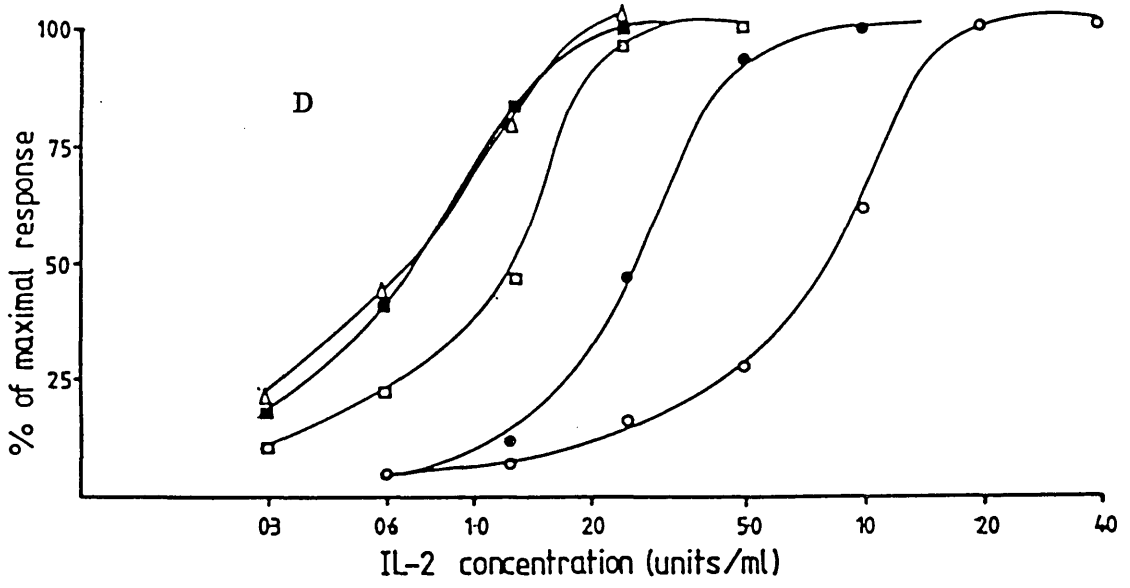


Figure 3.28 C,D Representative data for IL-2 dose-response curves for cells stimulated in the presence of unadsorbed (C, PP14 concentration 18.0 ug/ml) and immunoadsorbed (D, PP14 concentration 1.0 ug/ml) decidual extract. The number of cells per well was 1 x 10^5 (O), 5 x 10^4 (●), 2.5 x 10^4 (□), 1.25 x 10^4 (■) and 0.625 x 10^4 (Δ), n = 3.

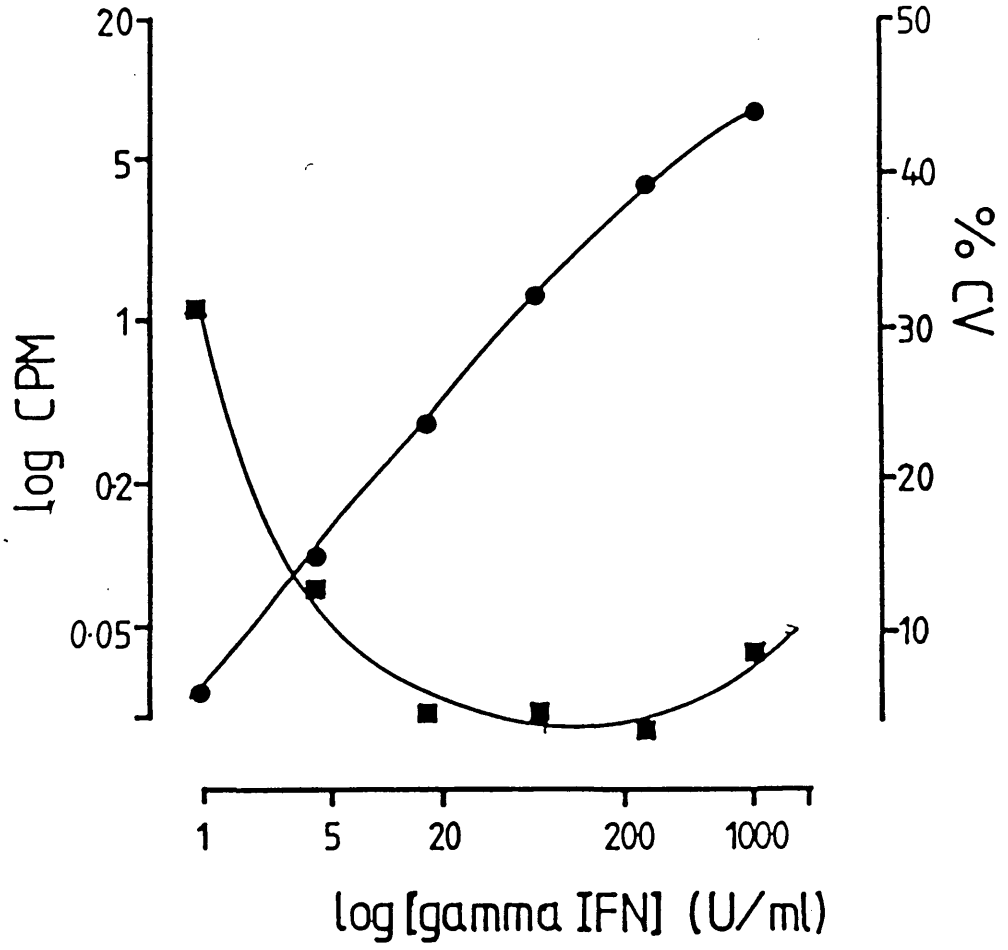


Figure 3.29 A typical standard curve (●) and error profile (■) for the IRMA of gamma IFN.

than 1.0 U/ml of gamma interferon. The minimum detection limit observed in this study was 0.5 U/ml. The working range of the assay was calculated to be 5.48 - 1024 U/ml. Detectable levels of gamma IFN (45 U/ml) were observed after 18 hours of PHA stimulation, with levels continuing to rise throughout the culture period investigated (0 - 90 hours, Fig 3.30). Gamma IFN was not detected in the supernatants of unstimulated cells at any time during the culture period investigated. Unadsorbed decidual extract inhibited gamma IFN production in the early stages of stimulation as compared to the immunoadsorbed extract control (0 - 42 hours), however no suppression was observed after 42 hours of stimulation (Fig 3.31).

IL-2 has been reported to cause the secretion of gamma interferon (Miedema et al., 1985). The observed suppression of IL-2 levels by PP14 are also partially reflected in an inhibition of gamma IFN level in culture supernatants of stimulated lymphocytes though the suppression of the gamma IFN levels are only observed at early stages of the culture period. This may be due to a threshold level of IL-2 being produced in the presence of PP14 , despite the fact that IL-2 levels are suppressed relative to the control. Alternatively there may be a pathway of IFN production which is distinct from an IL-2 dependency.

Exposure to IL-1 induces T cells to produce a number of lymphokines including gamma IFN and it may be that other factors produced during the mitogenic response may replace or substitute for IL-2 dependent gamma IFN production. Leukotrienes and cyclic GMP can substitute for IL-2 action to induce gamma IFN production (Johnson et al., 1985) and a number of laboratories have reported that various

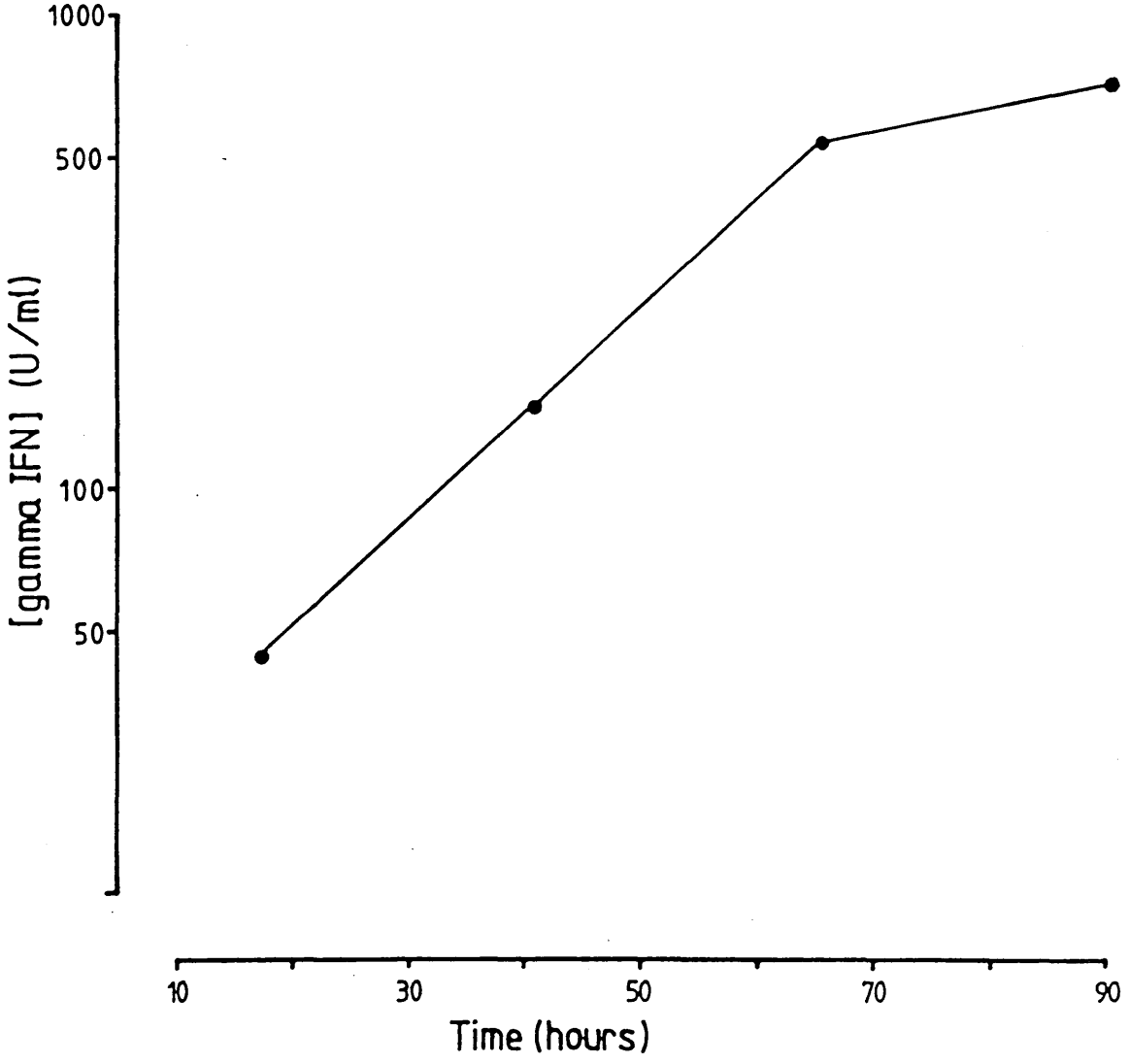


Figure 3.30 The kinetic analysis of the release of gamma IFN in the culture supernatants of PHA stimulated lymphocytes, results are means of duplicate experiments which were concordant in their interpretation.

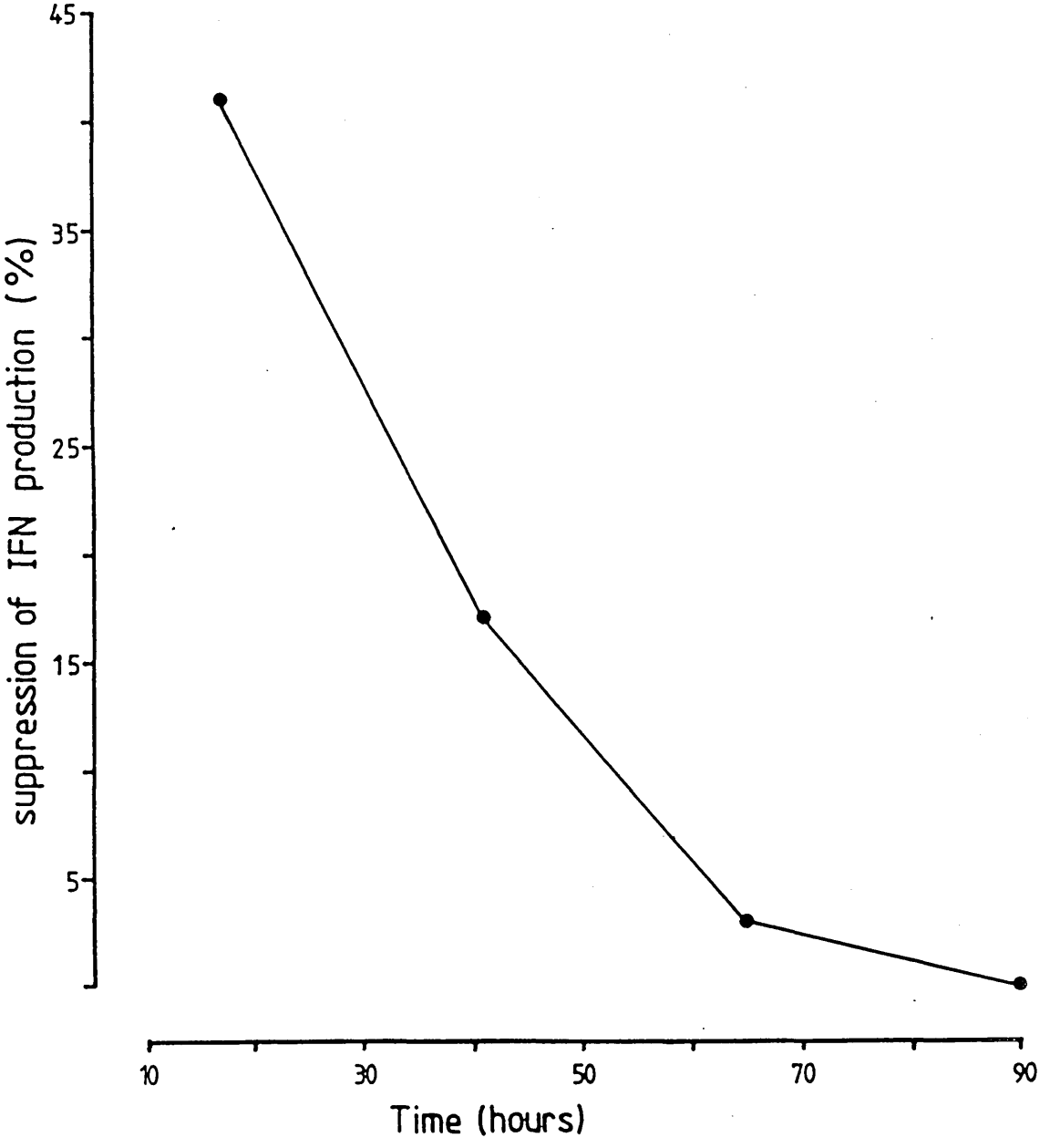


Figure 3.31 The suppression of gamma IFN production from PHA stimulated lymphocytes by unadsorbed decidual extract (PP14 concentration 8.0 ug/ml) as compared to the control (immunoadsorbed decidual extract, PP14 concentration 1.0 ug/ml), results are means of duplicate experiments which were concordant in their interpretation.

mitogens increase the levels of cyclic GMP in lymphocytes. PHA activates both membrane and soluble forms of lymphocyte guanylate cyclase from intact stimulated lymphocytes (Coffey et al., 1981) and it may be that this mechanism of gamma IFN induction leads to an apparently normal level of IFN in the presence of suppressed levels of IL-2.

3.21 The Effect of PP14 on Cell Surface Marker Expression.

The maximum number of cells expressing the transferrin receptor (TFR) on stimulation with PHA occurred after 3 days of incubation. The levels fell slightly after 4 days and maintained the reduced level for the period of culture investigated (Fig 3.32). The peak TFR density on the stimulated cells coincided with the peak number of cells expressing the antigen (3 days), with the levels falling towards the end of the culture period (Fig 3.32). Detectable levels of TFR occurred after 24 hours of culture which was the earliest time point investigated (Fig 3.32). The presence of unadsorbed or immunoadsorbed decidual extract had no effect on either the number of cells expressing the TFR (Fig 3.33) nor on the density of the receptor expression (Fig 3.34) as compared to the control.

Expression of the transferrin receptor (TFR), which is essential for the stimulated cells to enter the S phase of cell division, is unaffected by PP14. RNA production for the TFR has been shown to be increased by IL-2 which suggests a similar situation to that observed for the expression of the Tac antigen (section 3.16), where despite a lower level of IL-2 normal transferrin receptor expression is observed. An alternative mechanism for TFR expression may be present

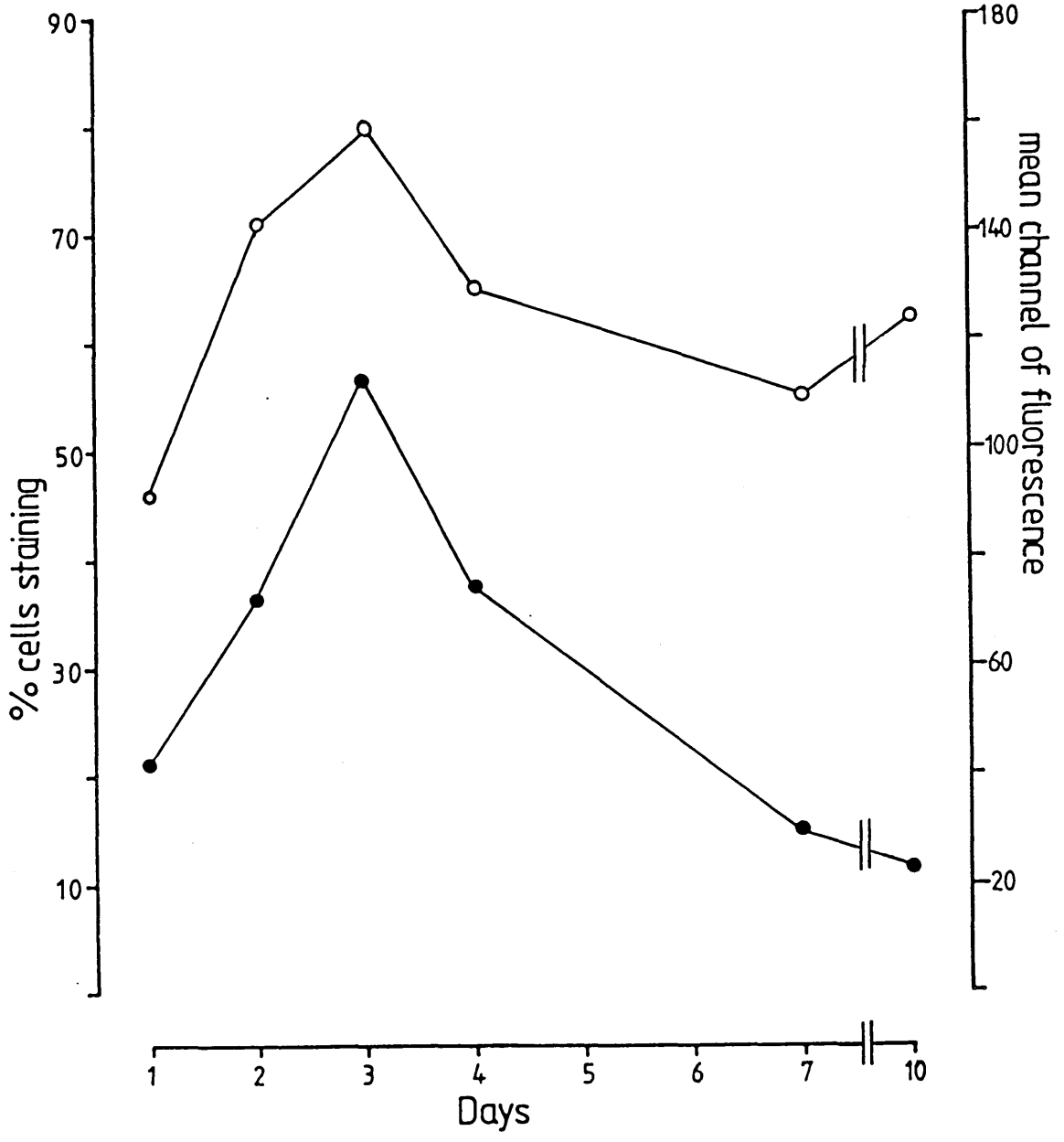


Figure 3.32 The kinetics of transferrin receptor (TFR) expression (O) and TFR density (●) on PHA stimulated lymphocytes. Data are summarised from control data presented in Figs 3.33 and 3.34.

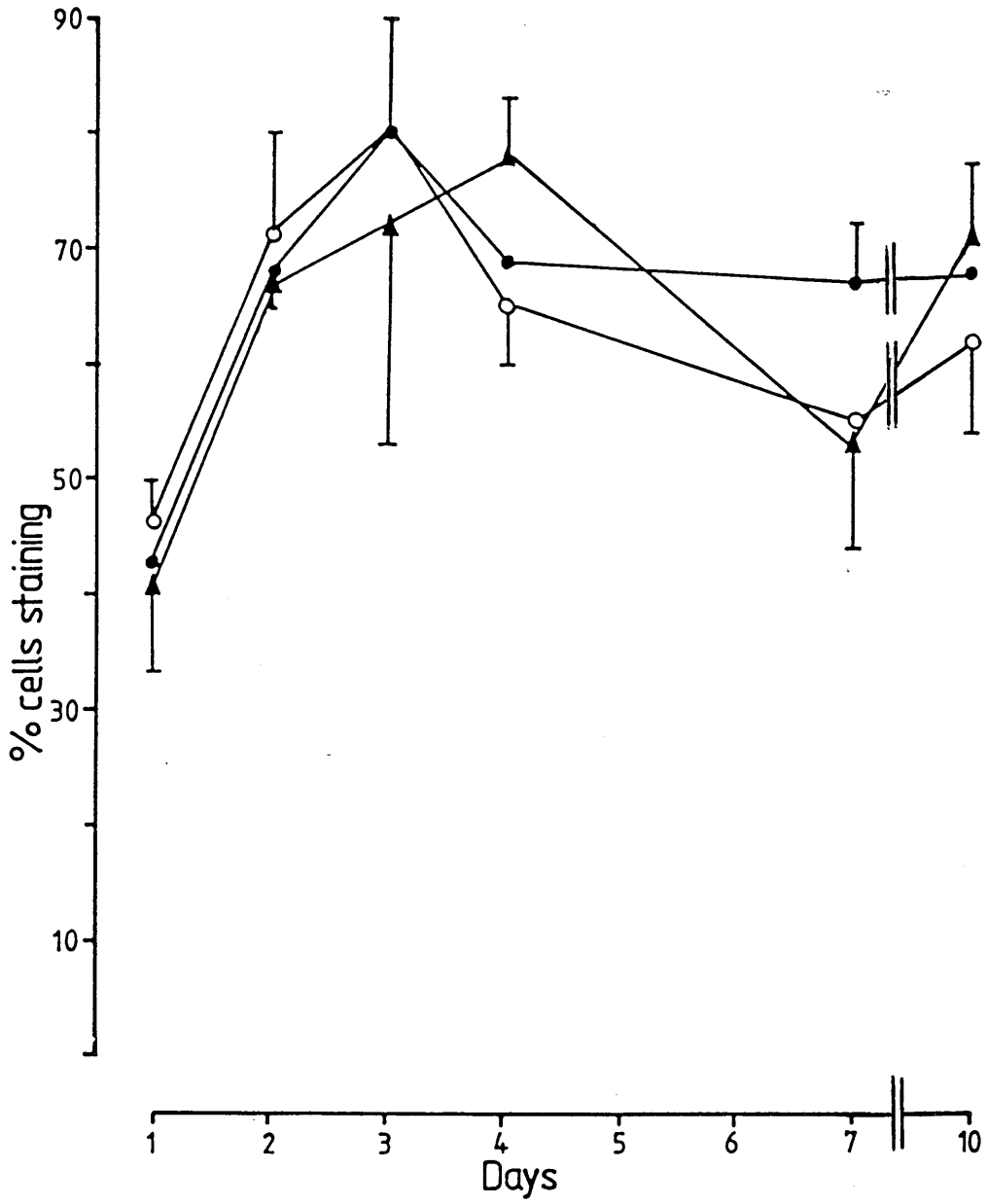


Figure 3.33 The effect of unadsorbed (▲, PP14 concentration 2.0 - 8.0 ug/ml) and immunoadsorbed (●, PP14 concentration 0 - 0.1 ug/ml) decidual extract on expression of the transferrin receptor on PHA stimulated lymphocytes, as compared to the control (O). Results are expressed as means ± sd, n=5.

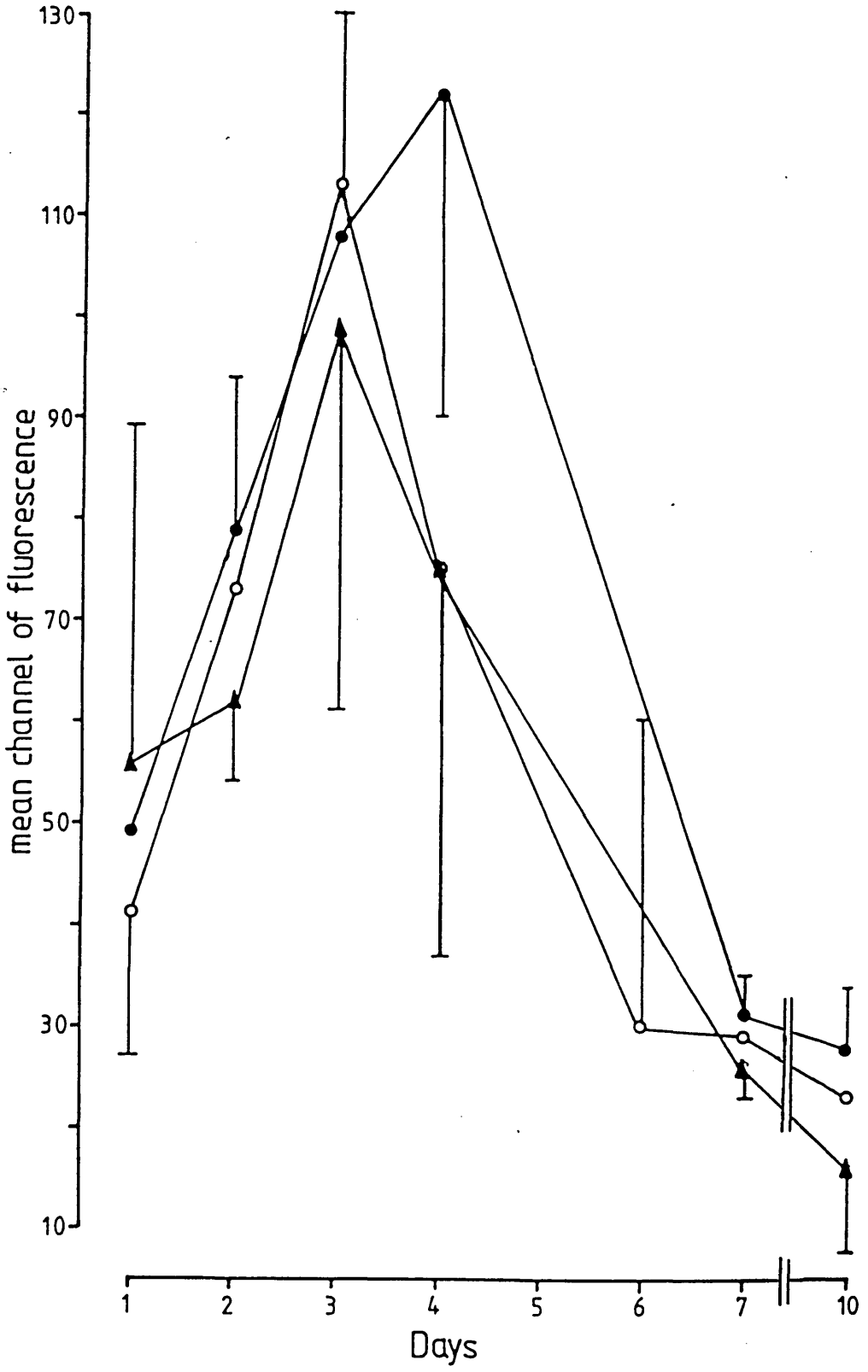


Figure 3.34 The effect of unadsorbed (▲, PP14 concentration 2.0 - 8.0 ug/ml) and immunoadsorbed (●, PP14 concentration 0 - 0.1 ug/ml) decidual extract on the transferrin receptor density on PHA stimulated lymphocytes, as compared to the control (○). Results are expressed as mean ± sd, n=5.

or possibly a threshold has been attained. The normal expression of the TFR has, in part, been confirmed by DNA cell cycle analysis which indicated no difference in cell cycle distribution in the presence of PP14 as compared to the control.

Detectable levels of HLA-DR antigen were observed after only 24 hours of culture with PHA with the number of HLA-DR$^+$ cells peaking initially at 48 hours. The levels declined thereafter and rose after 4 days and continued to rise towards the end of the culture period (Fig 3.35). There was a decrease in antigen density which coincided with the initial peak in the number of HLA-DR$^+$ cells. Antigen density peaked at 72 hours and declined thereafter towards the end of the culture period (Fig 3.35). The presence of unadsorbed or immunoadsorbed decidual extract had no effect on either the number of HLA-DR$^+$ cells (Fig 3.36) nor on HLA-DR antigen density (Fig 3.37) over the culture period investigated.

Gamma IFN has been shown to be implicated in the expression of HLA-DR antigens on cell surfaces (Nicholas et al., 1986). It is therefore somewhat surprising that PP14 had no apparent effect on the expression of HLA-DR on stimulated cells when the protein did appear to inhibit gamma IFN secretion during the early stages of the mitogenic response. It may be that the initial rise in HLA-DR expression is a direct result of PHA activation and independent of gamma IFN. Normal levels of IFN during the later stages of the culture would not be expected to affect HLA-DR expression.

The kinetic analysis of HLA-DR expression following mitogenic stimulation somewhat differs from that reported by Konttinen et al., (1986) where a maximum HLA-DR expression was observed after 1 day of

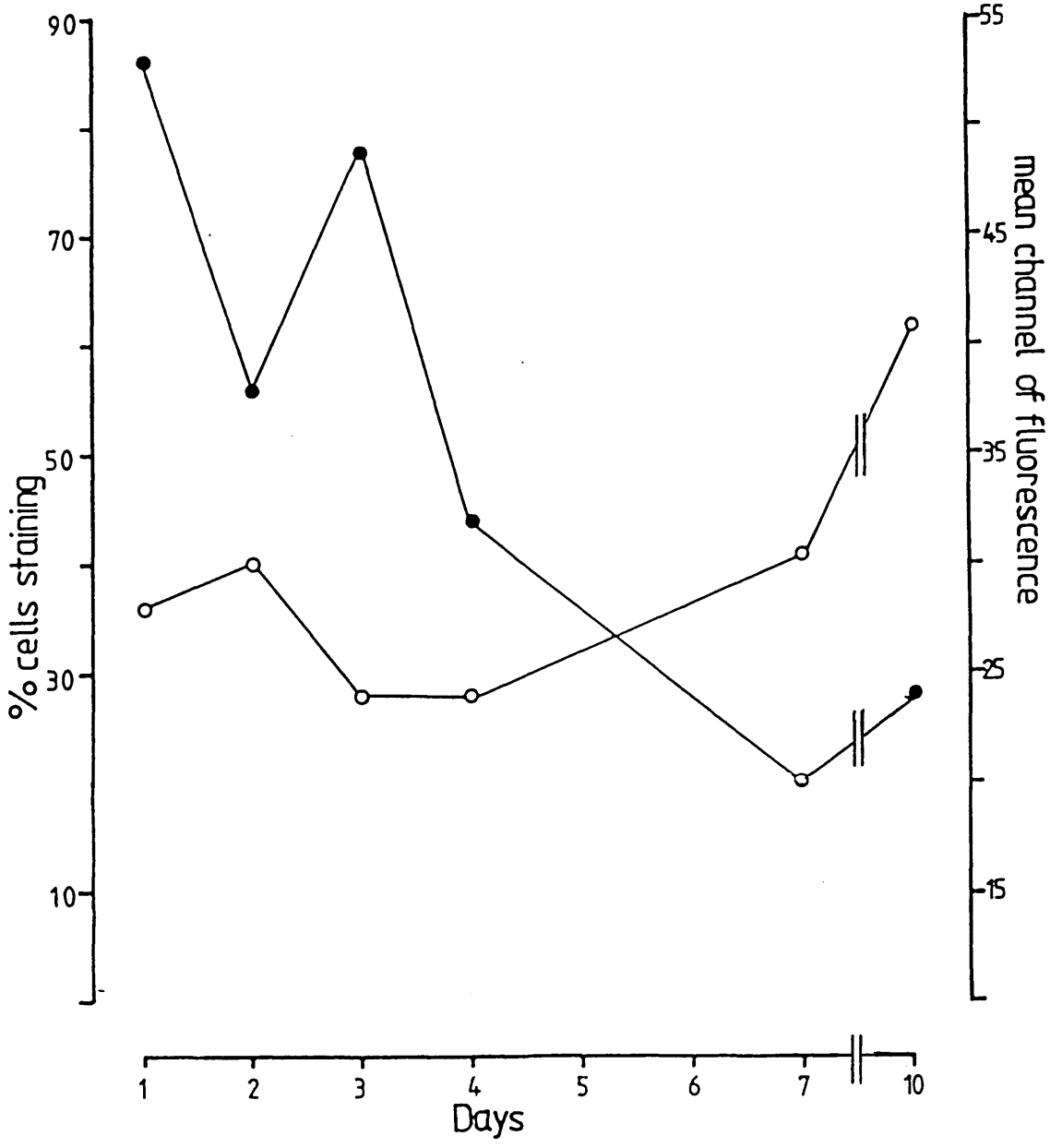


Figure 3.35 The kinetics of HLA-DR antigen expression (O) and antigen density (●) on PHA stimulated lymphocytes. Data are summarised from control data presented in Figs 3.36 and 3.37.

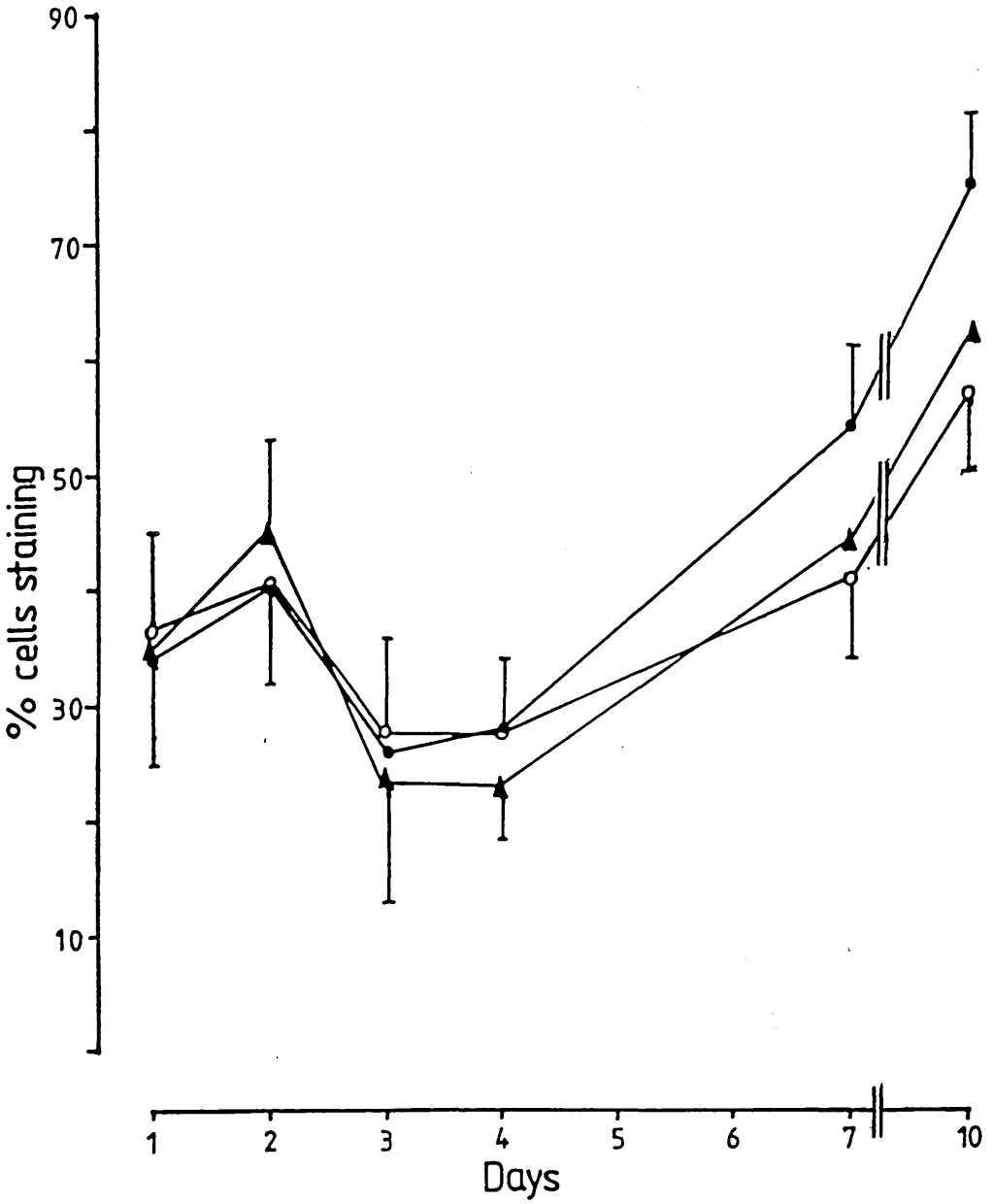


Figure 3.36 The effect of unadsorbed (▲, PP14 concentration 2.0 - 8.0 ug/ml) and immunoadsorbed (●, PP14 concentration 0 - 0.1 ug/ml) decidual extract on the expression of HLA-DR on PHA stimulated lymphocytes, as compared to the control (O). Results are expressed as means ± sd, n=5.

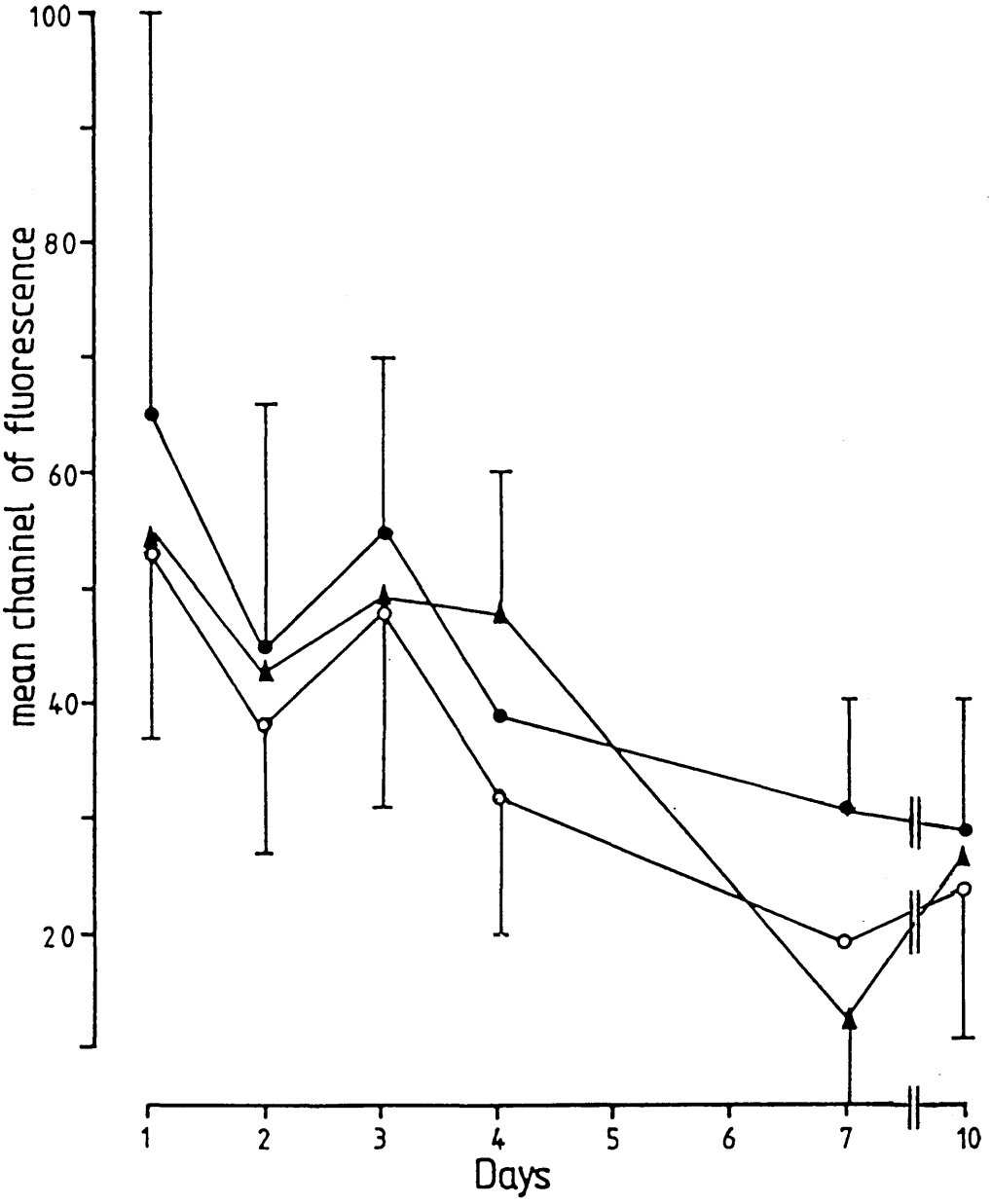


Figure 3.37 The effect of unadsorbed (▲, PP14 concentration 2.0 - 8.0 ug/ml) and immunoadsorbed (●, PP14 concentration 0 - 0.1 ug/ml) decidual extract on HLA-DR antigen density on PHA stimulated lymphocytes, as compared to the control (O). Results are expressed as means ± sd, n=5.

culture with the expression becoming insignificantly different from unstimulated control expression after 3 days of culture. In that study the culture period investigated was 5 days which, from the results obtained here, would not detect the secondary rise in HLA-DR observed after 4 - 5 days.

3.22 Cell-surface Associated PP14

PHA stimulated lymphocytes incubated in the presence of decidual extract were analysed by flow cytometry for cell-surface associated PP14 using monoclonal anti-PP14 antibody. After 72 hours of culture unstimulated control cells incubated in the absence of PP14 showed low levels of anti-PP14 immunoreactivity (2.5% of the cells staining) and also a low antigen density as determined by the mean channel of fluorescence (Table 3.15). Stimulated control cells showed an elevated level of reactivity to the monoclonal antibody (10% cells staining), however the staining was again of low intensity. The apparent increase in binding to stimulated cells could be accounted for, in part, by an observed increase in the non-specific binding of the FITC-conjugate. There was no observed difference in either the number or intensity of $PP14^{+}$ cells for cultures stimulated in either the absence of decidual extract or in the presence of unadsorbed (PP14 concentrations 2.0 - 8.0 ug/ml) or immunoadsorbed decidual tissue extracts (Table 3.15). This observation indicates that PP14 does not appear to exert its modulatory activity by binding to the surface of proliferating lymphocytes. These results do not exclude the possibility that the binding of PP14 to lymphocytes may mask the epitope recognised by the monoclonal antibody and thus give a false negative result.

	Cell-Surface Associated PP14	
	% Cells	Mean Channel
Unstimulated	3 ± 4.0	6.0 ± 7.0
Stimulated control	10 ± 5.0	21 ± 2.0
I/A DE	10 ± 2.0	23 ± 14
U/A DE	11 ± 6.0	32 ± 15

Table 3.15 The effect of immunoadsorbed decidual extract (I/A DE, PP14 concentration 0.10 ug/ml) and unadsorbed decidual extract (U/A DE, PP14 concentration 5.0 ug/ml) on the expression of cell-surface associated PP14 on PHA stimulated lymphocytes. Results are means ± sd, n = 5.

3.23 The Effect of PP14 on CD2 (T11) Antigen Expression

It has been reported that the blocking of the sheep red blood cell receptor (CD2, T11) on T lymphocytes abolished PHA stimulated Ca^{2+} fluxes (O'Flynn et al., 1985). Such an observation suggests that the mitogenic activity of PHA may in part be mediated via its binding to the CD2 molecule. Any molecule which binds to, or masks, such a receptor may modulate the cellular response to PHA. To investigate the possibility that PP14 may mask the CD2 molecule, cultured lymphocytes were analysed by flow cytometry using monoclonal anti CD2 antibodies.

Following 72 hours of mitogenic stimulation 98% of the cells were observed to be $CD2^+$ as compared to 89% of the unstimulated cells (Table 3.16). Antigen density also increased on stimulation with unstimulated cells having a mean channel of fluorescence of 157 as compared to 700 for stimulated cells. Incubation of stimulated cells with decidual extracts (both immunoadsorbed and unadsorbed) had no effect on the number of $CD2^+$ cells (Table 3.16). This makes unlikely the possibility that PP14 exerts its modulatory activity by binding and masking the CD2 receptor for PHA.

This observation was partially supported by the inability to demonstrate cell surface associated PP14 subsequent to incubation with decidual extract. However as stated earlier, binding of PP14 to the cell surface may mask the epitope recognised by the monoclonal antibody. Further investigations using monoclonal antibodies of differing specificities are required to confirm these data. Binding of PP14 to the CD2 antigen would not be expected to elicit a suppressive effect on the proliferative response to allogeneic stimulation.

CD2 Antigen Expression

	% Cells	Mean Channel
Unstimulated	89 ± 0.6	157 ± 31
Stimulated control	98 ± 3.0	700 ± 338
I/A DE	98 ± 4.0	650 ± 574
U/A DE	96 ± 6.0	656 ± 204

Table 3.16 The effect of immunoadsorbed decidual extract (I/A DE, PP14 concentration 0 - 0.10 ug/ml) and unadsorbed decidual extract (U/A DE, PP14 concentration 2.0 - 8.0 ug/ml) on the expression of the CD2 antigen on PHA stimulated lymphocytes. Results are means ± sd, n = 5.

3.24 The Influence of PP14 on T Cell Subset Distribution

Flow cytometry was used to assess the effect of PP14 on T cell subset distribution. Stimulation of lymphocytes with PHA caused a slight increase in $CD4^+$ and $CD8^+$ cell numbers whilst having no effect on antigen density (Table 3.17). Incubation of cells with both immunoadsorbed and unadsorbed decidual extract had no significant effect on either $CD4^+$ and $CD8^+$ cell numbers nor on CD4 and CD8 antigen density (Table 3.17). From Table 3.17 the total number of cells expressing the CD4 and CD8 antigens following mitogenic stimulation by PHA appeared to be over 100%, suggesting that the antibody used may cross react with other antigenic determinants on the cells present. The Leu 3a antibody used in this study has been shown to cross react with monocytes (J. Lawry, personal communication) which may explain the results obtained. An alternative possibility is that the stimulation of peripheral blood lymphocytes by PHA causes the cells to de-differentiate leading to co-expression of the CD4 and CD8 antigens. Such co-expression has been observed on actively proliferating leukaemic cells (J. Lawry, personal communication). Further work using double staining and double fluorescence studies would clarify the situation. The CD4/CD8 ratio of unstimulated cells (1.10, Table 3.18) was comparable to the quoted normal range of 1.65 ± 0.5 (Macey 1988). Stimulation of the cells by PHA reduced the ratio indicating a shift in the population distribution towards the $CD8^+$ subset (cytotoxic/suppressor). Incorporation of decidual extract into the cultures had no effect on the CD4/CD8 ratio nor on the ratio of the antigen density (Table 3.18). The increase in the relative proportions of lymphocytes

CD4 and CD8 Antigen Expression

	% Cells		Mean Channel.	
	CD4	CD8	CD4	CD8
Unstimulated	44 ± 2.0	40 ± 2.0	160 ± 55	413 ± 250
Stimulated	48 ± 8.0	57 ± 14	162 ± 42	389 ± 370
I/A DE	54 ± 7.0	71 ± 11	129 ± 25	248 ± 46
U/A DE	58 ± 3.0	77 ± 4.0	148 ± 16	201 ± 100

Table 3.17 The effect of immunoadsorbed decidual extract (I/A DE, PP14 concentration 0 - 0.10 ug/ml) and unadsorbed decidual extract (U/A DE, PP14 concentration 2.0 - 8.0 ug/ml) on the expression of CD4 and CD8 antigens on PHA stimulated lymphocytes. Results are means ± sd, n = 5.

CD4/CD8 Ratio.

	% cells	Mean Channel
Unstimulated	1.10 ± 0.05	0.45 ± 0.20
Stimulated control	0.85 ± 0.10	0.73 ± 0.50
I/A DE	0.78 ± 0.10	0.50 ± 0.20
U/A DE	0.77 ± 0.10	1.00 ± 0.70

Table 3.18 The effect of immunoadsorbed decidual extract (I/A DE, PP14 concentration 0 - 0.1 ug/ml) and unadsorbed decidual extract (U/A DE, PP14 concentration 2.0 - 8.0 ug/ml) on the CD4/CD8 ratio of PHA stimulated lymphocytes. Results are means ± sd, n = 5.

displaying the CD8 phenotype of cells of suppressor/cytotoxic character indicates that the proliferation evident in the ^{3}H-Tdr incorporation assay may be predominant in this fraction.

The observation that PP14 does not alter the CD4/CD8 ratio suggests that the protein has no preferential suppressive activity on the T cell subsets present.

3.25 DNA Analysis of PHA Stimulated Lymphocytes

DNA analysis of PHA stimulated cells using the nuclear dye propidium iodide allows the analysis of the DNA content of the cells and is able to identify the proportion of cells in the G_1, S and G_2 phases of the cell cycle. For all samples, the fraction of the cells in each phase was obtained by a computer fit of the fluorescence (which is proportional to the DNA) distribution patterns obtained in both unstimulated and PHA stimulated cells.

Representative distribution patterns from unstimulated and PHA stimulated cells are shown in Fig 3.38. The cells in the larger peak represent cells in G_1 phase (before DNA replication); the smaller peak represents cells in G_2 (following DNA replication) or M (mitosis), with the cells in S phase (during DNA replication) lying between the two peaks. The fraction of cells in the various phases of the cell cycle was derived by computer analysis.

The proportion of stimulated cells in the S phase peaked at around 44 to 68 hours of culture with the levels declining thereafter towards the end of the culture period investigated (0 - 137 hours, Fig 3.39). The cell cycle analysis of mitogenically stimulated

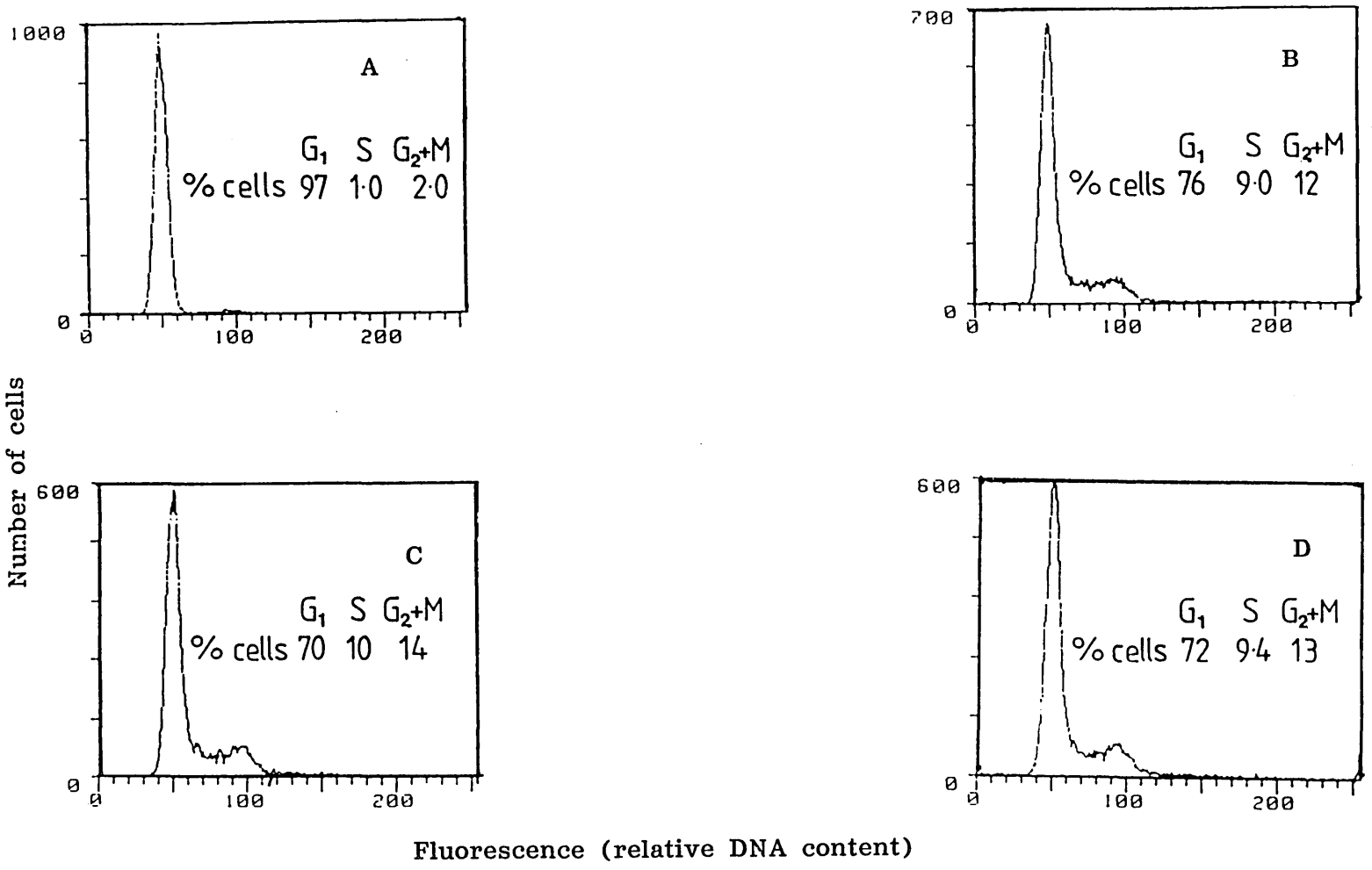


Figure 3.38 DNA distribution obtained by flow cytometric analysis of unstimulated (A) and PHA stimulated (B) lymphocytes and cells stimulated in the presence of immunoadsorbed (C, PP14 concentration 0.10 ug/ml) and unadsorbed (D, PP14 concentration 8.0 ug/ml) decidual extract. The percentage of cells in G_1, S and G_2+M were obtained by computer fit analyses of the DNA distribution curves.

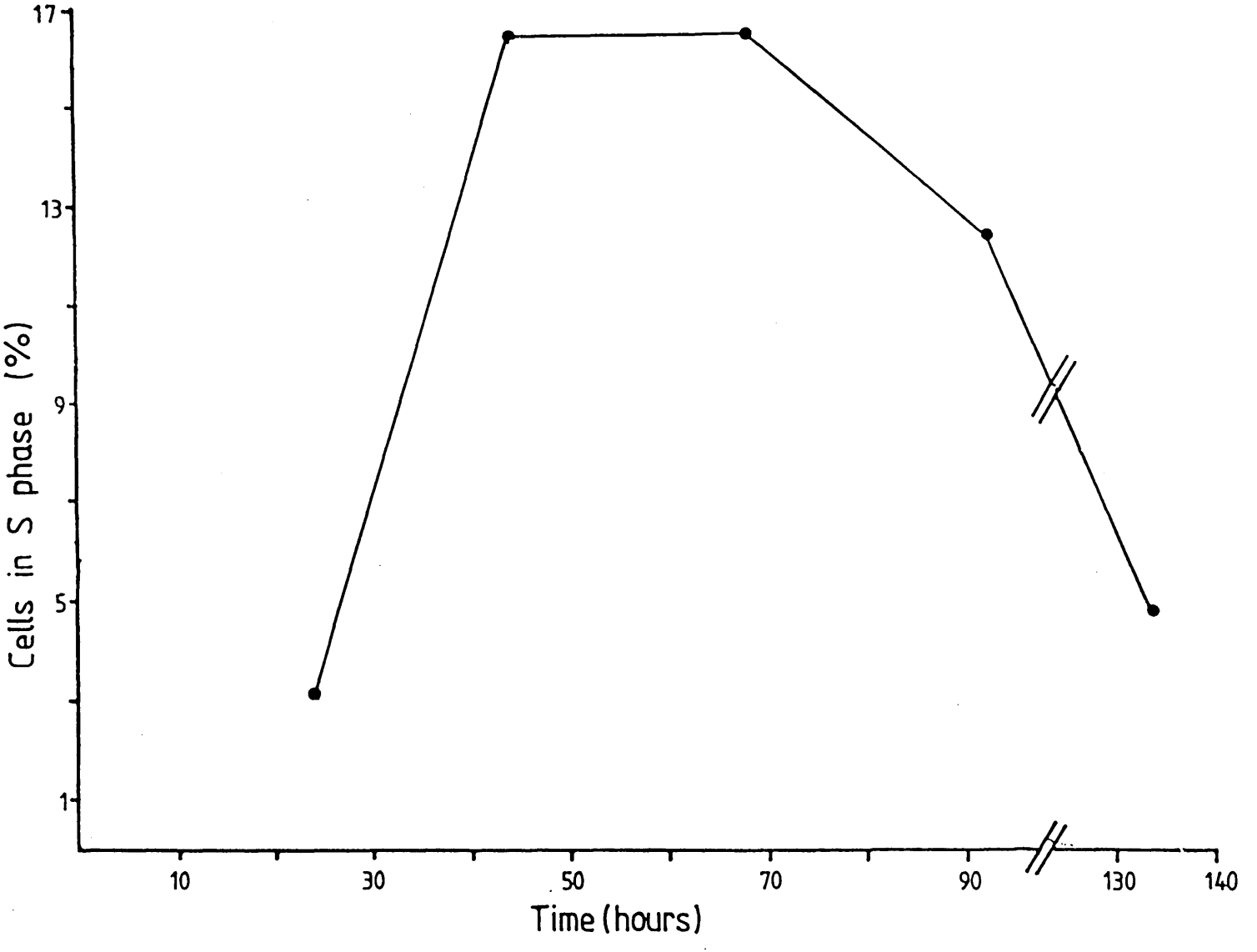


Figure 3.39 Kinetic analysis of the proportion of lymphocytes in the S phase of the cell cycle following stimulation with PHA. The percentage of cells in S phase was obtained by computer fit analyses of the DNA distribution curves.

lymphocytes confirmed the ^{3}H-Tdr uptake data presented earlier (Fig 3.8). Incubation of PHA stimulated lymphocytes in the presence of decidual extracts having a range of PP14 concentrations (2.0 - 8.0 mg/l) had no effect on the proportion of cells in S phase as compared to control cultures (Table 3.19). These data indicate that PP14 does not influence the distribution of stimulated cells through the cell cycle rather the protein may modulate the rate of passage.

	Proportion of Cells in the Cell Cycle (%)		
	G1	S	G2+M
Unstimulated	93 ± 6	2 ± 2	3 ± 2
Stim^{d} (control)	72 ± 7	12 ± 4	13 ± 3
+ DE	67 ± 6	14 ± 6	15 ± 3

Table 3.19 The effect of PP14-containing decidual extracts (PP14 concentrations 2.0 - 8.0 ug/ml) on the distribution of PHA stimulated cells in the cell cycle. Results are expressed as means ± sd, n = 5. Statistical analysis of data obtained at individual PP14 concentrations (paired Student t test) indicated no significant difference in cell cycle distribution in the presence of PP14. For clarity of presentation, data are expressed as the means of the results obtained at all PP14 concentrations investigated.

CHAPTER FOUR

FINAL DISCUSSION AND CONCLUDING REMARKS

4.1 Immunosuppression and Pregnancy

The whole area of immunosuppressive factors in pregnancy is contentious. The experimental basis for such studies depends on the use of lymphoproliferative assays and the inhibition of lymphoproliferation. All bioassays, by their very nature, have a high degree of variability and lymphoproliferation assays are no exception to this. Two factors have been identified as particularly contributing to variations in the latter assay, resulting in a high between-sample variation. These are differences in the frequency of responding units in the cell populations used and large differences in the gradient of the cell dose-response curves (Ross et al., 1987). The experimental data from the lymphoproliferation assays presented here were obtained using blood samples from different individuals taken on different days, which may be reflected in the variability observed in the experiments.

Much previous work on the effect of pregnancy-associated proteins on the lymphoproliferative response has been suspect due to the question of purity of the protein preparations used, particularly concerning the specificity of the reaction and the possibility of impurities in the preparations used exhibiting inhibitory effects. In an attempt to overcome such problems, the present work employed an approach involving the specific removal of PP14 from crude tissue extracts by immunoadsorption with a monoclonal anti-PP14 antibody.

Lymphoproliferation assays are as yet ill understood at a molecular level, and the designation of a material as an immunosuppressor or immunomodulator solely by its effect on the incorporation of tritiated thymidine during such an assay is simplistic. A more detailed exploration into the mode of action of a putative immunomodulator such as PP14 is more likely to indicate a specific rather than a non-specific immunomodulatory action.

4.2 Identification of Immunomodulatory Activities

There have been numerous reports as to the occurence of immunomodulatory activities, all of which appear to have specificity towards different levels of the immune response. The presence of PP14 may explain some of the observed activities described, however there are a large number of potentially immunosuppressive factors which are, as yet uncharacterised. Whether such activities are effective *in vivo* is unknown.

Daya *et al.*, (1987) have reported that decidual supernatants were able to suppress the proliferative response of interleukin-2 dependent cells with the peak suppressive activities being at molecular weights of 43 kDa and 21 kDa. The activity was specifically directed at IL-2 dependent cell lines and interfered with IL-2 action. The soluble factor was suggested to mediate its action via an interference with the binding of IL-2 to its receptor rather than a direct modification of the receptor affinity.

Human pregnancy serum inhibits the proliferation of T helper cells and their IL-2 synthesis in mixed lymphocyte cultures (Domingo *et al.*, 1985). The sera used in that study were obtained at 8 weeks

of gestation and so would contain measurable concentrations of PP14 though at concentrations unlikely to be sufficient to elicit the immunomodulatory activity reported. It has also been shown that pregnancy serum (at the 8th week of gestation) interferes with the capacity of IL-1 to promote the production of IL-2, by rendering IL-2 producer cells unable to synthesise IL-2 and subsequently to proliferate (Domingo et al., 1988). Other work has shown that the inhibition of the MLR by pregnancy sera could be reversed by the addition of supernatant from allogeneic MLR cultures and recombinant IL-2, but not IL-1 which suggests a level of control at the IL-2 synthesis level (Nicholas et al., 1984).

Suppression of the MLR has been demonstrated in the presence of human first trimester pregnancy endometrium (Nakayama et al., 1985). The suppression was inversely related to the gestational age of the tissue with the maximal suppression being observed at around 6 weeks of gestation. The decidual cells had no effect on the proliferative response of IL-2 dependent cells and it was suggested that the effect was at the antigen recognition phase of the MLR.

Human non-decidualised endometrium has been shown to release soluble immunosuppressive factors. The factors were capable of suppressing both allogeneic and mitogenic stimulation with the activity in the supernatants of secretory phase endometrial cultures being higher than that from proliferative phase (Wang et al., 1987). Addition of progesterone into the explant cultures of proliferative endometria significantly increased the suppressive activity of the culture supernatant (Wang et al., 1987). No enhancing of the suppressive activity of secretory phase endometria was observed which

was possibly due to saturation of progesterone binding capacity by the endogenous progesterone from the corpus luteum. Addition of oestrogen had no effect. Progesterone has been found in higher concentrations in secretory endometrium than in the proliferative phase (Porias et al., 1978), and is considered essential for promoting a decidual response capable of blocking the afferent arm of the immunologic reflex (Beer et al., 1975). Non-pregnant human secretory endometrium is capable of synthesising PP14 in vitro with the synthesis being related to the action of progesterone (Julkunen et al., 1986a). It may be that the observed immunosuppressive activity is in part due to the PP14 content of the preparations.

Mechanisms of suppressor factor release into the uterine lumen during peri-ovulatory periods may mediate a protective mechanism for sperm migration in the genital tract and the implantation of the semi-allogeneic foetus on the secretory phase endometrium. The observation may also in part explain why the abnormalities of progesterone secretion in women with corpus luteum defect are associated with a high rate of infertility. It can be envisaged that such a deficiency would lead to inadequate secretion of immunosuppressive factor(s) from the endometrium which would, in turn lead to failure of implantation or early embryonic loss.

A number of reports have suggested the release of immunoregulatory molecules from human decidual tissue cultures. Using a cell line derived from human decidual tissue (TTK-1), Tatsumi et al. (1987) have reported the release of an immunoregulatory factor. The factor was found to strongly suppress the MLR and had an estimated molecular weight of 43 - 67 kDa. The TTK-1 supernatant

suppressed IL-2 dependent T cell lines, but not IL-2 independent cell lines. The results suggested that the released factor inhibited the MLR via an inhibitory effect on the action of IL-2 and exerted its inhibitory activity primarily at the proliferative stage of the assay. The factor appeared to inhibit allogeneic stimulation more markedly that PHA stimulation.

Golander et al. (1981) have shown that explants of human decidual tissue obtained at 8 - 12 weeks of gestation and cultured for 24 hours secreted a factor which suppressed lymphocyte reactivities to lectins (PHA, Concanavalin A and pokeweed mitogen) and their activity in the MLR. In a similar observation to that reported by Tatsumi et al. (1987) the suppressive activity of the factor was more pronounced in the MLR than the mitogen stimulation assay (2 fold difference).

In addition to human decidual tissue secreting a number of immunomodulatory molecules, it is well known that the urine of pregnant women contains potent immunosuppressive factors which block mitogen (Cerni et al., 1977) and antigen-induced (Morse et al., 1976) lymphoproliferation of human blood lymphocytes in vitro. The immunosuppressive activity in both pregnancy serum and pregnancy urine was ascribed to a range of high and low molecular weight factors. One of the urine-derived pregnancy factors, termed uromodulin, has been purified to apparent homogeneity and appears to be an 85kDa glycoprotein (Muchmore and Decker 1985) with initial studies suggesting that uromodulin blocked antigen-induced lymphoproliferation at an early stage in the response. Results have since shown that uromodulin blocks the effects of recombinant IL-1 in

a thymocyte proliferation assay and appears to bind to IL-1 (Brown et al., 1986). Whether such IL-1 inhibitory activity underlies its suppressive activity on human lymphocytes remains to be established.

In addition to an effect at the proliferative stages of the immune response, there may also be a requirement for immunomodulatory activity at the effector stage. Chaouat and Kolb (1985) have reported the production of soluble factors from the spongiotrophoblast in the mouse which can interfere in a dose-dependent manner with the action of cytotoxic T lymphocytes. It has also been suggested that defective production may mediate some cases of immunologic abortions. An effect of PP14 at the effector stage of the immune response has not been investigated in this study. What appears to be a specific effect at the IL-2R level would suggest such an effect to be unlikely, however the possibility requires experimental confirmation.

4.3 The Immunomodulatory Activity of PP14

PP14 is present in measurable concentrations in the circulation in non-pregnant women, although the levels increase markedly during the first trimester of pregnancy and reach a peak at around week 10 of gestation (Julkunen et al., 1985). Higher levels are found in seminal plasma (Bolton et al., 1986a) with the highest levels of all being found in decidual tissue (Julkunen et al., 1985). Despite the high levels of PP14 found in the human reproductive system, little information has been published on the biological activities or physiological function of this protein.

Experimental results obtained in this study indicate that PP14, in purified form and in crude extracts of decidual tissue, exhibit immunosuppressive activity on both allogeneically and mitogenically stimulated lymphocytes possibly indicating a mode of action of this protein at a level common to both of these in vitro systems. Whilst it is tempting to suggest that PP14 inhibits allogeneic stimulation in a similar manner to its effects on mitogenic stimulation, it is not possible until a full investigation has been performed.

Difficulties were encountered in obtaining sufficient blood samples to perform more detailed investigations into the effect of PP14 on allogeneically stimulated lymphocytes. The degree of stimulation obtained using the MLR is highly dependent on and variable due to the MHC class differences of the cell donors. The degree of activation following allogeneic stimulation was observed to be much lower using the MLR system (typically 30% Tac^+ cells following 6 days of culture) as compared to the mitogenic system (typically 90% Tac^+ cells following 3 days of culture).

It appears from the results presented here that PP14 is involved in inhibiting T lymphocyte proliferation and may therefore be an immunoregulatory molecule. The data suggest that the level of the inhibitory activity for PHA stimulated lymphocytes is at the level of the IL-2R and functions by modulating the affinity of such receptors. The inhibitory effect appears to be at the induction stage of the high affinity receptor as PP14 does not inhibit the cellular response to IL-2 once the receptor has been expressed. It is unknown at the present whether the activity of PP14 is directed towards the induction mechanism itself or whether there is an effect

at an earlier stage in the induction of high affinity receptors. It has been reported that IL-1 induces receptivity to IL-2 possibly by increasing the receptor affinity rather than the receptor number (Oppenheim et al., 1986). Mizel (1987) has suggested that there may be a lymphokine produced by T cells which augments the ability of accessory cells to produce IL-1. Any effect at this level of the response would lead to a secondary effect at the IL-2R level in addition to modifying the secretion kinetics of IL-2 itself. A similar effect would also be observed by an effect at the IL-1 receptor level. An additional mechanism could be a control at the signal transduction level of the response. Results on the appearance of IL-2 and IL-2R may possibly suggest a control on the activation of protein kinase C or alternatively a modification of Ca^{2+} fluxes. The mechanism by which such an effect would be mediated is as yet unknown.

The lower levels of IL-2 in the culture supernatant of stimulated lymphocytes in the presence of PP14 seen in this study may be secondary to the apparent lowering of the receptor affinity. In addition to augmenting the proliferation of alloantigen-specific cytotoxic lymphocytes, IL-2 also activates and stimulates the proliferation of natural killer cells and lymphokine activated killer cells which are effective against targets which are resistant to specific cytotoxic T lymphocytes. The mechanism which appears to be observed with PP14 not only inhibits the proliferative response of the cytotoxic T cell population via an effect on the IL-2R, but also modulates, via IL-2, the functionality of a number of other potentially cytotoxic effector cells.

As indicated above, the distribution of PP14 in the decidualising endometrium and seminal plasma is compatible with an immunoregulatory role. At the concentrations present in seminal plasma (15 - 500 mg/l, Bolton et al., 1986a), human uterine luminal fluid (about 4 mg/l, S.C. Bell, personal communication) and deciduum (about 9 mg/g wet weight tissue, Bolton et al., 1986b), PP14 would be expected to exert an effect on T lymphocyte proliferation (Figs 3.6; 3.10). Although the maximal levels found in the peripheral circulation (up to 2.2 mg/l, median about 1 mg/l, Julkunen et al., 1985) may be expected to exhibit some inhibition, such high concentrations are present only for a period of about 4 weeks in the first trimester of pregnancy and even at these concentrations only a minimal effect on T lymphocyte proliferation would be anticipated. This suggests that any immunoregulatory response would be largely restricted to the lumen of the female tract and the decidual tissue by the localisation of the concentration of PP14 present.

There is the possibility that the immunomodulatory activity is mediated by a molecule associated with PP14 and is not due to PP14 itself. It has been suggested that the suppressive activity may be mediated by prostaglandin (S.C. Bell, personal communication). Prostaglandin synthesis by decidual tissue is well known (Antebi et al., 1975). In the mouse, supernatants from short-term cultures of decidual tissue produced prostaglandins which were capable of suppressing the mixed lymphocyte reaction (Matthews and Searle 1987). The suppression of the lymphocyte response appeared to be non-specific and was abrogated by dialysis.

Prostaglandins are known to regulate T cell lymphoproliferative responses by modulating the development of receptors for transferrin (Chaouat et al., 1985). Also IL-2 production and the expression of the IL-2R (as detected by anti-Tac) are inhibited by prostaglandin (PGE_2) at concentrations of around 10^{-9} M (Vercammen and Ceupens 1987). The addition of exogenous recombinant IL-2 and purified IL-1 was unable to reverse the inhibition of IL-2R expression, but IL-2 was able to partially reverse the inhibitory activity of PGE_2 on ^{3}H-Tdr uptake into stimulated lymphocytes (Vercammen and Ceupens 1987). PGE_2 does not affect the IL-2 driven proliferation of activated T cells, nor does it inhibit accessory cell function and it has its effects at an early step in T cell activation.

The dialysis of the decidual tissue extracts as used in this study would be expected to remove any PGE_2 loosely associated with PP14. Whilst certain elements of the immunosuppressive activity of PP14 are similar to those exhibited by PGE_2 such as the inhibition of IL-2 levels and the reversal of the inhibition of ^{3}H-Tdr uptake into proliferating cells by the addition of exogenous IL-2, the observation that PP14 does not inhibit Tac antigen expression or the expression of the transferrin receptor makes unlikely the possibility of a role for PGE_2 on PP14 induced immunosuppression.

The observation that PP14 may be progesterone controlled (Julkunen et al., 1986b) may suggest that it is progesterone which mediates the suppressive activity of PP14. Progesterone has been shown to exhibit immunosuppressive activities (Clemens et al., 1979) and directly interferes with cell-cell contact thus altering the local exchange of immunological information (Van Vlasselaar et al.,

1986). The observations made in this study that decidual tissue extracts do not interfere with cell-cell contact (Plate 3.2) indicate no role for progesterone in the immunosuppressive activity of PP14. This is supported by the finding that PP14 does not affect the activation stages of the *in vitro* response to both mitogenic and allogeneic stimulation as seen by the normal expression of activation antigens (Tac, HLA-DR and TFR) in the presence of PP14.

Whether such activities ascribed to PP14 are expressed *in vivo* and, if so, whether they are of physiological importance remains to be resolved. The mechanisms by which the mother and foetus immunologically tolerate each other during pregnancy are numerous and it would be fair to say that no single mechanism ensures the successful survival of the foetus in the potentially hostile environment of the mother. If PP14 does prove to be an important immunomodulatory factor associated with the reproductive tract, it will have exciting implications in the study of implantation and fertility. As such an activity is likely to be a highly localised phenomenon, it is perhaps less likely that the circulating levels of this protein will prove to be diagnostically useful. In a wider field, PP14 may prove a valuable tool in transplantation biology and the understanding of the cell mediated immune system and its control.

REFERENCES

Aarden L.A. et al. (1979). Revised nomenclature for antigen non-specific T cell proliferation and helper factors. J. Immunol. 123;2928-2929.

Adinolfi M. (1975). The human placenta as a filter for cells and plasma proteins. In (Edwards R.G., Howe C.W.S., and Johnson M.H., eds) Immunobiology of Trophoblast. Cambridge University Press, 193-210.

Albert F., Hua C., Truneh A., Pierres M. and Schmitt-Verhulst A.-M. (1985). Distinction between antigen receptor and IL-2 receptor triggering events in the activation of alloreactive T cell clones with calcium ionophore and phorbol ester. J. Immunol. 134;3649-3655.

Allen L.W., Svenson R.H. and Yachnin S. (1969). Purification of mitogenic proteins derived from Phaseolus vulgaris. Isolation of potent and weak phytohaemagglutinins possessing mitogenic activity. Proc. Natl. Acad. Sci. USA. 63;334-341.

Ando I., Hariri G., Wallace D. and Beverley P. (1985). Tumor promoter phorbol esters induce unresponsiveness to antigen and expression of interleukin 2 receptor on T cells. Eur. J. Immunol. 15;196-199.

Antebi S.O., Bauminger S., Zor U. and Lindner H.R. (1975). Prostaglandin synthesis in decidual tissue of the rat uterus. Prostaglandins 10;991-999.

Ascheim S. and Zondek B. (1927). Hypophysenvorderlapperhormon und ovarialhormon im harn von schwangeren. Klin. Wschr. 6;1322.

Ashwell J.D., Robb R.J. and Malek T.R. (1986). Proliferation of T lymphocytes in response to interleukin-2 varies with their state of activation. J. Immunol. 137;2572-2578.

Athanassakis I., Bleackley R.C., Paetkau V., Guilbert L., Barr P.J. and Wegmann T.G. (1987). The immunostimulatory effect of T cells and T cell lymphokines on murine foetally derived placental cells. J. Immunol. 138;37-44.

Auron P.E., Webb A.C., Rosenwasser L.J., Mucci S.F., Rich A., Wolff S.M., and Dinarello C.A. (1984). Nucleotide sequence of human monocyte interleukin-1 precursor cDNA. Proc. Natl. Acad. Sci. USA. 81;7907-7911.

Azer P.C., Braunstein G.D., Van De Velde R., Van De Velde S., Kogan R., and Engvall E. (1980). Ectopic production of pregnancy specific beta-1-glycoprotein by a non-trophoblast tumour in vitro. J. Clin. Endocrinol. Metab. 50;234-239.

Bankhurst A.D., Witemeyer S., and Williams R.G. (1978). A population of human cord blood mononuclear cells with surface alpha foeto-protein. Immunol. Commun. 7;187-208.

Beer A.E., Billingham R.E. and Scott J.R. (1975). Immunogenetic aspects of implantation, placentation and feto-placental growth rates. Biol. Reprod. 12;176-189.

Beer A.E. and Sio J.O. (1982). Placenta as an immunological barrier. Biol. Reprod. 26;15-27.

Beer A.E., Quebbeman J.F. and Zhu X (1986). Non-paternal leukocyte immunisation in women previously immunised with paternal leucocytes. In (Clark D.A. and Croy B.A. eds). Reproductive Immunology pp 261-268 Elsevier, Amsterdam.

Bell S.C. (1983). Decidualisation: Regional differentiation and associated function. Oxf. Rev. Reprod. Biol. 5;220-271.

Bell S.C. (1985). Comparative aspects of decidualisation in rodents and human; cell types, secreted products and associated function. In (Edwards R.G., Purdy J. and Steptoe P.C. eds) Implantation of the Human Embryo. pp 71-122. London Academic Press.

Bell S.C. and Billington W.D. (1980). Major anti-paternal antibody induced by murine pregnancy is non-complement fixing IgG_1. Nature 288;387-388.

Bell S.C. and Billington W.D. (1983). Humoral immune responses in murine pregnancy III. Relationship between anti-paternal alloantibody levels in maternal serum, placenta and foetus. J. Reprod. Immunol. 5;299-310.

Bell S.C. and Bohn H. (1986). Immunochemical and biochemical relationships between human pregnancy-associated endometrial $alpha_1alpha_2$-globulins ($alpha_1alpha_2$-PEG) and the soluble placental proteins 12 and 14 (PP12 and PP14). Placenta 7;283-294.

Bell S.C., Patel S., Hales M.W., Kirwan P.H., and Drife J.O. (1985). Immunochemical detection and characterisation of pregnancy-associated endometrial $alpha_1$- and $alpha_2$-globulins secreted by the human endometrium. J. Reprod. Fertil. 74;261-270.

Bell S.C., Patel S.R., Kirwan P.H. and Drife J.O. (1986). Protein synthesis and secretion by the human endometrium during the menstrual cycle and the effect of progesterone in vitro. J. Reprod. Fertil. 77;221-231.

Benacerraf B. and McDevitt H.O. (1972). Histocompatibility linked immune response genes. Science 175;273-279.

Benson R.C. (1977). Handbook of Obstetrics and Gynaecology. Lange Medical Publications, California, USA.

Benveniste J., Henson P.M. and Cochrane C.G. (1972). Leucocyte-dependent histamine release from rabbit platelets. The role of IgE, basophils and a platelet activating factor. J. Exp. Med. 136;1356-1364.

Benveniste E.N. and Merril J.E. (1986). Stimulation of oligodendroglial proliferation and maturation by interleukin-2. Nature 321;610-613.

Biddison W.E., Rao P.E., Talle M.A., Goldstein G. and Shaw S. (1982). Possible involvement of the OKT4 molecule in T cell recognition of class II HLA antigens. Evidence from studies of cytotoxic T lymphocytes specific for SB antigens. J. Exp. Med. 156;1065-1076.

Billington W.D. and Bell S.C. (1983a). Fetal histocompatibility antigens and maternal immune responses. In Fetal antigens and cancer. Ciba Found. Symp. Pitman London 96;69-88.

Billington W.D. and Bell S.C (1983b). Immunobiology of mouse trophoblast. In (Loke Y.W. and Whyte A. eds) Biology of Trophoblast. Elsevier/North Holland Biomedical Press 571-595.

Billington W.D. and Burrows F.J. (1986). The rat placenta expresses paternal class I major histocompatibility antigens. J. Reprod. Immunol. 9;155-160.

Bischof P., Duberg S., Sizonenko M.T., Schindler A.M., Beguin F., Herrmann W.L., and Sizonenko P.C. (1984). In vitro production of pregnancy associated plasma protein-A by human decidua and trophoblast. Am. J. Obstet. Gynaecol. 148;13-19.

Bischof P., Lauber K., Wurstemburger B., and de Girard J.P. (1982). Inhibition of lymphocyte transformation by pregnancy associated plasma protein-A (PAPP-A). J. Clin. Lab. Immunol. 7;61-65.

Bischof P., Meisser A., Geinoz A., and Schindler A.M. (1985). Origin and biological effects of pregnancy associated plasma protein-A. In (Bischof P. and Klopper A. eds) Proteins of the Placenta. 5th Int. Congr. on Placental Proteins, Annecy 1984. Karger, Basel. 142-158.

Bischof P., Meisser A., Haengelli L., Reber G., Bouvier C., Beguin F., Herrmann W.C. and Sizonenko P.C. (1983). Pregnancy associated plasma protein-A (PAPP-A) inhibits thrombin induced coagulation of plasma. Throm. Res. 32;45-55.

Blazsek I. and Mathe G. (1984). Zinc and immunity. Biomed. Pharmacother. 38;187-193.

Bohn H. (1972). Isolierung und charakterisierung des schwanger-schafts-spezifischen beta 1-glykoproteins. Blut 24;292-302.

Bohn H. (1985). Biochemistry of Placental Proteins. In (Bischof P. and Klopper A. eds) Proteins of the Placenta. 5th Int. congr. on Placental Proteins. Annecy 1984. Karger, Basel. 1-25.

Bohn H., Johannsen R. and Kraus W. (1980). Neues plazentaprotein (PP15) mit immunosuppressiver wirkung. Arch. Gynaecol. 230;167-172.

Bohn H., Kraus W. and Winckler W. (1982). New soluble placental tissue proteins: their isolation, characterisation, localisation and quantification. Immunology of Human Placental Proteins. Placenta (suppl 4) Praeger. 67-81.

Bolton A.E. (1985). Radioiodination Techniques (2nd edition). Amersham International, Amersham.

Bolton A.E., Chapman M.G., Stoker R.J., Andrew C.E., Wass D. and Bohn H. (1983). The radioimmunoassay of human placental protein 14 (PP14). Clin. Chim. Acta. 135;283-291.

Bolton A.E., Clough K.J., Bohn H., and Chapman M.G. (1986b). Placental protein 14 (PP14) in the human reproductive tract. In Pregnancy Proteins in Animals. Walter de Gruyter, Berlin. 165-171.

Bolton A.E., Pinto-Furtado L.G., Andrew C.E., and Chapman M.G. (1986a). Measurement of the pregnancy-associated proteins, placental protein 14 and pregnancy-associated plasma protein A in human seminal plasma. Clin. Reprod. Fertil. 4;233-240.

Bolton A.E., Pockley A.G., Clough K.J., Mowles E.A., Stoker R.J., Westwood O.M.R. and Chapman M.G. (1987). The identification of placental protein 14 (PP14) as an immunosuppressant factor involved in human reproduction. Lancet i;593-595.

Boyum A. (1968). Isolation of mononuclear cells and granulocytes from human blood. Scand. J. Clin. Lab. Invest. 21(suppl 97);77-89.

Braunstein G.D. et al., (1973). Secretory rates of human chorionic gonadotrophin by normal trophoblast. Am. J. Obstet. Gynecol. 115;447-450.

Braunstein G.D. (1976). Serum human chorionic gonadotrophin levels throughout normal pregnancy. Am. J. Obstet. Gynecol. 126;178-181.

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. Proc. Natl. Acad. Sci. USA. 83;9119-9123.

Bruserud O., Degre. M. and Thorsby E. (1986). Interleukin-2 and interferon production and expression of interleukin-2 receptors by human mononuclear cells stimulated with mumps virus or phytohemagglutinin. Acta. Path. Microbiol. Immunol. Scand. (sect C) 94;51-55.

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

Bulmer J.N. and Johnson P.M. (1986). The T-lymphocyte population in first trimester human decidua does not express the interleukin-2 receptor. Immunology 58;685-687.

Bulmer J.N. and Sunderland C.A. (1984). Immunohistological characterisation of lymphoid poulations in the early human placental bed. Immunology 52;349-357.

Camussi G., Tetta C. and Bussolino F. (1983). Inhibitory effect of prostacyclin (PGI_2) on neutropenia induced by intravenous injection of platelet activating (PAF) in the rabbit. Prostaglandins 25;343-351.

Cantor H. and Boyse E.A. (1977). Regulation of cellular and humoral immune responses by T-cell subclasses. Cold Spring Harbor Symp. Quant Biol. 41;23-32.

Carlson G.A. and Wegmann T.G. (1978). Paternal-strain antigen excess in semi-allogeneic pregnancy. Transplant. Proc. 10;403-407.

Cerni C., Tatra G. and Bohn H. (1977). Immunosuppression by human placenta lactogen (HPL) and the pregnancy-specific beta 1-glycoprotein (SP-1). Inhibition of mitogen-induced lymphocyte transformation. Arch. Gynaekol. 223;1-7.

Chaouat G. and Kolb J.P. (1985). Immunoactive products of placenta IV. Impairment by placental cells and their products of CTL function at effector stage. J. Immunol. 135;215-222.

Chaouat G., Kolb J.P. and Wegmann T.G. (1983). The murine placenta as an immunological barrier between the mother and the foetus. Immunol. Rev. 75;31-60.

Chopra R.K., Nagel J.E., Chrest F.J., Boto W.M., Pyle R.S., Dorsey B., McCoy M., Holbrook N. and Adler W.H. (1987). Regulation of interleukin 2 and interleukin 2 receptor gene expression in human T cells I; effect of Ca^{2+}-ionophore on phorbol myristate acetate co-stimulated cells. Clin. Exp. Immunol. 69;433-440.

Clark D.A., Brierley J., Slapsys R., Daya S., Damji N., Chaput A. and Rosenthal K. (1986a). Trophoblast-dependent and trophoblast-independent suppressor cells of maternal origin in murine and human decidua. In (Clark D.A. and Croy B.A. eds). Reproductive Immunology. pp 219-226. Elsevier, Amsterdam.

Clark D.A., Brierley J., Slapsys R., Daya S., Damji N., Chaput A. and Rosenthal K. (1986b). Local intrauterine suppressor cells and suppressor factors in survival of the foetal allograft. In (Chaouat G. ed). Reproductive Immunology. Materno-fetal Relationship. vol. 154, pp 77-88. Colloque INSERM. Paris, France.

Clark D.A., Damji N., Chaput A., Daya S., Rosenthal K.L. and Brierley J. (1986c). Decidua-associated suppressor cells and suppressor factors regulating interleukin-2: Their role in the survival of the foetal allograft. In (Cinader B. and Miller R.G. eds). Progress in Immunology vol. VI pp1089-1099. Academic Press, New York.

Clark D.A., Slapsys R.M., Croy B.A. and Rossant J. (1984). Immunoregulation of host-vs-graft responses in the uterus. Immunology Today 5;111-115.

Clemens L.E., Siiteri P.K. and Stites D.P. (1979). Mechanism of immunosuppression of progesterone on maternal lymphocyte activation during pregnancy. J. Immunol. 122;1978-1985.

Coffey R.G., Hadden E.M. and Hadden J.W. (1981). Phytohaemagglutinin stimulation of guanylate cyclase in human lymphocytes. J. Biol. Chem. 256;4418-4424.

Cohen J. and Werret D.J. (1975). Antibodies and sperm survival in the female tract of the mouse and rabbit. J. Reprod. Fertil. 42;301-310.

Colvin R.B., Fuller T.C., MacKeen L., Kung P.C., Ip S.H. and Cosimi A.B. (1987). Plasma interleukin-2 receptor levels in renal allograft patients. Clin. Immunol. and Immunopath. 43;273-276.

Cornaby A., Simpson M.A., Dempsey R., Van Rice R., Madras P. and Monaco A.P. (1987). Interleukin-2 production in plasma and urine, plasma interleukin-2 receptor levels and urine cytology as a means of monitoring renal allograft recipients. Presented at the International Organ Transplant Forum, September.

Damber M.-G., von Schoultz B., Stigbrand T. and Tarnvik A. (1975). Inhibition of the mixed lymphocyte reaction by the pregnancy zone protein. FEBS Lett. 58;29.

Dattwyler R.J., Murgita R.A., and Tomasi T.B. (1975). Binding of alpha-foetoprotein to murine T cells. Nature 256;656-657.

Daya S., Rosenthal K.L. and Clark D.A. (1987). Immunosuppressor factor(s) produced by decidua-associated suppressor cells:- A proposed mechanism for fetal allograft survival. Am. J. Obstet. Gynecol. 156;344-350.

Depper J.M., Leonard W.J., Drogula C., Kronke M., Waldmann T.A. and Greene W.C. (1985). Interleukin-2 (IL-2) augments transcription of the interleukin-2 receptor gene. Proc. Natl. Acad. Sci. USA. 82;4230-4234.

Depper J.M., Leonard W.J., Kronke M., Naguchi P.D., Cunningham R.E., Waldmann T.A. and Greene W.C. (1984). Regulation of interleukin-2 receptor expression; effects of phorbol diester, phospholipase C, and re-exposure to lectin and antigen. J. Immunol. 133;3054-3061.

Diamantstein T. and Chen Z. (1982). The mechanism of induction of interleukin-2 receptors: An IL-2 receptor inducing factor, the prerequisite of generation of the receptor. Immunobiol. 162;403.

Diamantstein T. and Osawa H. (1984). Studies on the interleukin-2 receptor, its generation and dynamics using monoclonal anti interleukin-2 receptor antibodies. Mol. Immunol. 21;1229-1236.

Dinarello C.A., Clowes G.H. Jr., Gordon A.H., Saravis C.A. and Wolff S.M. (1984). Cleavage of human interleukin-1; isolation of a peptide fragment from plasma of febrile human and activated monocytes. J. Immunol. 133;1332-1338.

Domingo C.G., Domenech N., Aparicio P. and Palomino P (1985). Human pregnancy serum inhibits proliferation of T8-depleted cells and their interleukin-2 synthesis in mixed lymphocyte cultures. J. Reprod. Immunol. 8;97-110.

Domingo C.G., Moreno A. and Palomino P. (1988). The effect of human pregnancy serum on the synthesis and action of interleukin-1. J. Reprod. Immunol. 13;17-30.

Dower S.K., Kronheim S.R., March C.J., Conlon P.J., Hopp T.P., Gillis S. and Urdal D.C. (1985). Detection and characterisation of high affinity plasma membrane receptors for human interleukin-1. J. Exp. Med. 162;501-515.

Dower S.K., Call S., Gillis S. and Urdal D.L. (1986a). Similarity between the interleukin-1 receptors on murine T-lymphoma cell line and on a murine fibroblast cell line. Proc. Natl. Acad. Sci. USA. 83;1060-1065.

Dower S.K., Kronheim S.R., Hopp T.P., Cantrell M., Deeley M., Gillis S., Henney C.S. and Urdal D.L. (1986b). The cell surface receptors for interleukin-1 alpha and interleukin-1 beta are identical. Nature 324;266-268.

Droge W. (1986). Protein kinase C in T-cell regulation. Immunology Today 7;340-343.

Duff G.W. and Alkins E. (1982). The detection of endotoxin by in vitro production of endogenous pyrogen: comparison with limulus amebocyte lysate gelation. J. Immunol. Methods. 13;323-331.

Durno A.G., Ho D.D., Schooley R.T., Hirsch M.S., MacKeen L. and Ip S.H. (1986). Serum interleukin-2 receptor (IL-2R) levels in human immunodeficiency virus (HIV) infection. Blood 68;124a

Durum S.K., Schmidt J.A. and Oppenheim J.J. (1985). Interleukin-1: an immunological perspective. Ann. Rev. Immunol. 3;263-287.

Ellis S.A., Sargent I.L., Redman C.W.G. and McMichael A.J. (1986). Evidence for a novel HLA antigen found on human extra-villous trophoblast and choriocarcinoma cell line. Immunology 59;595-601.

Enns C.A., Suomalainen H.A., Gebhardt J.E., Schroder J. and Sussman H.H. (1982). Human transferrin receptor: expression of the receptor is assigned to chromosome 3. Proc. Natl. Acad. Sci. USA. 79;3241-3245.

Fabris N. (1973). Immunological reactivity during pregnancy in the mouse. Experientia 29;610-612.

Faulk W.P. and McIntyre J.A. (1981). Trophoblast survival. Transplantation. 32;1-5.

Faulk W.P. and McIntyre J.A. (1983). Immunological studies of human trophoblast; markers, subsets and functions. Immunol. Rev. 75;139-175.

Farrar J.J., Benjamin W.R., Hilfiker M.L., Howard M., Farrar W.L. and Fuller-Farrar J. (1982). The biochemistry, biology and role of interleukin-2 in the induction of cytotoxic T cell and antibody-forming B cell responses. Immunol. Rev. 63;129-166.

Fleischer B. (1987). A novel pathway of human T cell activation via a 103 kDa T cell activation antigen. J. Immunol. 138;1346-1350.

Gay D., et al., (1987). Functional interaction between human T cell protein CD4 and the major histocompatibility complex HLA-DR antigen. Nature 328;626-629.

Gelfand E.W., Cheung R.K., Grinstein S. and Mills G.B. (1986). Characterisation of the role for calcium influx in mitogen-induced triggering of human T cells. Identification of calcium dependent and calcium independent signals. Eur. J. Immunol. 16;907-912.

Gelfand E.W., Cheung R.K., Mills G.B. and Grinstein S. (1987a). Role of membrane potential in the response of human T lymphocytes to phytohaemagglutinin. J. Immunol. 138;527-531.

Gelfand E.W., Mills G.B., Cheung R.K., Lee J.W.N. and Grinstein S. (1987b). Transmembrane ion fluxes during activation of human T lymphocytes: Role of Ca^{2+}, Na^{+}/H^{+} exchange and phospholipid turnover. Immunol. Rev. 95;59-87.

Germain R.N. and Benacerraf B. (1980). Helper and suppressor T cell factors. Springer Sem. Immunopathol. 3;93-127.

Gery I. and Lepe-Zunige J.L. (1984). Interleukin-1: uniqueness of its production and spectrum of activities. Lymphokines 9;109-125.

Gillis S. (1983). Interleukin-2: biology and biochemistry. J. Clin. Immunol. 3;1-13.

Godovac-Zimmerman J., Conti A., Liberatori J. and Braunitzer G. (1985). The amino acid sequence of beta lactoglobulin II from horse colostrum (Equus callalbus Penssodactyl): Beta lactoglobulins are retinol binding proteins. Biol. Chem. 366;601-608.

Goeken N.E. and Thompson J.S. (1977). Conditions affecting the immunosuppressive properties of human AFP. J. Immunol. 119;139-146.

Golander A., Zakuth V., Shechter Y., and Spirer Z. (1981). Suppression of lymphocyte reactivity *in vitro* by a soluble factor secreted by explants of human decidua. Eur. J. Immunol. 11;849-851.

Gottesman S.R. and Stutman O. (1980). Cellular immunity during pregnancy I. proliferative and cytotoxic reactivity in para-aortic lymph nodes. Am. J. Reprod. Immunol. 1;10-17.

Green D.R., Flood P.M. and Gershon R.K. (1983). Immunoregulatory T-cell pathways. Ann. Rev. Immunol. 11;439-464.

Greene W.C., Depper J.M., Crabtree G.R., Rudukoff S., Pumphrey J., Robb R.J., Kronke M., Svetlik P., Peffer N.J. and Waldmann T.A. (1985a). Isolation and expression of complementary DNAs encoding the human interleukin-2 receptor. Cancer Research 45 (9 suppl);4563s-4567s.

Greene W.C. *et al.*, (1985b). Stable expression of cDNA encoding the human interleukin-2 receptor in eukaryotic cells. J. Exp. Med. 162;363-368.

Greene W.C. and Leonard W.J. (1986). The human interleukin-2 receptor. Ann. Rev. Immunol. 4;69-95.

Grimm E.A., Ramsey K., Mazumbder A., Wilson D., Djev J. and Rosenberg S. (1983). Lymphokine activated killer cell phenomenon. II. Precursor phenotype is serologically distinct from peripheral T lymphocytes, memory cytotoxic thymus derived lymphocytes and natural killer cells. J. Exp. Med. 157;884-897.

Grosser O. (1927). Fruhentwicklung, Eihautbildung und Placentation des Menschen und der Saugetiere. In (von Jaschke R.T. ed) Deutsche Frauenheilkunde, Geburtshilfe, Gynacologie und Nachbargebiete in Einzeldarstellung. Bergmann, Munich.

Guillet J.-G., Lai M.-Z., Briner T.J., Smith J.A. and Geffer M.L. (1986). Interaction of peptide antigens and class II major histocompatibility complex antigens. Nature 324;260-262.

Hammer S.M. and Gillis J.M. (1986). Effects of recombinant interleukin-2 on resting human T lymphocytes. J. Biol. Resp. Modifiers 5;36-44.

Han T. (1974). Inhibitory effect of human chorionic gonadotrophin on lymphocyte blastogenic response to mitogen, antigen and allogeneic cells. Clin. Exp. Immunol. 18;529-535.

Head J.R. and Gaede S.D. (1986). Ia antigen expression in the rat uterus. J. Reprod. Immunol. 9;137-153.

Hefneider S.H., Conlon P.J., Henney C.S. and Gillis S. (1983). In vivo interleukin-2 administration augments the generation of alloreactive cytolytic T lymphocytes and resident natural killer cells. J. Immunol. 130;222-227.

Heikinheimo M., Saksela O., Lehtovirta P., Seppala M. and Bohn H. (1981). Cultured human syncytiotrophoblasts synthesise pregnancy specific beta-1-glycoprotein (SP1). Acta. Microbiol. Scandinav. 89;139-144.

Henney C.S., Kunbayashi K., Kern D.E. and Gillis S. (1981). Interleukin-2 augments natural killer cell activity. Nature 291;335-338.

Herrman F., Cannista S.A., Levine H. and Griffin J.D. (1985). Expression of interleukin-2 receptors and binding of interleukin-2 by gamma interferon-induced human leukemic and normal monocytic cells. J. Exp. Med. 162;1111-1116.

Horne C.H.W. and Towler C.M. (1978). Pregnancy specific beta-1-glycoprotein: a review. Obstet. Gynecol. Survey 33;761-768.

Hosang M. and Shooter E.M. (1985). Molecular characteristics of nerve growth factor receptors on PC12 cells. J. Biol. Chem. 260;655-662.

Hsi B.-L., Yeh C-J.L. and Faulk W.P. (1982). Human amniochorion: tissue specific markers, transferrin receptors and histocompatibility antigens. Placenta 3;1-12.

Huhtala M.L., Kalkkinen N., Palomaki P., Julkunen M., Bohn H. and Seppala M. (1986). Amino acid sequence similarity between placental protein 14 and beta lactoglobulins from various species. XIV Annual Meeting of the International Society for Oncodevelopmental Biology and Medicine. Poster Communication.

Hunig T. (1986). The ligand of the erythrocyte receptor of T lymphocytes: expression on white blood cells and possible involvement in T cell activation. J. Immunol. 136;2103-2108.

Hunig T., Trefenhaler G., Meyer zum Buschenfelde K.H. and Meuer S.C. (1987). Alternative pathway of activation of T cells by binding of CD2 to its cell-surface ligand. Nature 326;298-301.

Inaba N. et al. (1987). The immunocytochemical localisation of new soluble placental tissue proteins ($PP_{14,16,17,19,20}$, and PP_{21}) in human and Cynomolgus Monkey placentae. Arch. Gynaecol. 240;13-19.

Isakov N. and Altman A. (1985). Tumor promoters in conjunction with calcium ionophores mimic antigenic stimulation by reactivation of alloantigen primed murine T lymphocytes. J. Immunol. 135;3674-3680.

Isakov N., Bleackley C.R., Shaw J. and Altman A. (1985a). Teleocidin and phorbol ester tumor promoters exert similar mitogenic effects on human lymphocytes. Biochem. Biophys. Res. Commun. 130;724-731.

Isakov N., Bleackley C.R. and Shaw J. (1985b). The tumor promoter teleocidin induces IL-2 receptor expression and IL-2 dependant proliferation of human peripheral blood T cells. J. Immunol. 135;2343-2350.

Isakov N., Mally M.I., Scholz W. and Altman A (1987). T lymphocyte activation: The role of protein kinase C and the bifurcating inositol phospholipid signal transduction pathway. Immunol. Rev. 95;89-111.

Johnson H.M., Vallaso T. and Torres B.A. (1985). Interleukin 2-mediated events in gamma-interferon production are calcium dependent at more than one site. J. Immunol. 134;967-970.

Johnson P.M. and Molloy C.M. (1983). Localisation in human term placental bed and amniochorion of cells bearing trophoblast antigens identified by monoclonal antibodies. Am. J. Reprod. Immunol. 4;33-37.

Johnson P.M., Natvig J.B., Ystehede U.A. and Faulk W.P. (1977). Immunological studies of human placentae: The distribution and character of immunoglobulins in chorionic villi. Clin Exp. Immunol. 30;145.

Joshi S.G., Ebert K.M. and Swartz D.P. (1980). Detection and synthesis of a progestagen-dependant protein in human endometrium. J. Reprod. Fertil. 59;273-285.

Josimovich J.B. and MacLaren J.A. (1962). Presence in the human placenta and term serum of a highly lactogenic substance immunologically related to pituitary growth hormone. Endocrinology 79;209-220.

Julkunen M., Apter D., Seppala M., Stenman U.-H. and Bohn H. (1986b). Serum levels of placental protein 14 reflect ovulation in non-conceptual menstrual cycles. Fertil. Steril. 45;47-50.

Julkunen M., Koistinen R., Sjoberg J., Rutanen E.-M., Wahlstrom T. and Seppala M. (1986c). Secretory endometrium synthesises placental protein 14. Endocrinology 118;1782-1786.

Julkunen M., Raiker R.S., Joshi S.G., Bohn H. and Seppala M. (1986a). Placental protein 14 and progestagen-dependant endometrial protein are immunologically indistinguishable. Human Reproduction 1;7-8.

Julkunen M., Rutanen E.-M., Koskimies A., Ranta T., Bohn H. and Seppala M. (1985). Distribution of placental protein 14 in tissue and body fluids during pregnancy. B. J. Obstet. Gynaecol. 92;1145-1151.

Julkunen M., Wahlstrom T. and Seppala M. (1986d). Human fallopian tube contains placental protein 14. Am. J. Obstet. Gynecol. 1545;1076-1079.

Julkunen M., Wahlstrom T., Seppala M., Koistinen R., Koskimies A., Stenman U.-H., and Bohn H. (1984). Detection and localisation of placental protein 14-like protein in human seminal plasma and in the male genital tract. Arch. Androl. 12(suppl);59-67.

Kanellopoulos J.M., De Petris S., Leca G. and Crumpton M.J. (1985). The mitogenic lectin from Phaseolus vulgaris does not recognise the T3 antigen of human T lymphocytes. Eur. J. Immunol. 15;479.

Kaplan D.R., Braciale U.L. and Braciale T.J. (1984). Antigen dependant regulation of interleukin-2 receptor expression on cloned human cytotoxic T lymphocytes. J. Immunol. 133;1966-1969.

Karmani-Arab V., Salehnioghoddam S., Danovitch G., Hirji K. and Rezai A. (1985). Mediation of the anti-proliferative effect of cyclosporin on human T lymphocytes by blockade of interleukin 2 biosynthesis. Transplantation 39;439-442.

Kimball E.S., Pickeral S.F., Oppenheim J.J. and Rossio J.L. (1984). Interleukin 1 activity in normal human urine. J. Immunol. 133;256-260.

King N.J.C., Drake B.L., Maxwell L.E. and Rodger J.C. (1987). Class I major histocompatibility complex antigen expression on early murine trophoblast and its induction by lymphokines in vitro. II The role of gamma interferon in the responses of primary and secondary giant cells. J. Reprod. Immunol. 12;13-21.

Koide S.L., Inaba K. and Stenman R.M. (1987). Interleukin 1 enhances T-dependent immune responses by amplifying the function of dendritic cells. J. Exp. Med. 165;515-530.

Konttinen Y.T., Nordstrom D., Bergroth V., Leirisala-Repo M. and Skrifvars B. (1986). Cell-mediated immune response in the diseased joints in patients with reactive arthritis. Scand. J. Immunol. 23;685-691.

Kristensen F., Walker C., Bettens F., Joncourt F. and de Weck A.L. (1982). Assessment of interleukin-1 and interleukin-2 effects on cycling and non-cycling murine thymocytes. Cell. Immunol. 74;140-149.

Kroner E.E., et al. (1981). Control of arachidonic acid accumulation in bone marrow derived macrophages by acyl transferases. J. Biol. Chem. 256;3690-3697.

Kronke M., Leonard W.J., Depper J.M., and Greene W.C. (1985). Sequential expression of genes involved in human T lymphocyte growth and differentiation. J. Exp. Med. 161;1593-1598.

Kuno M. and Gardner P. (1987). Ion channels activated by inositol 1,4,5-triphosphate in plasma membrane of human T lymphocytes. Nature 326;301-304.

Kupfer A., Singer S.J., Janeway C.A. Jnr. and Swain S.L. (1987). Co-clustering of CD4 (L3T4) molecule with the T cell receptor is induced by specific direct interaction of helper T cells and antigen presenting cells. Proc. Natl. Acad. Sci. USA 84;5888-5892.

Kurt-Jones E.A., Beller D.I., Mizel S.B. and Unanue E.R. (1985a). Production of a membrane-associated interleukin-1 in macrophages. Proc. Natl. Acad. Sci. USA. 82;1204-1208.

Kurt-Jones E.A., Virgin IV H.W. and Unanue E.R. (1986). In vivo and in vitro expression of macrophage membrane interleukin-1 in response to soluble and particulate stimuli. J. Immunol. 137;10-14.

Lachman L.B., Hacker M.P. and Handschumaker R.E. (1977). Partial purification of human lymphocyte activating factor by ultrafiltration and electrophoretic techniques. J. Immunol. 119;2019-2023.

Larsson E.-L., Iscove N.N. and Coutinho A. (1980). Two distinct factors are required for T cell growth. Nature 283;664-666.

Liang S.-M. Allet B., Rose K., Hirschi M., Liang C.M. and Thatcher D.R. (1985). Characterisation of human interleukin 2 derived from Eschericia coli. Biochem. J. 229;429-439.

Liang S.-M., Thatcher D.R., Liang C.-M. and Allet B. (1986). Studies of structure-activity relationships of human interleukin-2. J. Biol. Chem. 261;334-337.

Licastro F., Chiricolo M., Tabacchi P. and Franceschi C. (1987). Simple sugars inhibit proliferation of human T lymphocytes in autologous and allogeneic mixed lymphocyte reactions. Cell. Immunol. 107;15-23.

Lin T.M. and Halbert S.P. (1976). Placental localisation of human pregnancy associated plasma proteins. Science 193;1249-1252.

Lin T.M., Halbert S.P., Kiefer D. and Spellacy W.N. (1974). Three pregnancy-associated human plasma proteins: purification, monospecific antisera and immunological identification. Int. Archs. Allergy App. Immunol. 47;35-53.

Littman B.H., Alpert E. and Rocklin R.E. (1977). The effect of purified alpha-foetoprotein on in vitro assays of cell mediated immunity. Cell. Immunol. 30;35-42.

Loh T.T., Higuchi D.A., Van Bockxmeer F.M., Smith C.H. and Brown E.B. (1980). Transferrin receptors on the human placental microvillous membrane. J. Clin. Invest. 65;1182-1191.

Loke Y.W. (1986). Female Reproductive Immunology. In (Philipp E., Barnes J. and Newton M. eds) Scientific Foundations of Obstetrics and Gynaecology. pp 66-77. Heineman Medical Books.

Lotze M.T., Frana L.W., Sharrow S.O., Robb R.J. and Rosenberg S.A. (1985). In vivo administration of purified interleukin-2. I: Half-life and immunologic effects of the Jurkat cell line derived interleukin-2. J. Immunol. 134;157-166.

Lowenthal J.W., Zubler R.H., Nabholz M., and MacDonald H.R. (1985). Similarities between interleukin-2 receptor number and affinity on activated B and T lymphocytes. Nature 315;669-672.

Lowenthal J.W., Zubler R.H., Sabe H., Ishida N. and Honjo T. (1986). Structure and function of the interleukin-2 receptor: Affinity conversion model. Immunol. Rev. 92;103-119.

Lowry O.H., Rosebrough N.J., Farr A.L. and Randall R.J. (1951). Protein measurement with the Folin Phenol reagent. J. Biol. Chem. 193;265-275.

Lozzio C.B. and Lozzio B.B. (1975). Human chronic Myelogenous leukaemia cell line with positive Philadelphia chromosome. Blood 45;321-324.

Lu C.Y., Changelian P.S. and Unanue E.R. (1984). Alpha-fetoprotein inhibits macrophage expression of Ia antigens. J. Immunol. 132;1722-1727.

Macey M.G. (1988). Flow cytometry: principles and clinical applications. Med. Lab. Sci. 45;165-173.

Male D., Champion B. and Cooke A. (1987). Advanced Immunology. Gower Medical Publishing, London.

Malek T.R., Schmidt J.A. and Shevach E.M. (1985). The murine IL-2 receptor III: Cellular requirements for the induction of IL-2 receptor expression on T cell subpopulations. J. Immunol. 134;2405-2413.

Malek T.R., Ashwell J.D., Germain R.N., Shevach E.M. and Miller J. (1986). The murine interleukin-2 receptor, biochemical structure and regulation of expression. Immunol. Rev. 92;81-101.

Mann D.L. and Sharrow S.O. (1980). HLA-DRw alloantigens can be detected in peripheral blood T lymphocytes. J. Immunol. 125;1889-1896.

March C.J. et al. (1985). Cloning, sequence and expression of two distinct human interleukin-1 complementary DNAs. Nature 315;641-646.

Marrack P. and Kappler J. (1986). The T cell and its receptor. Scientific American Feb. 28-37.

Matthews C.J. and Searle R.F. (1987). The role of prostaglandins in the immunosuppressive effects of supernatants from adherent cells of murine decidual tissue. J. Reprod. Immunol. 12;109-124.

McIntyre J.A. and Faulk W.P. (1979a). Antigens of human trophoblast. Effects of heterologous anti-trophoblast sera on lymphocyte responses in vitro. J. Exp. Med. 149;824-836.

McIntyre J.A. and Faulk W.P. (1979b). Trophoblast modulation of maternal allogeneic recognition. Proc. Natl. Acad. Sci. USA. 76;4029-4033.

McIntyre J.A. and Faulk W.P. (1982). Allotypic trophoblast lymphocyte cross-reactive (TLX) cell surface antigens. Human Immunology 4;27-35.

McIntyre J.A., Hsi B., Faulk W.P., Klopper A. and Thompson R. (1981). Immunological studies of the human placenta. Functional and morphological analysis of pregnancy-associated plasma protein A (PAPP-A). Immunology 44;577-584.

McPhail L.C., Clayton C.C. and Snyderman R. (1984). A potential second messenger role for unsaturated fatty acids; activation of Ca^{2+}-dependant protein kinase. Science 224;622-625.

Mendelsohn J., Multer M.M. and Bernheim J.L. (1977). Inhibition of human lymphocyte stimulation by steroid hormones: Cytokinetic mechanisms. Clin. Exp. Immunol. 27;127-134.

Merluzzi V.J., Welte K., Savage D.M., Last-Barney K. and Mertelsmann R. (1983). Expansion of cyclophosphamide-resistant cytotoxic precursors in vitro and in vivo by purified human interleukin 2. J. Immunol. 131;806-809.

Metcalf J.C., Pozzan T., Smith G.A. and Hesketh T.R. (1980). A calcium hypothesis for the control of cell growth. Biochem. Soc. Symp. 45;1-26.

Meuer S.C., Acuto O., Hussey R.E., Hodgdon J.C., Fitzgerald K.A., Schlossman S.F. and Reinherz E.L. (1983). Evidence for the T3 associated 90kD heterodimer as the T cell antigen receptor. Nature 303;808-810.

Meuer S.C., Hussey R.E., Cantrell D.A., Hodgdon J.C., Schlossman S.F., Smith K.A. and Reinherz E.L. (1984a). Triggering of the T3-Ti antigen receptor complex results in clonal T cell proliferation through an interleukin-2 autocrine pathway. Proc. Natl. Acad. Sci. USA. 81;1509-1513.

Meuer S.C., Hussey R.E., Fabbi M., Fox D., Acuto O., Fitzgerald K.A., Hodgdon J.C., Protentis J.P., Schlossman S.F. and Reinherz E.L. (1984b). An alternative pathway of T cell activation: A functional role for the 50 kd T11 sheep erythrocyte receptor protein. Cell 36;897-906.

Meuer S.C. and Meyer K.-H. (1986). T cell receptor triggering induces responsiveness to interleukin-1 and interleukin-2 but does not lead to T cell proliferation. J. Immunol. 136;4106-4112.

Miedema F. Van Oostreen J.W., Sauerwein R.W., Terpstra F.G., Aarden L.A. and Melief C.J. (1985). Induction of immunoglobulin synthesis by interleukin 2 is $T4^+/T8^+$ cell dependent. A role for interleukin 2 in the pokeweed mitogen-driven system. Eur. J. Immunol. 15;107-112.

Mills G.B., Cheung R.K., Grinstein S. and Gelfand E.W. (1985). Interleukin 2 induced lymphocyte proliferation is independent of increases in cytosolic-free calcium concentrations. J. Immunol. 134;2431-2435.

Mire A.R., Wickremasinghe R.G., Michalevicz R. and Hoffbrand A.V. (1985). Interleukin-2 induces rapid phosphorylation of a 85 kDa protein in permeabilised lymphocytes. Biochem. Biophys. Acta. 874;159-163.

Mitchison V.A., Rajewsky K. and Taylor R.B. (1970). Co-operation of antigenic determinants and of cells in the induction of antibodies. In (Stertzl J. and Riha I. eds.) Developmental Aspects of Antibody Formation and Structure Vol 2. Academic Press, New York pp. 547-561.

Mizel S. (1987). Interleukin 1 and T cell activation. Immunology Today 8;330-331.

Mizel S.B. and Mizel D. (1981). Purification to apparent homogeneity of murine interleukin-1. J. Immunol. 126;834-837.

Modlin R.L., Brenner M.B., Krangel M.S., Dulby A.D. and Bloom B.R. (1987). T cell receptors of human suppressor cells. Nature 329;541-545.

Morgan D.A., Ruscetti F.W. and Gallo R.C. (1976). Selective in vitro growth of T lymphocytes from normal human bone marrow. Science 193;1007-1008.

Mori T., Kobayashi H., Nishimoto H., Suzuki A. and Nishimura T. (1977). Inhibitory effect of progesterone and 20 alpha-hydroxy pregn-4-en-3-one on the phytohaemagglutinin-induced transformation of human lymphocytes. Am. J. Obstet. Gynecol. 127;151-157.

Morisada M., Yamaguchi H. and Iizuka R. (1976). Immunobiological function of syncytiotrophoblast; a new theory. Am. J. Obstet. Gynecol. 125;3-16.

Morris P.J. and Ting A. (1982). Studies of HLA-DR with relevance to renal transplantation. Immunol. Rev. 66;103-131.

Morse J.H., Stearns G., Arden J., Agosto G.M. and Canfield R.E. (1976). The effects of crude and purified human gonadotrophin on in vitro stimulated human lymphocyte cultures. Cell. Immunol. 25;178-188.

Morton H., Rolfe B. and Cavanagh A. (1982). Early pregnancy factor, biology and clinical significance. In (Grudzinskas J.G., Teisner B. and Seppala M. eds.) Pregnancy Proteins, Biology, Chemistry and Clinical Application. Academic Press, London pp 391-405.

Mowbray J.F. (1986). Effect of immunisation with paternal cells on recurrent spontaneous abortion. In (Clark D.A. and Croy B.A. eds). Reproductive Immunology. pp 269-276. Elsevier, Amsterdam.

Mowbray J.F., Gibbings. C.R., Sidgwick A.S., Ruszkiewicz M. and Beard R.W. (1983). Effects of blood transfusion in women with recurrent abortion. Transplant. Proc. 10;896-899.

Muchmore A.V. and Decker J.M. (1985). Uromodulin: A unique 82-kilodalton immunosuppressive glycoprotein isolated from urine of pregnant women. Science 229;479-481.

Muchmore A.V., Decker J.M. and Blaese R.M. (1980). Evidence that specific oligosaccharides block early events necessary for the expression of antigen-specific proliferation by human lymphocytes. J. Immunol. 125;1306.

Mukherjee A.B., Ulane R.E. and Agrawal A.K. (1982). Role of uteroglobulin and transglutaminase in masking antigenicity of implanting embryos. Am. J. Reprod. Immunol. 2;135-141.

Munoz C.G., Keusch G.T. and Dinarello C.A. (1987). A new micromethod for determination of interleukin-1 production from frozen human blood mononuclear cells. J. Immunol. Methods 99;123-127.

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

Nancarrow C.D., Evison B.M., Scaramuzzi R.J. and Turnbull K.E. (1979). Detection of induced death of embryos in sheep by rosette inhibition test. J. Reprod. Fertil. 57;385-389.

Nebel -L., Rudak E., Mashiah S., Dor J. and Goldman B. (1985). Mal-implantation caused by trophoblastic insufficiency resulting in failure of gestation following in vitro fertilisation-embryo transfer. In (Toder V. and Beer A.E. eds). Contributions to Obstetrics and Gynaecology. vol 14. pp170-175. Karger, Basel.

Neckers L.M. and Cossman J. (1983). Transferrin receptor induction in mitogen stimulated human T lymphocytes is required for DNA synthesis and cell division and is regulated by interleukin-2. Proc. Natl. Acad. Sci. USA. 80;3494-3498.

Nedwin G.E., Svedersky L.P., Bringman T.S., Palladino M.A. and Goeddel D.V. (1985). Effect of interleukin 2, interferon-gamma, and mitogens on the production of tumor necrosis factors alpha and beta. J. Immunol. 135;2492-2497.

Neufeld E. and Ashwell G. (1979). Carbohydrate recognition systems for receptor mediated pinocytosis. In (Lennarz W. ed.) Biochemistry of Glycoproteins and Proteoglycans. N.Y. Plenum pp 241s.

Nicholas N.S., Panayi G.S. and Nouri A.M.E. (1984). Human pregnancy serum inhibits interleukin-2 production. Clin. Exp. Immunol. 58;587-595.

Nicholas N.S., Panayi G.S., Murphy J. and Pitzalis C. (1986). Human retroplacental sera inhibits the expression of class II major histocompatibility antigens. J. Reprod Immunol. 9;95-102.

Nicola N.A. and Peterson L. (1986). Identification of distinct receptors for two haemopoietic growth factors (granulocyte colony-stimulating factor and multipotential colony-stimulating factor) by chemical cross linking. J. Biol. Chem. 261;12384-12389.

Ninio E., Mencia-Huerta J.M., Heymans F. and Benveniste J. (1982). Biosynthesis of platelet activating factor I. Evidence for an acetyl transferase activity in murine macrophages. Biochim. Biophys. Acta. 710;23-31.

O'Flynn K., Krensky A.M., Beverley P.C.S., Burakoff S.J. and Lunch D.C. (1985). Phytohaemagglutinin activation of T cells through the sheep red blood cell receptor. Nature 313;686-687.

Ohnishi S.T. and Barr J.K. (1978). A simplified method of quantitating protein using the Biuret and Phenol reagents. Analytical Biochemistry 86;193-200.

O'Neill C., Gidley-Baird A.A., Pike I.L., Porter R.N., Sinosich M.J. and Saunders D.M. (1984). Maternal blood platelet physiology and luteal phase endocrinology as a means of monitoring pre- and post-implantation embryo viability following in vitro fertilisation. J. in Vitro Fertil. Embryo Transfer 2;59-65.

Oppenheim J.J., Kovacs E.J., Matsushima K. and Durum S. (1986). There is more than one interleukin-1. Immunology Today 7;45-56.

Orozco C., Perkins T. and Clarke F.M. (1986). Platelet activating factor induces the expression of early pregnancy factor activity in female mice. J. Reprod. Fertil. 78;549-555.

Osawa H., Josimovic-Alasevic O. and Diamantstein T. (1986a). Enzyme-linked immunosorbent assay of mouse interleukin-2 receptors. J. Immunol. Methods 92;109-115.

Osawa H., Josimovic-Alasevic O. and Diamantstein T. (1986b). Interleukin-2 receptors are released by cells in vitro and in vivo I: Detection of soluble IL-2 receptors in cell culture supernatants and in the serum of mice by an immunoradiometric assay. Eur. J. Immunol. 16;467-469.

Pakac F. and Siegert R. (1982). Chemical characterisation of a rabbit leukocytic pyrogen. Eur. J. Biochem. 127;375-380.

Palacios R. (1982). Mechanisms of T cell activation: Role and functional relationship of HLA-DR antigens and interleukins. Immunol. Rev. 63;73-110.

Papiz M.Z. et al. (1986). The structure of beta-lactoglobulin and its similarity to plasma retinol binding protein. Nature 324;383-385.

Parmely M.J. and Thompson J.S. (1976). Effect of AFP and other serum factors derived from hepatoma-bearing rats on the mixed lymphocyte culture. J. Immunol. 117;1832-1836.

Pavia C.S. and Stites D.P. (1981). Humoral and cellular regulation of alloimmunity in pregnancy. J. Immunol. 123;2194-2200.

Pearlstein K.T., Palladino M.A., Welte K. and Vilcek J. (1983). Purified human interleukin-2 enhances induction of immune interferon. Cell. Immunol. 80;1-9.

Pervais S. and Brew K. (1985). Homology of lactoglobulin, human serum retinol binding protein, and protein HC. Science 228;335-337.

Petrunin D.D., Gryaznova I.M., Petrunina I.A. and Tatarinov Y.S. (1976). Immunological identification of organ specific human placental alpha2-globulin and its content in amniotic fluid. Bull. Exp. Biol. Med. USSR 82;994-996.

Petrunin D.D., Gryaznova I.M., Petrunina I.A. and Tatarinov Y.S. (1978). Comparative immunochemical and physicochemical characteristics of human chorionic alpha1- and alpha2-microglobulins. Bull. Exp. Biol. Med. USSR 85;658-661.

Pinto-Furtado L.G., Bolton A.E., Grudzinskas J.G., Chapman M.G., Sinosich M.J. and Sharma V. (1984). The development and validation of a radioimmunoassay for human pregnancy-associated plasma protein A (PAPP-A). Arch. Gynecol. 236;83-91.

Pockley A.G., Barratt C.L.R. and Bolton A.E. (1988a). The immunosuppressive effects of human cervical mucus. B. J. Obstet. Gynaecol. in the press.

Pockley A.G., Barratt C.L.R. and Bolton A.E. (1988b). Placental protein 14 content and immunosuppressive activity of human cervical mucus. In Soc. Exp. Biology Seminar series, Cold Spring Harbor. in the press.

Porias H., Sojo I., Carranco A., Gonzales-Martinez R. and Cortes-Gallegos V. (1978). A simultaneous assay to quantitate plasma and endometrial hormone concentrations. Fertil. Steril. 30;66-69.

Power D.A. et al. (1983). The fetus is an allograft; evidence for protective antibodies to HLA-linked paternal antigens. Lancet ii;701-704.

Redman C.W.G., McMichael A.J., Stirrat G.M., Sunderland C.A. and Ting A. (1984). Class I major histocompatibility complex antigens on human extra villous trophoblast. Immunology 52;457-468.

Reem G.H. and Yeh N.H. (1984). Interleukin-2 regulates expression of its receptor and synthesis of gamma-interferon by human T lymphocytes. Science 225;429-430.

Reske-Kunz A.B., Von Steldern D., Rude E., Osawa H. and Diamantstein T. (1984). Interleukin-2 receptors on an insulin-specific T cell line: Dynamics of receptor expression. J. Immunol. 133;1356-1361.

Ritz J., Campen T.J., Schmidt R.E., Royer H.D., Hercend T., Hussey R.E. and Reinherz E.L. (1985). Analysis of T-cell receptor gene rearrangement and expression in human natural killer clones. Science 228;1540-1543.

Robb R.J. (1984). Interleukin 2; the molecule and its function. Immunology Today 5;203.

Robb R.J. and Greene W.C. (1987). Internalisation of interleukin 2 is mediated by the beta chain of the high affinity interleukin 2 receptor. J. Exp. Med. 165;1201-1206.

Robb R.J., Greene W.C. and Rusk C.M. (1984). Low and high affinity cellular receptors for interleukin-2; implications for the level of Tac antigen. J. Exp. Med 160;1126-1146.

Robb R.J., Mayer P.C. and Garlick R. (1985). Retention of biological activity following radioiodination of human interleukin-2: Comparison with biosynthetically labelled growth factor in receptor binding assays. J. Immunol. Methods 81;15-30.

Robb R.J., Munck A. and Smith K.A. (1981). T cell growth factor receptors. Quantitation, specificity and biological relevance. J. Exp. Med. 154;1455-1474.

Robb R.J. and Smith K.A. (1981). Heterogeneity of human T cell growth factor due to glycosylation. Mol. Immunol. 18;1087-1094.

Rocklin R.E., Kitzmiller J.L. and Kaye M.D. (1979). Immunobiology of the maternal-fetal relationship. Ann. Rev. Med. 30;375-404.

Rolfe B.E., Morton H. and Clarke F.M. (1983). Early pregnancy factor is an immunosuppressive contaminant of commercial preparations of human chorionic gonadotrophin. Clin. Exp. Immunol. 51;45-52.

Rosen S.W., Kaminska J., Calvert I.S. and Aaronson S.A. (1979). Human fibroblast produce 'pregnancy specific' beta-1-glycoprotein in vitro. Am. J. Obstet. Gynecol. 134;734-738.

Ross G., Dixon J.B. and Veevers A (1987). Variation in the lymphocyte transformation assay- slope analysis of cell dose-response curves. J. Immunol. Methods. 98;189-193.

Rozengurt E. (1981). Stimulation of Na^+ influx, Na-K pump activity and DNA synthesis in quiescent cultured cells. Adv. Enzyme Regul. 19;61-85.

Rubin L.A., Kurman C.C., Fritz M.E., Biddison W.E., Boutin B., Yarchoan R. and Nelson D.L. (1985). Soluble interleukin-2 receptors are released from activated human lymphoid cells in vitro. J. Immunol. 135;3172-3177.

Sabe H., et al. (1986). Properties of human interleukin-2 receptors expressed on non lymphoid cells by DNA transfection. Mol. Biol. Med. 2;379-396.

Saizawa K., Rojo J. and Janeway C.A. (1987). Evidence for a physical association of CD4 and the CD3:alpha:beta T cell receptor. Nature 328;260-263.

Sargent I.L., Arenas J. and Redman C.W.G. (1987). Maternal cell mediated senstisation to paternal HLA may occur, but it is not a regular event in normal human pregnancy. J. Reprod. Immunol. 10;111-120.

Sargent I.L. and Redman C.W.G. (1985). Maternal cell mediated immunity to the fetus in human pregnancy. J. Reprod. Immunol. 7;95-104.

Scatchard D. (1949). The attractions of proteins for small molecules and ions. Ann. N.Y. Acad. Sci. 51;660

von Schoultz B. and Stigbrand T. (1973). Purification of the 'pregnancy zone protein'. Acta Obstet. Gynecol. Scand. 52;51-57.

von Schoultz B. and Stigbrand T. (1982). Pregnancy zone protein: Chemistry, biology and clinical studies. In (Grudzinskas J.G., Teisner B. and Seppala M. eds.) Pregnancy Proteins, Biology, Chemistry and Clinical Application. Academic Press, London pp 167-175.

von Schoultz B., Stigbrand T. and Tarnvik A. (1973). Inhibition of PHA induced lymphocyte stimulation by the pregnancy zone protein. FEBS Lett. 38;23-26.

Schroder J. (1975). Are fetal cells in maternal blood mainly B lymphocytes? Scand. J. Immunol. 4;279-285.

Seppala M., Rutanen E,-M., Koistinen R., Julkunen M., Koskimies A.I. and Wahlstrom T. (1985). Placental proteins in early events of human reproduction. In (Bischof P. and Klopper A. eds) Proteins of the Placenta. 5th Int. Congr. on Placental Proteins, Annecy 1984. pp 123-128. Karger, Basel.

Shackelford D.A. and Trowbridge I.S. (1984). Induction of expression and phosphorylation of the human interleukin-2 receptor by a phorbol diester. J. Biol. Chem. 259;11706-11712.

Shirakawa F., Tanaka Y., Ota T., Suzuki H., Eto S and Yamashita U. (1987). Expression of interleukin 1 receptors on human peripheral T cells. J. Immunol. 138;4243-4248.

Shohat B., Hirsch M., Jardena O., Henry J., Levy I. and Trainin N. (1986). Cellular immune aspects of the human fetal-maternal relationship. Am. J. Reprod. Immunol. Microbiol. 11;125-129.

Sinosich M.J., Davey M.W., Teisner B. and Grudzinskas J.G. (1983). Comparative studies of pregnancy-associated plasma protein-A and alpha2-macroglobulin using metal chelate chromatography. Biochem. Int. 7;33-42.

Sinosich M.J., King M., Bonifacio M.D., Nelson D. and Saunders D.M. (1984). Pregnancy-associated plasma protein-A in human seminal plasma. In (Peeters H. ed) Protides in Biological Fluids. vol 32. pp 289-292. Pergamon Press, Oxford.

Sinosich M.J., Teisner B., Davey M. and Grudzinskas J.G. (1981). Pregnancy-associated plasma protein-A; interaction with heparin in crossed affinity immunoelectrophoresis. Aust. N.Z. J. Med. 11;429-433.

Smith K.A. (1984). Interleukin 2. Ann. Rev. Immunol. 2;319-333.

Smith K.A. (1987). The two chain structure of high affinity IL-2 receptors. Immunology Today 8;11-13.

Smith K.A. (1988). The bimolecular structure of the interleukin 2 receptor. Immunology Today 9;36-37.

Smith K. and Cantrell D. (1985). Interleukin-2 regulates its own receptors. Proc. Natl. Acad. Sci. USA 82;864-868.

Smith R.N. and Sternlicht M. (1982). The alloantibody response in the allogeneically pregnant rat III: The localisation of the alloantibody forming cells. J. Immunol. 129;783-786.

Smithies O. (1959). An improved procedure for starch-gel electrophoresis: Further variations in the serum proteins of normal individuals. Biochem. J. 71;585-587.

Starkey P. (1987). Reactivity of human trophoblast with an antibody to the HLA class II antigen HLA-DP. J. Reprod. Immunol. 11;63-70.

Stimson W.H. (1976). Studies on the immunosuppressive properties of a pregnancy associated alpha macroglobulin. Clin. Exp. Immunol. 25;199-206.

Stites D.P. and Siiteri P.K. (1983). Steroids as immunosuppressants in pregnancy. Immunol. Rev. 75;117-137.

Strelkausas A.J., Wilson B.S., Dray S. and Dodsen M. (1975). Inversion of human T and B cell levels in early pregnancy. Nature 258;331-333.

Suciu-Foca N. et al. (1983). Anti-idiotype antibodies to anti-HLA receptors induced by pregnancy. Proc. Natl. Acad. Sci. USA 80;830-834.

Sunderland C.A., Naiem M., Mason D.Y., Redman C.W.G. and Stirrat G.M. (1981a). The expression of major histocompatibility antigens by human chorionic villi. J. Reprod. Immunol. 3;323-331.

Sunderland C.A., Redman C.W.G. and Stirrat G.M. (1981b). HLA-A,B,C antigens are expressed on non-villous trophoblast of the early human placenta. J. Immunol. 127;2614-2615.

Sutcliffe R.G., Bolton A.E., Sharp F., Nicholson L.V.B. and Mackinnon R. (1980). Purification of human alpha uterine protein. J. Reprod. Fertil. 58;435-442.

Sutcliffe R.G., Joshi S.G., Paterson W.F. and Bank J.F. (1982). Serological identity between human alpha uterine protein and human progetagen-dependant endometrial protein. J. Reprod. Fertil. 65;207-209.

Sutton L., Mason D.Y. and Redman C.W.G. (1983). HLA-DR positive cells in the human placenta. Immunology 49;103-112.

Swain S.L., Dutton R.W., Schwab R. and Yamamoto J. (1983). Xenogenic human anti-mouse T cell responses are due to the activity of the same functional T cell subsets responsible for allospecific and major histocompatibility complex-restricted responses. J. Exp. Med. 157;720-729.

Tada T. and Okumura K. (1979). The role of antigen-specific T cell factors in the immune response. Adv. Immunol. 28;1-87.

Tatarinov Y.S., Falaleeva D.M., Kalaschnikov V.V. and Toloknov B.O. (1976). Immunoflourescent localisation of human pregnancy-specific beta-globulin in placenta and chorioepithelioma. Nature 260;262-263.

Tatsumi K., Mori T., Mori E., Kanzaki H., and Mori T. (1987). Immunoregulatory factor released from a cell line derived from human decidual tissue. Am. J. Reprod. Immunol. and Microbiol. 13;87-92.

Taylor P.V. and Hancock K.W. (1975). Antigenicity of trophoblast and possible antigen masking effects during pregnancy. Immunology 28;973-982.

Terasaki P.I., Mickey M.R., Yamazaki J.N. and Vredevoe D. (1970). Maternal-fetal incompatibility I. Incidence of HLA-A antibodies and possible association with congenital anomalies. Transplantation 9;538-543.

Teshigawara K., Wang H.-M., Kato K. and Smith K.A. (1987). Interleukin 2 high affinity receptor expression requires two distinct binding proteins. J. Exp. Med. 165;223-238.

Tojo S., Ashitaki Y., Maruo T., Matsura S. and Nishimura R. (1982). The biology and chemistry of human chorionic gonadotrophin. In (Grudzinskas J.G., Teisner B. and Seppala M. eds.) Pregnancy Proteins, Biology, Chemistry and Clinical Application. Academic Press pp 25-38.

Tomasi T.B. (1978). Suppressive factors in amniotic fluid and newborn serum: Is alpha foeto-protein involved? Cell. Immunol. 37;459-466.

Tongio M.M., Berrebi A. and Mayer S. (1972). A study of lymphocytotoxic antibodies in multiparous women having had at least four pregnancies. Tissue Antigens 2;378-388.

Toop K.M. and Klopper A. (1983). Effect of anticoagulant on the measurement of pregnancy associated plasma protein A (PAPP-A). B. J. Obstet. Gynaecol. 90;150-155.

Truneh A., Albert F., Golstein P. and Scmitt-Verhulst A.-M. (1985). Calcium ionophore plus phorbol ester can substitute for antigen in the induction of cytolytic T lymphocytes from specifically primed precursors. J. Immunol. 135;2262-2267.

Unanue E.R. (1981). The regulatory role of macrophages in antigenic stimulation II: Symbiotic relationship between lymphocytes and macrophages. Adv. Immunol. 31;1-136.

Unanue E.R., Beller D.I., Lu C.Y. and Allen P.M. (1984). Antigen presentation: Comments on its regulation and mechanism. J. Immunol. 132;1-5.

Valentine M.A., Tsoukas C.D., Rhodes G., Vaughan J.H. and Carson D.A. (1985). Phytohaemagglutinin binds to the 20 kDa molecule of the T3 complex. Eur. J. Immunol. 15;851-854.

Vanderbeeken Y., Vlieghe M.P., Delespesse G. and Dauchateau J. (1982). Characterisation of immunoregulatory T cells during pregnancy by monoclonal antibodies. Clin. Exp. Immunol. 48;118-120.

Van Vlasselaer P., Goebels J. and Vandepulte M. (1986). Inhibition of lymphocyte aggregation by progesterone. J. Reprod. Immunol. 9;111-121.

Vercammen C. and Ceupens J.L. (1987). Prostaglandin E_2 inhibits human T-cell proliferation after cross-linking of the CD3-Ti complex by directly affecting T cells at an early step of the activation process. Cell. Immunol. 104;24-36.

Wagner D.K., Kiwanuka J., Edwards B.K., Rubin L.A., Nelson D.L. and Magrath I.T. (1987). Serum interleukin-2 receptor levels in patients with undifferentiated and lymphoblastic lymphomas: Correlation with survival. J. Clin. Oncology 5;1262-1274.

Waites G.T., Wood P.L., Walker R.A. and Bell S.C. (1988). Immunohistological localisation of human endometrial secretory protein, 'pregnancy-associated endometrial alpha2-globulin' (alpha2-PEG), during the menstrual cycle. J. Reprod. Fertil. 82;665-672.

Waldmann T.A., Goldman C.K., Robb R.J., Depper J.M., Leonard W.J., Sharrow S.O., Bonjiovanni K.F., Korsmeyer S.J. and Greene W.C. (1984). Expression of interleukin-2 receptors on activated human B cells. J. Exp. Med. 160;1450-1456.

Wang H.-M. and Smith K.A. (1987). The interleukin 2 receptor; functional consequences of its bimolecular structure. J. Exp. Med. 166;1055-1069.

Wang H.S., Kanzaki H., Yoshida M., Sato S., Tokushige M. and Mori T. (1987). Suppression of lymphocyte reactivity in vitro by supernatants of explants of human endometrium. Am. J. Obstet. Gynecol. 157;956-963.

Watson J.D. (1986). Cytokines and their receptors. Immunology Today 7;320-321.

Weiss A., Imboden J., Hardy J., Manger B., Terhorst C. and Stobo J. (1986). The role of the T3/antigen receptor complex in T cell activation. Ann. Rev. Immunol. 4;593-619.

Welte K., Andreef M., Platzer E., Holloway K., Rubin Y.Y., Moore M.A.S. and Mertelsman R. (1984). Interleukin-2 regulates the expression of Tac antigens on peripheral blood lymphocytes. J. Exp. Med. 160;1390-1403.

Welte K., Yi-Wang C., Mertelsman R., Venuta S., Feldman S.P. and Moore M.A.S. (1982). Purification of human interleukin-2 to apparent homogeneity and its molecular heterogeneity. J. Exp. Med. 156;454-464.

Werdelin O. (1987). T cells recognise antigen alone and not MHC molecules. Immunology Today 8;80-84.

Westwood O.M.R., Chapman M.G., Totty N., Philp R., Bolton A.E., and Lazarus N.R. (1988). N-terminal sequence analysis of placental protein 14, purified in high yield from decidual cytosol. J. Reprod. Fertil. 82;493-500.

Wilkinson M and Morris A. (1984). The E receptor regulates interferon-gamma production: Four receptor model for human lymphocyte activation. Eur. J. Immunol. 14;708-713.

Williams J.M., Deloria D., Hansen J.A., Dinarello C.A., Loertscher R., Shapiro H.M. and Strom T.B. (1985). The events of primary T cell activation can be staged by the use of Sepharose bound anti-T3 (64.1) monoclonal antibody and purified interleukin 1. J. Immunol. 135;2249-2255.

Wood D.D. (1984). Antigen non-specific factors elaborated by macrophages which stimulate lymphocytes: Interleukin-1. In (Bellanti J.A. and Herscovitz H.B. eds.) The Reticuloendothelial System: A Comprehensive Treatise. Vol. 6. Plenum Publishing Corp. New York p 201.

Wynn R.M. (1974). Ultrastructural development of the human decidua. Am. J. Obstet. Gynecol. 118;652-670

Yachnin S. and Svenson R. (1972). The immunological and physicochemical properties of mitogenic proteins derived from Phaseolus vulgaris. Immunology 22;871-873.

Zinkernagel R.M. and Doherty P.C. (1975). H-2 compatibility requirement for T cell mediated lysis of target cells infected with lymphocytic choriomeningitis virus. Different cytotoxic T cell specificities are associated with structures coded for in H-2K or H-2D. J. Exp. Med. 141;1427-1436.

APPENDIX I

Vacutainers (143 USP units LiHeparin).	Beckton Dickinson (UK) Ltd.
Dulbecco's modification of Eagle's medium (DMEM).	Flow Laboratories. Northumbria Biologicals Ltd.
Lymphopaque separation medium (relative density 1.084)	Nycomed UK Ltd.
Plastic universals	Sterilin Ltd. Northern Media Supplies.
Penicillin/streptomycin solution	Flow Laboratories. Northumbria Biologicals Ltd.
Cell culture grade biochemicals	Northumbria Biologicals Ltd.
SFI serum-free hybridoma growth medium.	Northumbria Biologicals Ltd.
Trypan blue dye	Flow Laboratories.
Cell culture grade PBS	Northumbria Biologicals Ltd.
96 well and 24 well tissue culture plates	Linbro, Flow Laboratories.
Tritiated thymidine (90 Ci/mmol)	Amersham International Ltd.
CO_2 incubator (Model 1500)	Flow Laboratories.
Laminar flow hood (Gelaire TC 48)	Flow Laboratories.
Semi-automated cell harvester	Skatron AS, Lier, Norway.
Trypsin EDTA	Flow Laboratories.
Liquid scintillation counter (Rackbeta 1212)	LKB Instruments Ltd.
Scintillation cocktail 'O'	BDH Chemicals Ltd.
LKB 'HiSafe' scintillant	LKB Instruments Ltd.
Phytohaemagglutinin (PHA)	Sigma Chemical Co. Ltd.
Phenyl methyl sulphonyl fluoride (PMSF)	Sigma Chemical Co. Ltd.
INTERTEST IL-2 ELISA test kit	Genzyme Corpn., USA.
CELL-FREE IL-2R test kit	T Cell Sciences Inc., USA.
SUCROSEP gamma interferon test kit	Boots-Celltech Diagnostics Ltd.
Recombinant IL-2 (ala-125)	Amersham International.
10mm x 64mm disposable polystyrene tubes (LP3)	Northern Media Supplies.
FITC-conjugated goat anti-mouse IgG antibody	Caltag Laboratories, USA.

Monoclonal antibodies for FACS analysis	DAKO Ltd.
Monoclonal anti IL-2 antibody	Genzyme Corpn., USA.
RPMI 1640 growth medium	Northumbria Biologicals Ltd. Flow Laboratories.
Foetal calf serum	Northumbria Biologicals Ltd. Flow Laboratories.
^{125}I-IL-2	Amersham International.
12mm x 75mm disposable polystyrene tubes (LP4)	Northern Media Supplies.
Silicone fluid DC 550	BDH Chemicals Ltd.
Liquid paraffin	BDH Chemicals Ltd.
Gamma counter (LKB Multigamma)	LKB Instruments Ltd.
Na^{125}I (IMS 30)	Amersham International.
Horse serum	Sigma Chemical Co. Ltd.
Concanavalin-A-Sepharose	Sigma Chemical Co. Ltd.
Non-immune rabbit serum	Welcome Reagents Ltd.
Donkey anti-rabbit serum	Immunodiagnostics Ltd.
Polyethylene glycol (PEG 6000)	Sigma Chemical Co. Ltd.
Cyanogen-bromide activated Sepharose-4B	Sigma Chemical Co. Ltd.
Bovine serum albumin (BSA fraction V)	Sigma Chemical Co. Ltd.
Folin and Ciocalteau phenol reagent	Sigma Chemical Co. Ltd.
Freund's complete/incomplete adjuvant	Sigma Chemical Co. Ltd.
LKB Multiphor electrophoresis equipment	LKB Instruments Ltd.
ELISA plate reader (CLS 962)	Cambridge Life Sciences plc.
Glass fibre filter paper (for manual cell harvesting)	Whatman Ltd.

All other chemicals used were of reagent grade and obtained from either Sigma Chemical Co. Ltd. or BDH Chemicals Ltd.

APPENDIX II

Buffers and Reagents

For two-dimensional crossed immunoelectrophoresis.

Tris/Barbitone buffer (pH 8.6)	22.4 g Diethylbarbituric acid 44.3 g Tris 0.533 g Ca-lactate 0.650 g sodium azide (NaN_3) Add distilled water to 1 litre to give a concentrated buffer. Dilute 1:4 before use.
Agarose gel layer	1% agarose in barbitone buffer. Gel formed by steaming at 100°C.
Washing solution	0.1 M NaCl.
Staining solution	1 g Coomassie Brilliant Blue 90 ml ethanol 20 ml glacial acetic acid 90 ml H_2O.
Destaining solution	The same as the staining solution but without Coomassie Brilliant Blue.

For Protein assay.

BS7 Reagent	1 volume of Biuret reagent in 7 volumes of 2.3% sodium carbonate.
Biuret Diluent	5 g KI in 0.25 mol NaOH/l

Stock Biuret Reagent	15.0 g of finely pulverised $CuSO_4.5H_2O$ were dissolved in 70 - 80 ml water. A solution of 45.0 g potassium sodium tartrate, tetrahydrate in 600 - 700ml biuret diluent was prepared and slowly to which was added the $CuSO_4$ solution with stirring. Both solutions were at room temperature when mixed to prevent reduction of Cu^{2+} by the tartrate. Biuret diluent was added to a volume of 1000ml. Before use the solution was filtered through qualitative paper to remove any deposited cuprous oxide. For the working biuret, the stock biuret was diluted 5 fold with biuret diluent.