INVESTIGATION OF IMMUNOREGULATORY FACTORS FROM HUMAN DECIDUA

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ABSTRACT

During normal pregnancy, the fetus is not rejected by the maternal immune system despite bearing paternal MHC antigen. It has been proposed that this is due to the local production of immunoregulatory factors by the maternal decidua. This study was designed to investigate immunosuppressive factors derived from early human decidua.

In the first part of the study, assays were standardised in order to monitor the immunoregulatory activity of factors derived from human decidua. These included mitogen induced lymphocyte proliferation, mixed lymphocyte culture, and the proliferation of human monocytic and T lymphoid cell lines.

Using these assays, two fractions from the FPLC separation of the 10-100 KDa crude decidual supernatant with immunosuppressive activity have been identified and partially characterised. One contained molecules of 183 KDa under non-reducing SDS-PAGE analysis, and 70 and 29 KDa under reducing conditions (large molecular weight fraction; LMWF). Another had molecules of 7-14 KDa under non-reducing conditions (small molecular weight fraction; SMWF). Both of them inhibited mitogen-induced lymphoproliferation and the mixed lymphocyte reaction. In addition they inhibited the growth of a monocytic cell line (Mono Mac 6), but not that of a T lymphoblastic cell line (Jurkat E6.1). The suppression of cellular growth by the LMWF, but not the SMWF, was due to the arrest of the cells in the G0/G1 phase of the cell cycle. Neither fraction affected the expression of MHC class II on Mono
Mac 6 cells.

The decidua-derived suppressive factors showed some similarity to human transforming growth factor type β (hTGFβ), but their activity could not be eliminated by neutralising antibodies to hTGFβ1 and β2. In contrast to the decidua factors, hTGFβ1 was able to significantly suppress the expression of MHC class II molecules. In addition, acidification of LMWF decreased their suppressive activity, whereas others have shown that similar treatment of TGFβ isolated from decidua enhanced its activity. Although some similarity to human decidua-associated protein hDP200 could be demonstrated with LMWF, IgG-like molecules could not be demonstrated in the latter. A low molecular weight protein with immunosuppressive activity-PP14- has been demonstrated in human decidua. Despite similarity, the SMWF clearly showed a distinct M.w pattern distribution to PP14 when analysed by Western blot. This suggests that SMWF was not PP14.

In the final part of the study, the supernatants of decidual mononuclear cell cultures (non-fractionated or fractionated) were used to investigate the cells which may produce the factors. The preliminary results suggest that non-leukocytic decidua cells are unlikely to be responsible for the activity. Either T lymphocytes or NK cells are the more likely source, as the suppressive activity was largely increased in populations enriched for these cells, and concanavalin A enhanced their suppressive activity. However, the precise cellular source of these factors needs to be elucidated further.
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<tr>
<td>Ab</td>
<td>antibody</td>
</tr>
<tr>
<td>ADCC</td>
<td>antibody-dependent cell-mediated cytotoxicity</td>
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<tr>
<td>Ag</td>
<td>antigen</td>
</tr>
<tr>
<td>Ab</td>
<td>antibody</td>
</tr>
<tr>
<td>APC</td>
<td>antigen presenting cell</td>
</tr>
<tr>
<td>Con A</td>
<td>Concanavalin A</td>
</tr>
<tr>
<td>CDPF</td>
<td>crude decidual protein fractions</td>
</tr>
<tr>
<td>CPM</td>
<td>counts per minute</td>
</tr>
<tr>
<td>CR</td>
<td>complement receptor</td>
</tr>
<tr>
<td>CTL</td>
<td>cytotoxic T lymphocyte</td>
</tr>
<tr>
<td>DCS</td>
<td>Supernatant of decidual cell culture</td>
</tr>
<tr>
<td>DMNC</td>
<td>decidual mononuclear cells</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>DF</td>
<td>decidual chromatographic fractions</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>FC</td>
<td>fragment crystallizable of Ig</td>
</tr>
<tr>
<td>FCS</td>
<td>foetal calf serum</td>
</tr>
<tr>
<td>FITC</td>
<td>fluoresceine isothiocyanate</td>
</tr>
<tr>
<td>FL</td>
<td>fluorescence</td>
</tr>
<tr>
<td>FPLC</td>
<td>Fast protein liquid chromatography</td>
</tr>
<tr>
<td>HLA</td>
<td>human leukocyte antigen</td>
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<tr>
<td>ICAM</td>
<td>intercellular adhesion molecule</td>
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</table>
Ig  immunoglobulin
LFA  leucocyte function associated antigen
IFN  Interferon
LGL  large granular lymphocyte
IL  interleukin
LMWF  large molecular weight fraction
       (decidual chromatograpgy fraction No. 6-11)
MACS  magnetic activated cell sorter
MoAb  monoclonal antibody
MHC  major histocompatible complex
MLR  mixed lymphocyte culture reaction
MTT  3-4,5-Dimethylthiazol -2-y]-2, 5-diphenyltetrazolium
       bromide
NK  natural killer cell
NMWC  normal molecular weight cut-off membrane
OD  optical density
PBMNC  peripheral blood mononuclear cells
PBS  phosphate buffered saline
PE  phycoerythrin
PG  prostaglandin
PHA  phytohemagglutinin
PI  Propidium iodide
PR-  without phenol red
P.S.I pounds per square inch
Rf relative electrophoretic mobility
RPMIc complete RPMI (RPMI supplemented with 10% FCS, 2mM L-glutamine and 100 μg/ml kanamycin
SDS Sodium dodecyl sulphate
SMWF small molecular weight fraction (decidual chromatography fraction No. 38-44)
TCA trichloroacetic acid
TCR T-cell antigen receptor
Th T helper
TGF transforming growth factor
TNF Tumour necrosis factor
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CHAPTER 1

INTRODUCTION
1.1. RELATIONSHIP BETWEEN THE DECIDUA AND THE FETUS DURING EARLY PREGNANCY

1.1.1. DEVELOPMENT OF THE DECIDUA

In the late stage of the menstrual cycle, the uterine endometrium proliferates and secretes under the influence of female hormones: oestrogens and progesterone. The thickened endometrium is known as the decidua. If fertilization and implantation does not occur, the swollen endometrium undergoes necrosis and is shed. However, if these events do occur, the endometrium becomes thicker, spongier, and more vascular, forming specialised gestational tissue, known as pregnant decidua (in this thesis the term "decidua" is used as a synonym for pregnant decidua). Throughout gestation, the decidua maintains direct physical contact with the developing fetus, providing the means by which it absorbs nourishment from the mother. Decidua by itself is composed of three distinct regions classified according to their position relative to the fetus. The decidual tissue beneath the site of implantation is called the decidua basalis; that lying over the fetus is known as the decidua capsularis; and the still unoccupied part is known as the decidua vera (Pritchard et al., 1985). The decidua basalis is of particular interest since it forms the maternal part of the hemochorial placenta (see below). Fig 1.1-1 depicts a picture of early pregnant human decidua in the uterus.
Fig. 1.1-1. Early pregnant human decidua in uterus containing an embryo (Adopted from Pritchard et al, 1985).

1). Decidua is composed of three portions: basalis, capsularis, and vera.
2). The uterine cavity is bounded by decidua vera and capsularis during the early months of pregnancy. By the fourth month, this cavity is filled by the growth sac.
3). Chorion frondosum (chorionic villi) proliferate into the decidua basalis; and atrophic chorion laeve are within decidua capsularis.
1.1.2 FORMATION OF THE FETUS.

The fetus develops as a result of fertilisation of a mature oocyte by a spermatozoan in the middle of the menstrual cycle. Both the spermatozoan and oocyte contain only half the normal complement of chromosomes as a result of a meiotic division during their maturation. Following the fusion of a spermatozoan and an oocyte in the fallopian tube, the two sets of 23 chromosomes unite together, resulting in a cell containing the normal diploid complement of 46 chromosomes, half being derived from the mother, and half from the father.

The fertilized oocyte begins to divide in the fallopian tube, and soon forms a solid clump of cells with an amniotic cavity appearing amongst them. The latter is termed the blastocyst, which is the precursor of the fetus. The blastocyst continues to grow and divide in the uterus vera (Pritchard et al., 1985).

1.1.3 DEVELOPMENT OF THE HEMOCHORIAL PLACENTA

The blastocyst implants in the superficial layer of the decidua. Its outermost cells, i.e. the trophoblast, secrete digestive enzymes which break down the surrounding cellular structure of the decidua. Gradually the blastocyst burys itself completely within the decidua basalis.
After the invasion of the blastocyte, the trophoblast and mesoderm become organised into chorionic villi. Each villus is composed of a thick outer layer of syncytiotrophoblast (multinucleated cells; in which the nuclei are scattered in a mass of cytoplasm) with trophoblastic lacunae, an underlying layer of cytotrophoblast (mononuclear) and a central core of mesenchyme.

Meanwhile, some cytotrophoblast cells are located in the extravillous space and comprise the extra-villous population of the trophoblast. The villi branch and form tree-like structures (Fig 1.1-2).

As the trophoblast develops its villi, the decidua basalis grows thicker and more vascular. The enlarged maternal vessels surround the villi and connect with one another to form a continuous system of vascular, intervillous space. As chorionic villi continuously erode the decidua and penetrate the maternal capillaries within the decidua basalis, the lacunar clefts become filled with maternal blood. Thus, the distinct tissue, hemochorial placenta, is formed; and the syncytiotrophoblasts and trophoblasts come into direct contact with maternal circulating lymphocytes.

1.1.4. THE ROLE OF MATERNAL-FETAL SURFACE IN THE MAINTENANCE OF PREGNANCY

One characteristic of the immune response is its ability to discriminate between "self" and "non-self". Once "foreignness" has been established, the immune response proceeds to eliminate the foreign material, including
Fig. 1.1-2. Chorionic villi (Adopted from Llewellyn-Jones, 1990).

1). Each villus is composed of syncytiotrophoblast with trophoblastic lacunae, cytotrophoblast, and mesoblastic core of villus.
2). The lacunar clefts are filled with maternal blood (which for clarity is not shown).
incompatible allografts. An exception occurs in pregnant mothers; they do not reject their fetus which carries the father's histocompatibility antigens. So far, there is little evidence for a systemic tolerance of the mother to paternal alloantigens during pregnancy. Thus, the mechanisms for protection of the fetus have been sought locally at the fetal-maternal interface.

1.1.4.1. The regulation of the expression of MHC antigens on trophoblast.
The fetal membranes are the fetal tissues in closest contact with the maternal decidua. The fetal cells in direct contact with maternal blood or tissue are the syncytiotrophoblast and cytotrophoblast. The regulation of major histocompatibility complex (MHC) antigen expression on these cells is thought to be one of the mechanisms involved in the prevention of recognition of the semi-allogenic graft by the maternal immune system. The regulation includes preventing the expression of class II HLA-D antigens, and reducing steady-state levels of mRNA for the Class I MHC genes HLA-A, B on both syncytiotrophoblast and cytotrophoblast (review by Hunt J., 1992a, 1992 b). Instead, one non-classic, class I antigen, HLA-G, has been detected on cytotrophoblast. The HLA-G antigen is a non-polymorphic 39 KDa heavy chain (smaller than classic MHC I antigens with M.w. of 43 kDa) associated with β-microglobulin on the cell surface. According to the 'missing self' hypothesis, one function of natural killer (NK) cells is to recognise and eliminate cells that fail to express self MHC class I molecules. Cytotrophoblast do not express polymorphic MHC class I antigens but express HLA-G. So, it has been proposed that HLA-G may act to protect the trophoblast from attack by maternal NK cells (Reviewed by Johnson, 1993).
Recently, it has been reported that cytotrophoblast is resistant to NK cell mediated cytolysis (Loke et al, 1991; Ferry et al, 1991). However, the finding that β2-microglobulin deficient mice (also defective in CD4-8+ T cells) are capable of reproducing readily with normal litter size (Zilstra et al, 1990) suggests that HLA-G may functionally redundant (Reviewed by Johnson, 1993).

1.1.4.2 The expression of complement-regulatory proteins on trophoblast
The complement cascade may be activated by the presence of either maternal anti-fetal antibody attack or a response to microbial infection at the fetal-maternal surface. The human trophoblast express high levels of complement regulatory proteins on their surface. These proteins can protect trophoblast cells from potential complement-mediated damage. There are three kinds of complement-regulatory proteins: (a). CD46. It was previously described as trophoblast-leucocyte common (TLX) antigen and membrane co-factor protein (MCP). (b). Decay-accelerating factor (DAF, CD55). These two act at the level of C3 convertase enzymes which activate C3 to C3b. (c). CD59, which inhibits the formation and function of the terminal cytolytic membrane attack complex by interacting with C8 and C9 (reviewed by Holmes et al, 1992; Johnson, 1993). Thus, these proteins appears to play an important role in the maintenance of normal human pregnancy.

1.1.4.3. The maternal immune responses to fetal antigens
Specific antibodies were found in the serum and placental eluates of pregnant female mice. These antibodies eluted from the placentae can attach to an eluted placenta, and bind to paternal (but not maternal)
lymphocytes and thymocytes. Antibodies eluted from the placenta were able to block Fc receptors of B cells, suppress the mixed lymphocyte reaction (MLR) and other T cell function. When injected into maternal strain recipients, these antibodies prevent rejection of paternal-strain tumour allograft (reviewed by Viosin, 1983). However, agammaglobulinaemic patients (lacking blocking antibodies) reproduce normal, it suggests that the antibodies are probably the consequence of pregnancy rather than important for its maintenance (Riches, personnel communication).

Pregnancy serum also contains more than 30 pregnancy-associated proteins. One third of them have been proposed as immunoregulatory factors. For instance, pregnancy-associated α2-glycoprotein and α-fetoprotein were shown to be inhibitory to MLR, and T cell mitogen responses. Sex steroid hormones also exerted a inhibitory influence over maternal immunological activity (Stimpson, 1983).

1.1.4.4. The role of decidua in the maintenance of pregnancy.
Recently, it has been reported that HLA-G could bind to the CD8 molecule on T cytotoxic / suppressor lymphocytes (Sanders, 1991). There is some evidence that the cytotrophoblast may also express an unusual form of MHC class II (Starkey, 1987). Moreover, some minor histocompatibility antigens or other polymorphic membrane proteins on the trophoblast may be recognised as non-self by the maternal immune cells and subsequently provoke a local immune responses (Starkey, 1992).
Recently, it was found that a subset of $\gamma\delta$ T lymphocytes were considerably increased in murine decidua. These maternal T cells can specifically recognise trophoblasts and produce IL-2 in a non MHC-restricted fashion. It suggests a novel form of T cell recognition in decidua, although the effector functions that result from the trophoblast recognition remain to be determined (Heyborne et al., 1994). The accumulated evidence reveals another possible protective mechanism, i.e. the decidua may locally produce soluble factors which regulate the maternal response to the cytotrophoblast and the semi-allogeneic fetus. For example:

1). When experimental allogeneic grafts (skin grafts) were transplanted in the pregnant uterus, their survival was prolonged compared to that of identical grafts transplanted systemically (Beer et al., 1974; Glass et al., 1978).

2). When murine embryonic tissue was implanted elsewhere in the mother's body, it was promptly rejected (Streilein et al., 1987).

3). The competence of the immune system of pregnant women was similar to that of non-pregnant women. Comparing the phenotype of PBMNC from these two groups showed no significant differences (Mincheva-Nilsson et al., 1992). Maternal antibodies to paternally-derived, fetal-HLA are common in the sera of multiparous women (reviewed by Hunt J., 1992a).
4). Numerous studies have demonstrated that a large variety of immunocompetent cells infiltrate the murine and human decidua, including large granular lymphocytes with natural killer activity, macrophages, and T lymphocytes. It has been shown that a subpopulation of these cells from both human and mice, or supernatants from their culture, exhibit suppressive activity on the immune response (Wood et al., 1988; Ferry et al., 1990; Parr et al., 1990; Golander et al., 1981; Nakayama et al., 1985; Matsui et al., 1989a).

1.1.5. SPONTANEOUS ABORTION

Abortion means the loss of a fetus before it reaches viability. Spontaneous abortion is the commonest complication of an established pregnancy. A substantial proportion occur without an explanation. A large number of these abortions occur in the first trimester of pregnancy; 55% of recurrent abortions (three or more successive abortions) were found to occur in this period (Llewellyn-Jones, 1990).

The possibility that abortion might be mediated by the abnormality of maternal immunoregulation has attracted considerable attention (Scott et al., 1987; Clark et al., 1991a). One speculation is that an allogeneic incompatibility is necessary at an MHC or a closely linked locus for maternal recognition and development of fetal acceptance. If such a recognition is lacking, cell-mediated, allograft rejection or antibody response to foreign fetal
antigen may lead to fetal loss (reviewed by Landers et al., 1991). Recently, it was found that the percentage of decidual CD3+/HLA-DR+ cells, which are characteristic of activated T cells, was significantly higher in spontaneous abortions than in normal pregnancies (Maruyama et al., 1992). It suggests that abnormal immune regulation in decidua may be involved in this process.

1.2. CELLS AND THEIR INTERACTION IN THE IMMUNE RESPONSE

1.2.1. MONOCYTES AND MACROPHAGES

1.2.1.1. Morphology and distribution
Monocytes are differentiated from myelomonocytic progenitor cells. After maturation, the cells are released from the bone marrow into the peripheral blood. They are large cells (12-15 \( \mu \text{m} \)) with an large eccentric nucleus (occupying more than 50% of the cell space). The cytoplasm contains fine and large azurophilic granules, and vacuoles (Auger & Ross, 1992). When monocytes migrate to different tissues, they become macrophages. Resident macrophages locate in lungs, liver, spleen, bone marrow, intestine, decidua and other areas of the body, and display structural and functional heterogeneity. For instance, dendritic cells in peripheral blood and peripheral lymphoid organs have an irregular shape and are the most potent immunostimulatory cells.
1.2.1.2. Surface antigens

Monocytes and macrophages interact with their environment by antigens expressed on their surface membrane. CD14, a receptor that binds lipopolysaccharide (LPS), is characteristic of these cells being expressed at high density on both monocytes and tissue macrophages.

These cells also express MHC class II molecules (see Section 1.2.5.1) at a high surface density. About 90% of human blood monocytes are HLA-DR positive, 32% are DQ positive and 15% DP positive (Asherson & Golizi, 1989). In addition, monocytes and macrophages (along with other cell types such as B cells and granulocytes) express type I and II receptors for the Fc region of IgG (CD64 and CD32 respectively) and type 3 complement receptors (CD11b/CD18).

1.2.1.3. Functional characteristics

Macrophages carry out a wide range of functions. These include:

1). Phagocytosis. Various stimuli including foreign material, LPS, antigen-antibody complex and mitogen can stimulate monocytes and macrophages. Activated macrophages contain many lysosomes. They phagocytose and kill ingested bacteria. They also display tumoricidal activity.

2). Antigen processing and presentation. Exogenous protein antigens are phagocytosed by macrophages and degraded into polypeptides. These bind
to MHC class II molecules; the peptide-MHC complex on the cell surface can be recognised by specific T cell receptors (see Fig. 1.2-1). Thus, as antigen presenting cells, they play an important role in initiating and regulating immune responses (Benjamini & Leskowitz, 1991).

3). Immunoregulation. Activated macrophages secret prostaglandin (PG) E1 and E2 which mediate the suppression of lymphocytes and NK cells (Pryjma et al., 1989). In addition, these cells can secret cytokines such as TNFα, IL-1, and TGFβ. These cytokines allow mononuclear phagocytes to activate many other cells, both immune and non-immune, and play a role in tumor cell killing, T cell proliferation and activation.

Functions and surface antigen expression vary according to the state of activation and differentiation of the cells, e.g. macrophages can phagocytose but unlike monocytes cannot kill the phagocyted organism unless activated by factors such as gamma interferon.

1.2.2. T CELLS

1.2.2.1. Distribution

T cells are derived from lymphoid stem cells in either the bone marrow or fetal liver. They migrate to the thymus and further differentiate into antigen-specific lymphocytes. The mature cells leave the thymus and enter the blood and peripheral lymphoid tissues.
Fig. 1.2-1. The processing of protein antigens by antigen presenting cells (Adopted from Benjamini & Leskowitz, 1991).

The exogenous protein antigens are phagocytosed by macrophages. The enzymes in lysosomes join phagosome and degrade the antigens into polypeptides. These peptides bind to MHC class II molecules synthesised by rough endoplasmic reticulum. The peptide-MHC complex on the cell surface can be recognised by specific T cell receptor.
Mature T cells comprise 65-80% of lymphocytes in peripheral blood. In addition, some non-lymphoid tissues also contain a variable proportion of T cells, such as the mucosal immune system (including gut, mammary gland, female reproductive tract, etc). The sites of graft rejection and autoimmune processes also show T cell infiltration (Hunt S., 1987).

1.2.2.2. Surface antigen expression

T cells commonly express the CD3 molecule on their surface which is closely associated with the T cell antigen receptor (TcR). TcRs are responsible for the recognition and response to specific antigens. The CD3 molecule transduces signals from the TcR when it recognises specific antigen. There are two kinds of T cell receptors, the αβ receptor which mediates MHC-restricted antigen recognition, and the γδ receptor which recognises antigen in an non 'classic' MHC-restricted fashion.

T cells express other accessory molecules which are involved in activation and interaction with other types of cell. The most important surface antigens expressed on T cells are summarised in Table 1.2-1 (adopted from Austyn & Wood, 1993).

1.2.2.3. Subsets and their function

T cells are divided into two subpopulations according to the expression of the CD4 and CD8 antigens. They represent ~70% (CD4+) and ~25% (CD8+) of the total T cell population in the blood and peripheral lymphoid tissues.
Table 1.2-1. Surface molecule expression on T cells


<table>
<thead>
<tr>
<th>Molecule</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>TcR αβ</td>
<td>Specific recognition of peptide-MHC complex</td>
</tr>
<tr>
<td>TcR λδ</td>
<td>Recognition functions</td>
</tr>
<tr>
<td>CD2</td>
<td>Interact with LFA-3 (CD58); mediates T cell activation</td>
</tr>
<tr>
<td>CD3</td>
<td>Signal transduction from TcR</td>
</tr>
<tr>
<td>CD4</td>
<td>Binds MHC class II; mediates cell adhesion (indirectly?) and signal transduction</td>
</tr>
<tr>
<td>CD5</td>
<td>T cell activation</td>
</tr>
<tr>
<td>CD8</td>
<td>Binds MHC class I; mediates cell adhesion (indirectly?) and signal transduction</td>
</tr>
<tr>
<td>CD11a/CD18</td>
<td>Binds ICAM-1 (CD54), ICAM-2, others? mediates cell adhesion</td>
</tr>
<tr>
<td>CD28</td>
<td>Binds B7/BB1; regulates cytokine gene expression</td>
</tr>
<tr>
<td>CD45 A</td>
<td>Protein tyrosine phosphatase involved in the regulation of T cell activation</td>
</tr>
<tr>
<td>Thy1</td>
<td>T cell activation; cell adhesion</td>
</tr>
</tbody>
</table>
According to the pattern of cytokines secreted after stimulation, CD4+ T cells can be further classified into subsets. Th1 cells secrete interleukin 2 (IL-2), interferon gamma (IFNγ), tumor necrosis factor (TNFα, TNFβ) and granulocyte-macrophage colony-stimulating factor (GM-CSF), all of which are primarily involved in helping inflammatory-type and cell mediated immune responses. Th2 cells produce IL-4, IL-5, IL-6, and IL-10, which are primarily involved in antibody-mediated immune responses. Both of them produce IL-3. These subsets have been clearly established in mice; the distinction is less obvious in humans. Recently, similar subsets have been demonstrated for CD8+ cells.

CD4+ T cells are central to the initiation of the immune response. They recognise exogenous peptide antigen which has been processed and associated with self-MHC class II molecules. In the presence of IL-1, a product of activated APC, the activated CD4+ T cells are able to 'help' the immune response by secreting cytokines. Th1 cells synthesize IL-2, and IFNγ which activate CD8+ killer T cells, natural killer (NK) cells, and macrophages. Th2 cells produce IL-4 and IL-5 which activate B cells to release the antigen-specific immunoglobulins. The interaction of CD80 (B7) on APC with the CD28 receptor on T cells supplies an 'accessory signal' for CD4+ cell effector function. A diagrammatic representation of the process T cells response is illustrated in Fig 1.2-2.
Fig 1.2-2. A diagram of the process of immune cell response (modified from Goodman, 1991). See text for explanation.
CD8+ T cells mediate antigen-specific cytotoxicity. They recognise processed endogenous peptide antigens bound to self MHC class I molecule on the surface of the target. By releasing the cytotoxic contents of their granules they kill the target cells. A proportion of the CD8+ cells can suppress immune responses, possibly by the release of soluble factors that interfere with the function of other immune cells.

1.2.3. B CELLS

12.3.1. Surface antigens

B cells originate from hemapoietic stem cells in the bone marrow. They further differentiate into mature B cells either in the bone marrow itself or in peripheral lymphoid organs. B cells comprise 8-15% of peripheral blood lymphocytes.

The characteristic marker on mature B cells is the presence of membrane-bound immunoglobulin on their surface (slg), which act as the B cell antigen receptor. In addition these cells possess receptors for polyclonal B cell activators (e.g. LPS), cytokines, and complement (CR1, CD35; CR2, CD21). They also express CD22 which is involved in cell adhesion and CD19 which is involved in signal transduction. B cells also express MHC class II molecules.

1.2.3.2. Functional characteristics
The major function of B cells is the production of specific antibodies to antigens. This function is usually controlled by CD4+ T helper cells. The latter cells express a 39 KDa membrane protein that induces T cell-dependent B cell activation. The receptor on B cells is CD40 (reviewed by Noelle & Snow, 1992). Under the influence of IL-4 and IL-5, the activated B cells proliferate and differentiate into plasma cells which secrete antibodies (see Fig 1.2-2).

1.2.3.3. Classes of immunoglobulins

Immunoglobulins consist of one or more basic units, each having two heavy (H) and two light (L) chains. There are five classes of Ig: IgG, IgM, IgA, IgD and IgE, each having structurally different H chains: γ, μ, α, δ, and ε respectively. All of the Igs share two classes of L chains: κ and λ. There are four subclasses of IgG: IgG1, IgG2, IgG3, IgG4.

Each immunoglobulin molecule has two antigen bind sites (Fab) each made up by the combination of the variable region of one heavy and one light chain. The biological function of the molecule is associated with the constant region of the heavy chains (Fc region).

The molecular weights of IgG, IgM, IgA, IgD and IgE are 150 KDa, 900 KDa, 165 KDa, 180 KDa, and 200 KDa respectively. Mercaptoethanol treatment, which reduces the disulfide bonds between the H and L chains shows that
IgG consists of two 53 KDa H chains and two 22 KDa L chains (Benjamini & Leskowitz, 1991).

In some autoimmune diseases, the body produces antibodies against normal Ig. For example, rheumatoid factor (an IgM or IgG antibody) binds to the Fc portion of normal IgG. These immune complexes are involved in the inflammation of joints and the pathologic characteristic of rheumatoid arthritis.

1.2.4. NATURAL KILLER CELLS

1.2.4.1. Morphology and distribution
Natural killer cells (NK) are thought to be derived from precursor cells in the bone marrow. Most are part of a discrete subset of mononuclear cells - the large granular lymphocytes (LGL). These cells are 10-14 μm in diameter, have a typical, often kidney-shaped nucleus, and abundant pale staining cytoplasm with distinct azurophilic granules. A small portion of NK cells are of medium size and agranular.

About 5-10% of peripheral blood lymphocytes are NK cells. These cells are also distributed throughout a variety of body tissues, such as the lungs, liver, spleen, intestinal mucosa, and peritoneal cavity (reviewed by Lotzova, 1992). Recently, a large proportion of LGL were also found in human and murine decidual tissue (Starkey, 1992).
1.2.4.2. Surface antigen expression

The major feature of NK cells is the expression of CD56 (an isoform of neural cell adhesion molecule) and receptors for the Fc region of IgG (FcγR), in particular the type III receptor - CD16. These cells do not express the T cell antigen receptors (αβ, γδ) or CD3, or surface immunoglobulin. However, NK cells do express some myelomonocytic (e.g. CD11) or T cell (e.g. CD2, CD8, IL-2R) markers (Austyn & Wood, 1993).

1.2.4.3. Functional characteristics

NK cells are considered to be part of the innate immune system since they do not express classical receptors for specific antigen. NK cells have spontaneous, MHC-unrestricted, cytotoxic activity against a number of tumour or virally infected cells. This activity may be enhanced by cytokines such as IFNγ. However, activation of NK cells does not involve secondary or memory responses.

Cells expressing CD16 exhibit high levels of antibody-dependent cellular cytotoxicity (ADCC). However, they do not use this mechanism to kill tumours, since NK cell-mediated tumour killing can still occur even when their Fc receptors are blocked (Benjamini & Leskowitz, 1991). The exact mechanism by which NK cells recognize and kill their targets is still not clear.
NK cells are involved in allograft rejection, since they can recognize and lyse some foreign cell types without prior sensitization. Recently their role in reproductive immunology has attracted considerable attention.

NK cells also display immunoregulatory activity through the production of a variety of cytokines, including IL-1, IL-2, IL-4, IFNs, and TNFα, β, etc (Lotzova, 1992). Through these cytokines they play a general regulatory role in humoral and cellular responses.

1.2.5. ALLOREACTIVITY

1.2.5.1. Major histocompatibility complex (MHC)

Products of major histocompatibility complex (or its equivalent) are expressed in all vertebrate species. In man they are called the human leukocyte associated antigens (HLA). Human MHC class I and class II (HLA) genes are located on chromosome 6. There are three independent MHC class I loci (A, B, C) each of which codes for a transmembrane polypeptide of molecular weight 43 KDa, which is expressed on the surface of cells associated with an 12 KDa polypeptide, called β2-microglobulin. MHC class I molecules are found on every nucleated cell in the body.

The genetic regions coding for MHC class II molecules are DP, DQ, DR. Each of them codes for a two-chain membrane anchored glycoprotein, the chains being called α (34 KDa) and β (28 KDa) respectively. Unlike MHC
class I molecules, class II molecules have a limited cellular distribution. They are found on immunocompetent cells such as B lymphocytes, macrophages, dendritic cells, thymic epithelial cells and activated human T cells.

One of the important characteristics of MHC molecules is their polymorphism. Within each locus, a large number of variants exists in the population as a whole. As a result, different individuals express different MHC molecules on their cell surface (Benjamini & Leskowiyz 1991).

In addition to MHC antigens, minor histocompatibility antigens, encoded by minor histocompatibility genes on different chromosomes, may be immunogenic in different individuals (Austyn & Wood, 1993).

1.2.5.2. Alloreactivity
An allograft is a transplant from one individual to a genetically dissimilar individual of the same species. A large proportion of T cells from any individual can recognize and respond to the foreign MHC molecules. This reactivity against allogeneic MHC molecules is called alloreactivity (Austyn & Wood 1993).

The T cells that recognize foreign MHC molecules expressed on grafted cells are known as alloreactive T cells. Among them, CD4+ T cells recognize foreign class II molecules (which resemble foreign Ag associated with self MHC class II molecules) and CD8+ T cells recognize class I molecules.
1.2.5.3. Immunological tolerance

Immunological tolerance refers a state of the immune system that is manifested by a diminished or absent capacity to express either cell-mediated or humoral immunity. Normally, the body's immune system does not respond to self-antigens. The main theories to explain this phenomenon are clonal selection and clonal anergy (i.e. APC fail to deliver co-stimulatory signals making the specific T cell unable to respond to the antigen). Suppressor T cells may also play a role in T-cell tolerance. In in vitro experiments, immunological tolerance of adult T and B cells to foreign antigens have been induced by the use of relatively large doses or small doses of antigens, given repeatedly over long periods of time. The weaker the immunogenicity of an antigen, the easier to induce tolerance to it in mice (Benjamini & Leskowlyz 1991).

Maternal immunological tolerance to the fetus may be due to inefficient antigenic stimulus due to the regulation of the expression of MHC antigens on trophoblast, or due to the immunosuppression resulting from the various suppressive factors derived from fetal-maternal interface (see 1.1.4).
(which resemble foreign Ag associated with self MHC class I molecules). APC in grafts secrete IL-1 which aids in triggering lymphocyte activation. Once CD4+ T cells are activated, they release IL-2 which activates CD8+ T cells and B cells. As a result, the effector cells can directly destroy graft cells by cytotoxic T cell mechanisms (see Table 1.2-2). An in vitro assay, the mixed lymphocyte culture reaction (MLR), is used to assess the extent of this allogeneic reaction.

1.2.6. POTENTIALLY IMMUNOCOMPETENT CELLS IN THE DECIDUA

There are three major types of cells found in first trimester human decidua: stromal cells, glandular epithelium, and leukocytes. They are interspersed with stromal fibroblasts and blood vessels (Parhar, 1988). The leukocytes (including lymphocytes, macrophages and neutrophils) are present either in aggregates situated adjacent to the glandular epithelium, or scattered amongst other cells (Mincheva-Nilsson, 1992). After disruption of decidual tissue by enzymatic or mechanical methods, a higher proportion of potentially immunocompetent cells are obtained in the mononuclear cell layer. Phenotypic and functional analyses of these cells have provided some information about their composition.

1.2.6.1. Large granular lymphocytes (LGL).

A large proportion of decidual mononuclear cells (DMNC) are LGL. They comprise between 40% to 70% of DMNC population (Mincheva-Nilsson et al.,
1992; Ferry et al., 1990; King et al., 1991). The main characteristic of these cells is that they express a natural killer (NK) cell marker, CD56. Although the majority of CD56+ cells express the early T-lineage markers, CD2 and CD7, they do not express the mature T-cell antigens such as CD3, CD5 and CD4/8. They also express CD11a/CD18 (integrins) and CD38. Unlike peripheral blood NK cells, only a few decidua LGL express CD16. Decidua LGL have low NK activity against the K562 cell line compared with their peripheral blood CD56+ counterparts (Reviewed by King et al, 1991). These cells express only the p75 chain of the IL-2 receptor (IL-2Rβ), and have a low proliferative capacity in response to IL-2 (Christmas et al, 1990).
1.2.6.2. Macrophages.

Macrophages have been shown to constitute a variable but significant proportion of human decidual cells. Estimates vary from 14% to 20% (Bulmer, 1989; Loke et al., 1991; Mincheva-Nilsson et al, 1992). These cells express CD14, and the majority of them express the class II MHC gene products, HLA-DR, or DP, DQ (Lessin et al., 1988; Bulmer, 1989). It was reported that these cells can suppress lymphocyte alloreactivity in vitro through the release of prostaglandin E2 (Parhar et al., 1988).

1.2.6.3. T lymphocytes.

Mature decidual T lymphocytes comprise a population of between 10% to 23% of DMNC (Loke et al., 1991; Mincheva-Nilsson et al., 1992). They are CD3 positive cells. The majority of them are αβ T cells, and express CD4 or CD8. Both CD4+ and CD8+ subsets express significantly higher CD69, HLA-DR, IL-2Rα and IL-2Rβ antigens than their peripheral blood counterparts, indicating they have been activated (Saito et al., 1992). A proportion of γδ T cells has been found in murine decidua; they seem to be able to recognise trophoblast in an MHC-non-restricted fashion (Heyborne et al., 1992, 1994). B cells and polymorph cells are very rare in decidua tissue.

1.2.6.4. Non-leukocyte decidual cells.

Some non-leukocytic cells found in the decidua have been shown to have immunomodulatory activity. Decidual stromal cells, for example, were reported to be inhibitory in mixed lymphocyte culture reaction and in the generation of cytotoxic T cells (Parhar et al., 1988). In murine decidua, it has been shown that decidual stromal cells inhibit several macrophage functions (Redline et al., 1990).
As mentioned above, these immonocompetent cells have potentially immunomodulatory activity (Summarised in Table 1.2-2). However, their immunoregulatory mechanisms during normal pregnancy are still far from clear. Severe combined immunodeficient (SCID, deficient in NK cell and lacking T cells and B cells), and beige mutant (depletion of NK cell function) mice were found to reproduce normally. It has been suggested that the inhibitory cytokine derived from decidua may not be from classical mature NK cells, T cells, or B cells (Croy et al, 1990).

1.3. IMMUNOREGULATORY FACTORS

1.3.1. CYTOKINES

Cytokines are a group of soluble, polypeptides or glycoproteins with molecular weights (M.w) of < 30 KDa. They are induced by specific stimuli and regulate the function of effector cells. Cytokines produced by lymphocytes are called lymphokines; cytokines produced by monocytes or macrophages are called monokines. Cells of the immune system can interact with each other via cytokines. They can act on cells other than those which produced them (paracrine activity) or on the cells by which they were secreted (autocrine activity).

1.3.1.1. Immunostimulatory cytokines

A variety of cytokines have the capacity to stimulate the immune response. This information is summarised in Table 1.3-1.
Among these stimulatory cytokines, IL-2 plays a central role in the immune response. It is secreted by CD4+ Th1 cells (and others), and exerts numerous immunological effects by stimulating cellular proliferation and the production of other cytokines by T cells, B cells, and NK cells. The biological effects of IL-2 are mediated through the binding of the growth factor to specific receptors present on cell targets. There are three forms of the IL-2 receptor: one is low affinity (IL-2Rα); the second is of intermediate affinity (IL-2Rβ); and the third is a heterodimetric complex of the previous two with high-affinity for IL-2 (Kuziel & Greene, 1991).

1.3.1.2. Immunosuppressive cytokines

Cytokines with immunosuppressive activity include transforming growth factor β (see Section 1.3.2) and IL-10. The former is closely associated with this study, and is emphasised in Section 1.3.2.

IL-10 was initially discovered as an inhibitor of cytokine synthesis (TNF, IL-1 and IL-6) and was called cytokine synthesis inhibitory factor. Under different conditions of immune activation, CD4+ T cells, as well as monocytes, macrophages and B cells are capable of secreting it. IL-10 is an acid-sensitive (pH < 5.5) protein which is stable in basic conditions (pH up to 11) with a M.w. of 30-40 KDa. It has been reported to be exhibit a wide range of
Table 1.2-2. Immunoregulatory cells in human decidua.

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Proposed Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Large granular lymphocytes</td>
<td>NK activity</td>
</tr>
<tr>
<td>Macrophages</td>
<td>Immunosuppression</td>
</tr>
<tr>
<td></td>
<td>Antigen presentation</td>
</tr>
<tr>
<td>T lymphocytes</td>
<td>Immunorecognition</td>
</tr>
<tr>
<td></td>
<td>Immunosuppression</td>
</tr>
<tr>
<td>Non-Leukocytic cells</td>
<td>Immunosuppression</td>
</tr>
</tbody>
</table>
Table 1.3.1. Characteristic properties of immunostimulatory cytokines
(modified from Oppenheim, 1991 & Roitt, 1991)

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>M.w</th>
<th>Principle Cell Sources</th>
<th>Stimulator Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1</td>
<td>17.5</td>
<td>Macrophages &amp; others</td>
<td>Immunoaugmentation.</td>
</tr>
<tr>
<td>IL-2</td>
<td>15.5</td>
<td>T cells &amp; LGL</td>
<td>T &amp; B cell growth factor; Activates T &amp; NK cells.</td>
</tr>
<tr>
<td>IL-4</td>
<td>20</td>
<td>CD4+ T cells</td>
<td>T &amp; B cell growth factor.</td>
</tr>
<tr>
<td>IL-5</td>
<td>18</td>
<td>CD4+ T cells</td>
<td>Stimulates B cells; Production of Ig.</td>
</tr>
<tr>
<td>IL-6</td>
<td>22-30</td>
<td>CD4 T cells</td>
<td>Growth factor for B cell; Polyclonal immunoglobulin production.</td>
</tr>
<tr>
<td>IFNα</td>
<td>18-20</td>
<td>Leukocytes</td>
<td>Antiviral;</td>
</tr>
<tr>
<td>IFNβ</td>
<td>25</td>
<td>Fibroblasts</td>
<td>Expression MHC I.</td>
</tr>
<tr>
<td>IFNγ</td>
<td>20-25</td>
<td>T cells</td>
<td>Stimulates macrophages &amp; NK cells; Induce cell membrane antigens (eg, MHC)</td>
</tr>
<tr>
<td>TNFα</td>
<td>17</td>
<td>Macrophages &amp; others</td>
<td>Immunoenhancing &amp;</td>
</tr>
<tr>
<td>TNFβ</td>
<td>18</td>
<td>T lymphocytes</td>
<td>Tumoricidal</td>
</tr>
</tbody>
</table>

29
immunosuppressive activity including inhibition of cytokine production and the killing of intracellular pathogen by macrophages, suppression of MHC class II expression on macrophages, and due to its action on macrophages and other APC, inhibition of the proliferation and/or cytokine production by T cells, Th1 clones and NK cells (reviewed by Howard et al., 1992 a, b & Rennick et al., 1993).

1.3.2. TRANSFORMING GROWTH FACTOR β (TGFβ)

Originally, TGFβ was defined as a cytokine which was able to induce certain non-neoplastic cells to express a transformed phenotype and to undergo anchorage-independent growth (Roberts et al., 1983). So far, five members of it have been identified in mice: TGF β1, β2, β3, β4 and β5. The first three types have also been identified in human cells and show a high degree of homology at the level of amino-acid sequence. For example, TGFβ2 shares 71% homology with β1; TGFβ3 shares 72% with β1 and 76% with β2. The most studied molecules in this family is TGFβ1. In most instances, the effect of the various TGFβ isoforms are indistinguishable. One notable exception is the relative lack of inhibitory effect of TGFβ2 on growth of endothelial cells (Robert & Sporn, 1992).

1.3.2.1. synthesis of TGFβ

TGFβ is produced by a variety of tissue and cell types, such as human placenta, platelets, activated macrophages and lymphocytes (Frolik et al.,
1983; Assoian, 1983; Kehrl, 1986; Kehrl, 1987; Assoian 1987). TGFβ is released in a latent form (391 amino acids) and is subsequently activated by some physiological factors which may result in alteration of the glycosylation of the amino-terminal glycopeptide (Lyons et al., 1990). In vitro, activation of latent TGFβ is commonly achieved by acidification of cell-conditioned medium (Lyons et al., 1990). The purified TGFβ with biological activity is a polypeptide of 25 KDa (112 amino acids) which is composed of two 12.5 KDa subunits held together by disulfide bonds. It has been demonstrated that the dimeric nature of this molecule is very important; the disruption of the structure resulting in a total loss of biological activity (Assolain et al., 1983).

1.3.2.2. Biological activity

TGFβ is involved in many cell and tissue functions. So far, the range of its known biological activity has extended to nearly all types of cell. It exhibits multiple modulating functions on these cells. It stimulates some cell types, and inhibits the proliferation and function of others. It is involved in tumour development, extracellular matrix protein synthesis, wound healing, embryogenesis, and bone formation/fracture healing. In the immune system it enhances the functions of monocytes, i.e. migration and the respiratory burst. However, it may affect the growth of all lymphocytic cells as demonstrated by its ability to inhibit IL-1 dependent thymocyte proliferation in the presence of a suboptimal concentration of phytohemagglutinin (Wahl et al., 1988); the IL-2 dependent proliferation of Con A-stimulated T cells (Kehrl et al., 1987); IL-2 dependent B cell proliferation, and Ig secretion by B cells.
(Kehrl et al., 1986); allospecific cytotoxic T cell development (Ranges et al., 1987); NK cell activity and the cytotoxic activity of peripheral blood LGLs (Rook et al., 1986).

1.3.2.3. Mechanism of action

In spite of their similarity in biological activity, TGFβ1 and β2 have different receptors on their target cells. Briefly, they share a 280 KDa receptor; but only β1, not β2, interacts specifically with a 65 KDa and an 85 KDa receptor (Cheifetz et al., 1987). The intracellular signalling pathway, after the occupancy of these receptors, is not clear yet. Although GTP-binding protein has been shown to be involved in the process, no evidence of intracellular Ca++ fluxes, inositol phosphate, or kinase activity has been found (Lyons et al., 1990). It has been shown that blockage of DNA synthesis in target cells by TGFβ is due to its arresting the cells in the middle to late G1 phase, delaying enter into S phase. This inhibition is reversible and thus not the result of cytotoxicity (Shipley et al., 1986).

1.3.3. PROSTAGLANDIN

1.3.3.1. Synthesis and classification

Prostaglandins (PGs) are a family of lipid-soluble molecules. The major precursor of PG is arachidonic acid, which is derived from the metabolism of membrane phospholipids by the action of the enzymes phospholipase A2 and C. Arachidonic acid has a short half-life and can be metabolised by two major
routes, the cyclo-oxygenase and lipoxygenase pathways. The products of the former are PGs; the latter are leukotrienes mainly involved in inflammatory process (reviewed by Pruzanski et al., 1991). Fig 1.3-1 illustrates this metabolic process.

So far, at least 16 different prostaglandins have been identified. According to the difference in their chemical substituents they can be divided into nine different classes, designated PGA to PGI.

1.3.3.2. Regulation of PG production

PGs are found in many human tissues. Almost all mammalian cells, except red blood cells, produce PGs and their related compounds. Different cell types produce different PGs. For example, heart muscle makes PGI2, PGF2α, and PGE2, monocytes and macrophages synthesise PGE2 and PGF2, mast cells produce PGD2, and lymphocytes may produce PGE2 but this is controversial.

PGs do not exist free in tissues, but have to be synthesized and released in response to an appropriate stimulus. For example, PGE2 can be induced by IL-1, TNFα, the cross-linking of Fc receptors, lipopolysaccharide (LPS) and components of the complement cascade. These molecules have a short half-life and act in the same environment in which they are synthesized (Phipps et al, 1991; Austyn et al, 1993).
Fig 1.3-1. The diagram of the synthesis of prostaglandins
(Modified from Austyn et al, 1993).

Phospholipids in Cell Membranes

\[ \text{Phospholipase A2 and C} \]

Arachidonic acid

\[ \text{Cyclo-oxygenase} \quad \text{Lipoxygenase} \]

Prostaglandins Leukotrienes
Some nonsteroidal anti-inflammatory drugs, such as aspirin and indomethacin can inhibit the synthesis of the prostaglandins by inhibiting or inactivating cyclooxygenase activity (Voet et al., 1990). Therefore, these inhibitors have been used to elucidate the biosynthesis of PGs, and their functions.

1.3.3.3. Biological activity

The biological activity of PGs is incredibly diverse. They are intimately involved in the inflammatory response, regulate blood pressure, control several reproductive functions such as the induction of labor, induce blood clotting, and regulate sleep/wake cycle (Voet et al., 1990). The immunoregulatory effect of PGs has been extensively studied.

In general, PGEs are non-specific suppressors of the immune response. They inhibit a variety of lymphocyte functions including proliferation, IL-2 and other interleukin production, the synthesis of Ab by B cells, CTL and NK cytotoxicity, leukocyte locomotion and cell and cell interactions (Austyn et al., 1993; Phipps et al., 1991).

Recently, it has been shown that PGEs may enhance the synthesis of certain isotypes of immunoglobulin and selected cytokines and cytokine receptors (reviewed by Phipps et al., 1991).
1.3.4. CYTOKINES AND OTHER IMMUNOREGULATORY FACTORS DERIVED FROM DECIDUA

1.3.4.1. Cytokines
A variety of cytokines have been found in human or murine decidua and placenta (Table 1.3-2).

1.3.4.2. TGFβ
There have been several reports about the generation of TGFβ-like molecules and their immunosuppressive activity from murine decidua (Clark et al., 1988, 1990). This molecule inhibits allospecific CTL responses by the inhibition of the action of IL-2 on CTL development, and lymphokine-activated killer cell generation. The supernatant of decidual cell cultures also stimulates anchorage-independent growth of the NRK cell, which is defined as a function of TGFβ (Clark et al., 1985). In addition, TGFβ-like molecules have been detected at high concentration in amniotic fluid (Altman et al., 1990). The effects of the factors in both decidual supernatants and in amniotic fluid could be neutralized by anti-TGFβ2, but not β1, monoclonal antibodies (Altman et al., 1990; Lea et al., 1992).

1.3.4.3. TUMOR NECRISIS FACTOR α (TNFα)
TNF was originally identified as a product of activated macrophages (TNFα) and lymphocytes (TNFβ) that caused a wasting syndrome in tumour-bearing animals, regression of some tumors, and hemorrhagic necrosis in certain
Table 1.3.2. Production of cytokines by uterine and decidual cells.
(adopted from Robertson et al., 1994)

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Uterus/decidua</th>
<th>Placenta</th>
</tr>
</thead>
<tbody>
<tr>
<td>CSF-1</td>
<td>Yes (H,R)*</td>
<td>Yes (H), No</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Yes (H,R)</td>
<td>Yes (H,R)</td>
</tr>
<tr>
<td>IL-3</td>
<td>Yes/No (R)</td>
<td>No (R)</td>
</tr>
<tr>
<td>G-CSF</td>
<td>Yes (H)</td>
<td>Yes (H)</td>
</tr>
<tr>
<td>IL-1</td>
<td>Yes (H,R)</td>
<td>Yes (H,R)</td>
</tr>
<tr>
<td>IL-2</td>
<td>Yes (R)</td>
<td>Yes (H,R)</td>
</tr>
<tr>
<td>IL-4</td>
<td>Yes (R)</td>
<td>Yes (H,R)</td>
</tr>
<tr>
<td>IL-5</td>
<td>Yes (R)</td>
<td>Yes (H), No (R)</td>
</tr>
<tr>
<td>IL-6</td>
<td>Yes (H,R)</td>
<td>Yes (H,R)</td>
</tr>
<tr>
<td>IFNα</td>
<td>Yes (H,R)</td>
<td>Yes (H,R)</td>
</tr>
<tr>
<td>IFNγ</td>
<td>Yes (H,R)</td>
<td>Yes (H,R)</td>
</tr>
<tr>
<td>TNFα</td>
<td>Yes (H,R)</td>
<td>Yes (H,R)</td>
</tr>
<tr>
<td>TGFβ</td>
<td>Yes (H,R)</td>
<td>Yes (H,R)</td>
</tr>
</tbody>
</table>

H=human; R= rodent
tumor tissues. TNF could stimulate T and B lymphocyte function and proliferation. In conjunction with IFNγ, it could up-regulate MHC antigen expression in several cell types.

Several groups have demonstrated biological activity of TNFα in human decidua (Jaattela et al., 1988; Casey et al., 1989; Chen et al., 1991; Vince et al., 1992). It appears that quite a few cell types are capable of producing this factor, i.e. decidual stromal cells, and macrophages. It has been proposed that TNFα may cause the immunosuppression seen in human decidua (Hunt, 1992b). However, since TNFα is usually recognised as an immunopotentiating agent which stimulates the proliferation of lymphocytes and enhances the functional response of lymphocytes and macrophages, it has been argued that its increased production in pregnancy may threaten fetal survival (Vince et al., 1992; Clark et al., 1991b).

1.3.4.4. Prostaglandins

Prostaglandins also have been demonstrated in early murine decidua (Matthews & Searle, 1987). In human decidua, PGE2 was identified as a primary source of suppression (Parhar et al., 1988). Addition of indomethacin, an inhibitor of prostaglandin synthesis, eliminated the suppression (Wood et al., 1988).
1.3.4.5. Human decidua-associated protein hDP 200 (hDP200)

Human decidua-associated protein hDP 200 was identified in 1990 (Halperin et al.) and named according to its molecular weight in non-reducing SDS-PAGE analysis (hDP200). SDS-PAGE analysis performed under reducing conditions showed that hDP200 is composed of two polypeptide chains of M.w of 55 and 25 KDa. From amino-terminal sequence analysis, hDP200 seems to be an immunoglobulin G-like molecule (Halperin et al., 1994). It has not been known if this molecule has any immunoregulatory function. Another group has reported that human decidua extracts inhibited T cell mitogen induced proliferation of PBMNC, and the causative factor (M.w 158 KDa) was IgG or a substance copurified with IgG as demonstrated by immunoelectrophoresis and double immunodiffusion (Kitano et al., 1990). The latter one may be identical with hDP200.
1.4. AIMS OF THIS STUDY

The mechanisms involved in the protection of the fetus from attack by the maternal immune response have not been elucidated clearly. Especially the factors responsible for maintenance of the fetus during early pregnancy in humans are poorly understood. An understanding of these mechanisms may provide an insight into the maintenance of tolerance to foreign antigens and thus the pathology of many immunological diseases. This would also have implications for the survival of therapeutic tissue and organ transplants.

The present study was designed to investigate potential immunosuppressive factors from first trimester human decidua using a variety of biochemical and immunological techniques. These factors were to be identified and characterised, an attempt was to be made to identify the cells which produce them in the human decidua.
CHAPTER 2

MATERIALS AND METHODS
2.1. PREPARATION OF DECIDUAL FRACTIONS

2.1.1. COLLECTION OF DECIDUAL SAMPLES

First trimester human decidua were obtained from women undergoing therapeutic abortion of healthy pregnancies. Gestational age was calculated from the date of the last menstrual period. All operations were carried out under sterile conditions. Maternal decidual tissue was separated from fetal tissue by the surgeon at the time of operation, and samples for the preparation of soluble decidual extracts were frozen at -20 °C immediately and transferred to -70 °C as quickly as possible. Samples to be used for the isolation of decidual mononuclear cells were transported to the laboratory in less than 3 hours and were processed immediately.

2.1.2. ULTRAFILTRATION OF SOLUBLE DECIDUAL EXTRACTS

2.1.2.1. Preparation of extracts.

Samples were defrosted in a waterbath (37 °C) and when nearly thawed, a portion was diluted 1:8 (v/v) with pre-cooled PBS (4 °C) and homogenised (Polytron homogenizer, speed No. 6). An aliquot of the lysate (50 µl) was stained with 0.2% trypan blue to assess the extent of cell disruption.

Residual cells and cellular organelles were removed by transferring the homogenate to 250 ml tubes and centrifuging (26,000 g; 4 °C) for 35 minutes.
using a Beckman centrifuge (Model JA-21 equipped with rotor model JA-14). Some small fat droplets visible on the surface of the supernatant were removed carefully using a Pasteur pipette. The clarified supernatant was collected for subsequent separation.

2.1.2.2. Ultrafiltration of the extract

A Minitan ultrafiltration system (Millipore) was employed to concentrate and crudely fractionate the decidual extract. Four different types of Minitan normal molecular weight cut-off membranes (NMWC) were used with the nominal exclusion level of >10, >30, >100 and >300 KDa (Millipore, type PTGC, PTTK, PTHK, and PTMK). The decidual supernatant was initially filtered through two 10 KDa filters, generating <10 KDa fraction. The retentate was collected and filtered through two 30 KDa membranes, producing a fraction with approximate molecular weight range of 10-30 KDa. In a similar manner, 30-100 KDa, 100-300 KDa and >300 KDa fractions were collected by using the membranes described above. After ultrafiltration, the individual fractions of <10, 10-30, 30-100, 100-300, and >300 KDa were pooled, sterilised using a 0.2 μm filter (Millipore), and a portion stored at -70 °C for further purification. Smaller aliquots were also stored at -70 °C until assayed for their immunoregulatory activity.

2.1.3. FURTHER PURIFICATION OF DECIDUAL EXTRACTS.

2.1.3.1. Preparation of sample
Crude decidual protein fractions (CDPF) with M.W. 10-30 KDa and 30-100 KDa, which were found to have immunoregulatory activity in preliminary experiments, were mixed and loaded into a 10 KDa macrosep centrifugal concentrator (Filtron, Northborough, MA, USA). After centrifugation (3600 g; 50 min; 4 °C), the concentrate was collected and the process repeated. The protein concentration of the concentrated extract was approximately 3-4 mg/ml as assessed by the Ohnishi and Barr (1978) modification of the method described by Lowry (1951).

2.1.3.2. Fast performance liquid chromatography (FPLC)

Gel filtration chromatography was performed using a XK 2.6/70 column (Pharmacia, Sweden) packed with pre-swollen Sephacryl S-100 HR (Sigma; fractionation range: 1-100 KDa). The column was connected to a FPLC system (Pharmacia). Depending on the separation requirements, either PBS (pH 7.1), or 0.1 M acetic acid and 0.1 M KCl (pH 2.9) was used as eluent buffer. The column was pre-equilibrated with appropriate eluent buffers before application of the sample (3.5 ml). The column was run at 0.75 ml/min (8.5 ml/cm²/h) and the eluate (2.6 ml under neutral conditions, or 3 ml under acid conditions) collected automatically. Where appropriate, fractions were pooled, sterilised using a 0.2 µm filter (Millipore), aliquoted in Eppendorf tubes and stored at -70 °C.

In order to estimate the molecular weight of the decidual extracts, the column was calibrated using protein M.w standards (Sigma) under neutral conditions.
To estimate the protein concentration of FPLC fractions, a standard curve was obtained using bovine serum albumin solution of known concentration and a Multiskan MCC Spectrophotometer with a 280 nm test wavelength filter.

2.2. BIOCHEMICAL ANALYSIS OF DECIDUAL EXTRACTS

2.2.1. SODIUM DODECYL SULPHATE - POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

Decidual extracted proteins were resolved using the Laemmli SDS-discontinuous buffer system (1970). The composition of the stacking gel and resolving gels and all buffers used are given in Appendix.

Glass plates were washed with detergent, and rinsed in turn with MilliQ water, ethanol, and acetone before assembly in the gel holders. The resolving gel (either 12% or 7.5% depending on the samples) was poured between the glass plates, care being taken to prevent trapping air bubbles. The surface of gel was overlayed with 200 µl of water-saturated butanol to prevent oxygen-dependent inhibition of polymerization, and to ensure a smooth surface. The gel was allowed to set for 30-40 min at room temperature.
The overlay was poured off and the surface of the gel rinsed three times with resolving buffer to remove residual butanol. A 5% stacking gel was poured over the resolving gel. Its height was twice that of the samples to be applied, and the sample well comb inserted. Once the gel was set (30-40 min; room temperature), it was placed in the electrophoresis tank and the comb gently removed. The surface of the gel was washed with tank buffer. The samples (in sample buffer) were heated for 5 min in a boiling water bath and carefully loaded into the appropriate sample wells. Prestained M.w. markers (Bio-Rad) were included to calibrate the gels.

The proteins were electrophoresed in tank buffer at 150 V until the dye had passed the stacking gel whereupon the voltage was raised to 200 V for the resolution of the proteins. The process was terminated when the buffer dye arrived at the bottom of the resolving gel.

2.2.1.1. Detection of proteins

In order to visualise the resolved protein, SDS-PAGE gels were incubated with Coomassie brilliant blue at room temperature for 1 hour, the process was enhanced by gentle rocking. Excess stain was removed by incubation with fix/destain until the desired reduction in background was achieved. The composition of the stain and destain used are given in Appendix.
2.2.1.2. Density measurement of protein bands

To quantify the amount of protein in each band on the gels, the absorbance of the peak area of each band was measured using a densitometer (Shimadzu, Japan) and analysed with an on line computer. The results were expressed as arbitrary units.

2.2.2. WESTERN BLOTTING

2.2.2.1. Protein transfer

The mini western blot was transferred on to a Hybond-C super nitrocellular membrane (Amersham) using a semi-dry blotter. The gel / membrane were sandwiched between sheets of blotting paper which had been soaked in transfer buffer for 20 min prior to transfer. Electrophoresis was at 10V for 24 min. When electrotransfer was done, the membrane was stained immediately or dried and stored at 4°C for later use.

A trans-blot cell was used to transfer the proteins from standard sized gels. The trans-unit was assembled as follows: filter pad, 3 mm paper, hybond-C super nitrocellulose membrane, protein gel, 3 mm paper, and filter paper. The unit was placed into the Trans-blot cell which was already full of transfer buffer. The membrane was toward to positive electrode. The electrophoresis was 60V for 3 hours or 25V overnight at 4°C.
2.2.2.2. Immunostain

The immunodetection of TGFβ-like factors from relevant decidual fractions was performed using an enhanced chemiluminescence (ECL) procedure (Amersham). Horseradish peroxidase (HRP)-linked secondary antibody, in the presence of hydrogen peroxidase ($H_2O_2$), catalyses the oxidation of luminol. During this process the luminol is in an excited state which may decay to ground state via a light emitting pathway.

The Western blot was rinsed with MilliQ water to remove any transfer buffer, immersed in blocking buffer and soaked slowly for 1 hour to block non-specific binding sites. The blot was incubated with primary antibody on a roller incubator at room temperature for 2 hours. At the end of the incubation period, the primary antibody solution was decanted. Unbound antibody was washed by briefly rinsing the membrane in a large quantity of MilliQ water, and 3x10 min shaking in TTBS. The blot was then incubated with secondary Ab-HRP or biotinylated secondary antibody for 1 hour on a roller incubator. In the case of the latter, the blot was washed as described above, and then incubated with Extravidin (HRP-conjugated) for 1h. At the end of the incubation time, it was washed as described above. The plot was exposed using a mixture of equal volumes of ECL reagents 1 and 2 for 1 min and then wrapped in Saran-wrap and exposed to X-ray film for 1 min to 1 hour (see Appendix 7.4).
2.2.3. RADIAL IMMUNODIFFUSION

In order to ascertain whether any of the decidual fractions contained immunoglobulin, radial immunodiffusion was carried out using an Ouchterlony double diffusion test (Hudson et al., 1989). Noble agar (1.5% in 0.3 M phosphate buffer) was prepared in a glass tube, and boiled in a waterbath until the agar solution was clear. The tube was removed and allowed to cool to about 56 °C, when it was poured into petri dishes (15 ml / dish). The agar was allowed to set under sterile conditions and the dishes stored in a moist box at 4 °C until use.

Wells were punched in the gel using a stainless steel cutter (diameter 3 mm; well separation, 7.5 mm centre to centre) and a syringe needle was used to pierce the agar plug and lift it clear. Decidual fractions, and appropriate antibodies at various concentrations (5 μl of each) were carefully loaded into the wells. The dishes were stored in a moist box at 4 °C for 24 h. The immunoprecipitation lines were examined and photographed.

2.3. FUNCTIONAL ANALYSIS OF DECIDUAL EXTRACTS

2.3.1. CHARACTERISATION OF TRANSFORMED HUMAN CELLS

2.3.1.1. Human cell lines
Mono Mac 6 cells were purchased from the German collection of microorganism and cell cultures, Braunschweig, Germany. The cells were originally derived from a patient with myeloid metaplasia (Ziegler-Heitbrook et al., 1988).

THP1, U937 and Jurkat E6.1 cells were obtained from the European Collection of Animal Cell Culture (Porton Down, Wiltshire). THP1, a human monocytic cell line (ECACC No. 88081201) was originally derived from the peripheral blood of a patient with acute monocytic leukaemia (Tsuchiya et al., 1980). U937, as human histiocytic lymphoma (ECACC No. 85011440) originated from the pleural fluid of a patient with diffuse lymphoma (Sundstrom et al., 1976). Jurkat E6-1 cells (ECACC No. 88042803) are a leukaemic T lymphoblastoid cell line.

All cell lines were adjusted to $2-5 \times 10^5$ cells/ml in complete medium (RPMIc). To maintain them in an exponential growth phase, the cells were subcultured at 3-4 day intervals using fresh RPMIc to dilute the cells.

For storing, cells were pelleted (400 g, 10 mins) and resuspended at 5-10 $x10^6$ /ml in either 10% glycerol (BDH; THP1 and Jurkat E6.1 cells) or 10% DMSO (Sigma Chemical Ltd) in RPMIc. The cell suspension was transferred to cryo-tubes (Nunc; 1 ml/vial), and frozen overnight at -70°C freezer. The following day the cells were transferred to liquid nitrogen.
To recover cells from liquid nitrogen, they were thawed rapidly in a 37 °C water bath and the cell suspension slowly transferred to a centrifuge tube containing 10 ml of RPMIc using a sterile plastic Pasteur pipette. After washing (360 g, 3 min), the cells were resuspended in 10 ml of RPMIc and incubated at 37 °C (5% CO₂) in an humidified incubator.

2.3.1.2. Cell proliferation and cycling

Propidium iodide (PI) is a dye which binds specifically to nucleic acids by intercalating between the bases in double-stranded DNA and RNA. After being stained with PI, the dye may be excited by UV or blue light to emit red fluorescence. By using ribonuclease (RNase) to cleave RNA, PI can be used for visualisation of the cell cycle (Ormerod, 1994).

As PI is unable to pass through an intact cell membrane, it was used for assessing cell viability and to exclude the influence of dead cells on cell cycle analysis. For this purpose, PI was directly added to the cell suspension without previous fixing.

To assess cell cycle distribution, 10⁶ cells were transferred to a FACScan tube. After washing (400 g; 5 min; PBS), the cell pellets were resuspended in 200 μl PBS. Subsequently, 2 ml of ice cold, 70% ethanol in PBS was added and the samples left at 4 °C for 30 min. After washing (400 g; 5 min; PBS), the cells were resuspended in 800 μl of PBS, 100 μl RNase (30 μ/ml in PBS; Boehringer, Mannheim GMbH), and 100 μl PI (400 μg/ml in PBS; Sigma
After incubation at 37°C for 30 min, the samples were analysed using the FACScan and FACScan research software.

To assess cell viability, PI was added to an unfixed sample of the cell population, and analysed as described above.

2.3.1.3. Phenotypic analysis

Cells which were to be phenotypically characterised were treated as described below. An aliquot of cells (0.5-1 x10⁶ cells) in PBS was transferred to a FACScan tube (Becton Dickinson). After washing in PBS (400 g; 5 mins), the cell pellets were resuspended in PBS, and saturating volumes of the appropriate MoAb (10-20 μl; according to the manufacturers' instruction) were added. The samples were mixed well and kept on ice for 30 min. Thereafter they were washed twice with ice-cold PBS, resuspended in 1 ml cold PBS and placed on ice until analysed using the FACScan flow cytometer (Becton Dickinson, USA) equipped with an argon ion laser (488 nm, 15mV) and FACScan research software. Appropriate isotype controls were included in each experiment.

2.3.2. MITOGEN INDUCED LYMPHOCYTE PROLIFERATION

2.3.2.1. Isolation of peripheral blood mononuclear cells (PBMNC)

Samples of heparinized venous blood (10 μ/ml) were obtained with informed consent from healthy volunteers. The blood was diluted with an equal volume
of RPMI at room temperature and layered over 12 ml of lymphoprep (Nycomed, Birmingham, UK). After centrifugation (400 g; 30 minutes), the interface cells were harvested. After washing twice with RPMI (400 g; 10 minutes), the cell pellets were resuspended in RPMIc and counted using an improved Neubauer haemocytometer and trypan blue exclusion (0.2% v/v in PBS) as a measure of viability. Cells were diluted to the required concentration with RPMIc.

2.3.2.2. Mitogen-induced lymphocyte proliferation

In this assay, 100 or 200 μl aliquots of freshly isolated PBMNC in RPMIc (10^6/ml) were placed in each well of a 96-well tissue culture plate. To each well was added either 10 μl RPMIc (as control), or 10 μl of an appropriately diluted stock solution of phytohemagglutinin (PHA; ICN), or Concanavalin A (Con A; Sigma) in RPMIc. All experiments were set up with a minimum of three replicate wells. The plates were incubated at 37 °C in a humidified atmosphere (5% CO₂) for 3 days. The cellular proliferation was measured using either a tritiated thymidine incorporation assay or a 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide (MTT) assay.

2.3.2.3. Tritiated thymidine incorporation

In order to measure cellular proliferation, tritiated thymidine incorporation was assessed. In this assay, 1 μCi tritiated thymidine (Amersham, specific activity 25 Ci/mmol) was added to each well of a microtitre plate, 6 h (mitogen response), or 18 h (MLR) before the end of the culture period. The cells were
harvested on glass filter paper using an ILACON cell harvester and a distilled water / methanol washer. After drying the filter paper discs in air, they were placed into vials containing 4 ml of scintillation fluid (BDH) and analysed using a liquid scintillation β-counter equipped with a line printer (LKB). The results were expressed as counts per minute (CPM).

2.3.2.4. 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide (MTT) assay

The colorimetric MTT assay involves the selective ability of living cells to reduce the tetrazolium salt MTT to an insoluble product (formazan) via the action of the mitochondrial enzyme succinate dehydrogenase (Slater et al, 1963). This conversion takes place only in living cells and the amount of active formazan produced is proportional to the number of cells present (Mosmann, 1983).

The basic protocol used in this study is based on Mosmann's method (1983) as modified by Denizot et al (1986). However, in preliminary experiments it was found that the suggested solvent (ethanol) did not achieve maximal solubilization of formazan crystals, even after agitation for 20 min. Thus, dimethyl sulphoxide (DMSO) was used as solvent, which achieved much better solubilization as evidenced by the disappearance of the purple formazan crystals in the wells (Twentymann et al., 1987)
A stock solution was prepared at 5 mg/ml MTT in PBS and sterilised by passing through a 0.2 μm filter (Millipore) which also removed any insoluble crystals. The solution was stored in the dark at 4 °C until use when it was diluted in phenol red-free RPMI (RPMI-PR') medium (Gibco, Paisley, UK) containing 1 mg/ml pyruvic acid.

Cells cultured in 96-well, flat bottomed, tissue culture plates were pelleted by centrifugation (800 g; 5 min), and the supernatant removed by carefully inverting the plates. Fifty microlitres of the MTT solution in RPMI-PR' (1 mg/ml) was aliquotted into each well, and the plates incubated at 37 °C for 3 h. After centrifugation (800 g; 5 min), the non-reduced MTT was removed by carefully inverting the plates. Each well was supplemented with 50 μl of DMSO, and the plate agitated (plate shaker model R 100, Luckham) until complete solubilization of the formazan product occurred. The optical density of each well was measured using a plate reader (Multiskan MCC) fitted with a 540 nm test wavelength filter.

2.3.3. ONE-WAY MIXED LYMPHOCYTE RESPONSE (MLR)

The one-way, mixed lymphocyte culture response (MLR) was used to assess the allogeneic reaction resulting from the interaction between CD4+ T lymphocytes and MHC class II antigen-presenting cells from genetically distinct individuals. Responding cells (PBMNC, or purified T cells isolated as described in Section 2.4.2.), were counted and adjusted to 1-2x10^6 cells/ml
with RPMIc. Stimulating cells (either non-T PBMNC, or Mono Mac 6 cells) were treated in the dark for 1 h with 25 μg/ml (final concentration) of mitomycin C (37 °C). After washing three times in RPMI (400 g; 5 minutes) to remove residual mitomycin C, the cell suspension (100 μl) was added to the wells containing responder cells. Wells containing stimulator or responder cells alone with 100 μl RPMIc were set up as controls. The plate was incubated at 37 °C for 4-6 days and cellular proliferation assessed using the 

2.4. ISOLATION OF DECIDUAL MONONUCLEAR CELLS (DMNC)

Decidual tissue was collected into RPMIc immediately after abortion and kept cool until delivered to the laboratory (usually within 3 hours). Samples were washed several times with PBS until free from blood and the tissue minced with scissors and strained through a stainless steel mesh. The dissociated cells obtained in this way were filtered through a sterile gauze to remove any clumps, and washed twice with PBS (400 g, 10 minutes). The cell pellet was resuspended in PBS and gently layered over a percoll gradient (Pharmacia, Sweden) as described by Starkey et al. (1988) and Norwitz et al. (1991). The constituents of the percoll solution is given in Appendix A. Briefly, the cells were resuspended in 36% Percoll in RPMI and layered over 62.5% percoll. This was overlaid with 5ml of PBS and centrifuged (760 g) for 30 min. The top cell layer, which contained dead and many large cells, was
discarded. The other layer (which contained the decidual mononuclear cells; DMNC) was harvested and washed three times in RPMI (400 g; 5 minutes). The total number of cells was counted and their viability assessed using trypan blue. Only those DMNC with a viability of greater than 95% were used in subsequent experiments.

2.4.1. PLASTIC ADHERENCE

In order to deplete macrophages, an aliquot of unseparated DMNC in RPMIc (3x10^6 cells/ml) were incubated in a plastic tissue culture dish (37°C; 5% CO₂). The supernatant was aspirated and the plates washed. The aspirate and washing which contained the non-adherent cells were collected. Adherent cells were removed by cooling of the dish for 3-5 min at -20°C followed by vigorous washing. The cells were harvested, counted and adjusted to a desirable concentration.

2.4.2. CELL SEPARATION USING THE MACS

2.4.2.1. Preparation of buffer and column

A magnetic activated cell sorter (MACS) was purchased from Miltenyl Biotel GMBH (Germany) and appropriate columns and needles were selected on the basis of the expected total number of positive cells.
Although the standard buffer recommended by the manufacturer is PBS/0.01% sodium azide/5mM EDTA/1% FCS, the viability of cells separated in the presence of sodium azide has been reported to be decreased to 50-70% on subsequent incubation (Pflueger et al., 1990). Hence, sodium azide was omitted in all experiments.

2.4.2.2. Immunomagnetic staining and cell sorting

Mononuclear cells to be separated were washed with ice cold PBS twice and resuspended in buffer to which appropriate MoAbs conjugated to immunobeads were added. After incubation at 4 °C in the dark for 15 min, microbeads were removed by washing with ice cold buffer. Cells were resuspended in 1 ml of buffer and separated using the MACS.

Before operation, the column was autoclaved and rinsed several times with ice cold buffer, the final rinse being retained in the column for approximately 30 min. Immediately before the separation, the column was washed again with ice cold buffer.

The sample was loaded on the top of the column under sterile conditions and allowed to pass into the column bed. Ice cold buffer was run through to flush the non-magnetic cells through the system. After extensive washing, the column was removed from the magnetic field, and rinsed with 10 times the volume of the column matrix. The cells flowing through the column were harvested as the "magnetic fraction".
Immunobeads used in this study were CD3, CD4, CD8, CD14, plus an NK cell isolation kit. All immunobeads were purchased from Mittenyi Biotec, Germany. The efficiency of the separation procedure was assessed using the FACScan by labelling both the 'magnetic' and 'non-magnetic' fraction with appropriate MoAbs (Section 2.3.1.3.).

2.4.2.3. Depletion of T cells and macrophages from DMNC

NK cell-enriched populations were obtained using the MACS and the NK cell isolation kit microbeads. The kit includes a cocktail of modified CD3, CD4, CD19 and CD33 antibodies of mouse IgG1 isotype to label non-NK cells. CD14 microbeads were also included.

Mechanically isolated DMNC (10⁸ cells) were incubated with 625 µl of the buffer and 125 µl of reagent A (Cocktail of modified CD3, CD4, CD19, CD33 antibodies of mouse IgG1 isotype to label non-NK cells) in the dark at 4 °C for 15 min. The cells were washed and the same amount of reagent B (Colloidal superparamagnetic MACS microbeads recognising Reagent A) and buffer were added to the sample. An aliquot of CD14-conjugated microbeads (50 µl) was also added. After 15 min incubation, the cells were washed (400 g; 5 min), and immediately separated using the MACS as described in Section 2.4.2.2. The cells from both the magnetic and non-magnetic fractions were washed (400 g, 5 min), counted and their viability assessed using trypan
blue. Only those cell fractions showing greater than 95% viability were used in subsequent experiments.

2.5. CHARACTERISATION OF CELLS PRODUCING IMMUNOREGULATORY FACTORS IN CULTURE SUPERNATANT

2.5.1. PREPARATION OF SUPERNATANTS OF SHORT TERM INCUBATION

DMNC, or their subpopulations, at an appropriate concentration (1-4 x10⁶ /ml), were dispersed into 24 well tissue culture plates (Nunc). The concentration of cells used was in keeping with the literature (Searle et al., 1992; Wood et al., 1988; Norwitz et al., 1991). The cells, with or without stimulation (Con A or IL-2) were incubated at 37 °C for 3 days. At the end of the incubation time, the cell suspensions were transferred into Eppendorf tubes and microcentrifuged to pellet the cells. The supernatants were harvested, pooled and stored at -20 °C prior to use.

2.5.2. CHARACTERISATION OF CELLS PRODUCING IMMUNOREGULATORY FACTORS

The supernatants obtained were added in different volumes to the microcultures of Mono Mac 6 cells (2x10⁵ /ml) for three days. The growth of
the cells was measured using the MTT assay as described in Section 2.3.2.4. Control wells lacked decidual supernatants.

2.6. STATISTICS

Data were analysed using parametric and non-parametric tests where appropriate. Those tests used include the Student’s t-test, the Mann-Whitney U test, and the Wilcoxon’s rank sum test.
3.1 CORRELATION BETWEEN CELL DENSITY AND OD 540 nm

An MTT assay was used in this study to monitor the multiplication of cells, especially the growth of cell lines. To evaluate its reliability, a variable number of Jurkat E6.1 or Mono Mac 6 cells (in exponential growth phase) were aliquotted in a flat-bottomed microplate and the MTT assay performed as described in Section 2.3.2.4. Analysis within an experiment of the relationship between the cell density and the optical density (OD) of each well showed a linear correlation \(r = 0.98\). This is keeping with the work of others (Denizot et al., 1986; Sladowski et al., 1993). Although the amount of MTT-formasan produced by the cells varied between one experiment and another. Fig 3.1-1 shows a representative result obtained from Jurkat E6.1 cells.

An MTT assay was used to assess the mixed lymphocyte response (MLR) in four experiments. The response was detected in only one experiment. The low sensitivity of this assay to measure cell replication may account for this result. Hence, the \(^3\text{H}\)-thymidine incorporation assay was used for this experiment later on (see below 3.3, 3.4, 3.5).
**Fig. 3.1-1. Correlation between cell number and OD 540 nm.**

Various concentrations of Jurkat E6.1 cells in exponential growth phase were dispersed into microplates and incubated with 50 μl of 1 mg/ml of MTT. After 3 h incubation formazan precipitate was dissolved in 50 μl of DMSO. Each point is the mean and standard deviation from quadruplicate wells.
3.2. MITOGEN INDUCED LYMPHOCYTE PROLIFERATION

Concanavalin A (Con A) and Phytohaemagglutinin (PHA) are polyclonal activators primarily of T lymphocytes. They can stimulate a large proportion (sometimes as much as 70-80%) of susceptible lymphocytes. By interaction with specific sugar compounds on the lymphocyte membrane they trigger RNA and DNA replication, resulting in cell growth and proliferation (Sharm, & Lish, 1989). Whilst triggering T cells through a different route to antigen, mitogen stimulation provides a model which can be used to assess the immunoregulatory properties of decidual extracts. Thus, the response of human peripheral blood mononuclear cells (PBMNC) to Con A and PHA was standardised as described below.

3.2.1. TITRATION OF PHA AND Con A

A variety of concentrations of PHA (0.1-1 µg/ml) were added to PBMNC (200 µl; 10⁶ cells/ml in RPMI). After culture for three days, cellular proliferation was assessed using the MTT assay (Section 2.3.2.4). It was found that PHA caused a dose-dependent stimulation of lymphocytes up to a maximum at 1 µg/ml where the response plateaued (Fig 3.2-1). The mean increase at these concentrations was 55-60% compared with control cultures lacking PHA (mean +/- SD: 0.201 ± 0.021 in control; 0.311 ± 0.019, at 0.5 µg/ml; 0.321 ± 0.068, at 1µg/ml; and n=3). This is consistent with the literature which showed that 1 µg/ml was optimal concentration (Lang & Searle, 1994;
Fig. 3.2-1. Dose response of PHA on lymphocyte proliferation.
Human PBMNC (2x10^5 cells; 200 μl) were cultured in the absence or presence of various concentrations of PHA for 3 days. The cellular proliferation was measured with MTT assay. Each point represents the mean of quadruplicate cultures (standard deviation <20%) from three separate experiments.
Stoeck et al., 1989). In keeping with literature, 1 μg/ml of PHA was used in subsequent experiments.

When various concentration of Con A (1-20 μg/ml) were added into the culture of PBMNC, it also caused a dose-dependent stimulation of lymphocyte proliferation with a maximal effect at 10 μg/ml (Fig 3.2-2). In comparison with PHA, Con A exhibits a more potent stimulation of the cells. The mean increase in OD at the optimal concentration was 156% compared to controls (mean +/- SD: 0.182 ± 0.073 in control; 0.467 ± 0.246, at 10 μg/ml; n=3).

3.3. MLR WITH U937 CELLS AS STIMULATOR

The mixed lymphocyte culture reaction (MLR) is an important assay in the study of allogeneic reactivity. In this assay, CD4 molecules expressed on lymphocytes from one individual recognise and respond to MHC class II antigen bearing cells from another unrelated individual. The cells expressing MHC class II antigens on their surface from PBMNC are monocytes, dendritic cells and B lymphocytes.

Different approaches have been made to study the reaction. For instance, dendritic cells isolated from PBMNC can stimulate the reaction very effectively (Knight et al., 1986; Eales et al., 1988). As terminal cells they do not require irradiation or treatment with mitomycin C to prevent their proliferation. However, dendritic cells only account for approximately 1-5% of PBMNC.
Fig. 3.2-2. Dose response of Con A on lymphocyte proliferation.

Human PBMNC were cultured in the absence or presence of various concentrations of Con A for 3 days. The cellular proliferation was measured using an MTT assay. Each point represents the mean and standard deviation of quadruplicate cultures from three separate experiments.
Hence, the application of the protocol has been limited as a relatively large amount of blood from two individuals is required.

An alternative approach is to use an MHC class II expressing cell line as stimulator. Since IFN\(\gamma\) can induce class II antigen expression in many cell lines, including several human monocytic cell lines (Watanabe et al., 1990; Shaked et al., 1992), these cells have been reported to have the capacity to stimulate the proliferation of T lymphocytes (Shaked et al., 1992).

U937, a human myelomonocytic cell line, expresses many characteristics of monocytes, such as the release of lysozyme and myeloperoxidase (reviewed by Harris P et al., 1985). It has been reported that IFN\(\gamma\) treatment induced these cells to express MHC class II molecules (Watanabe et al., 1990; Shaked et al., 1992). Thus, the use of this cell line as stimulator in a MLR was investigated.

3.3.1. MODULATION OF HLA-DR EXPRESSION

U937 cells constitutively do not express HLA-DR antigens on their surface. To induce their expression, the cells (5x10\(^5\) /ml) were treated with 0, 100, 300, and 500 \(\mu\)g/ml of IFN\(\gamma\) for 24 h and 48 h respectively. Cell surface antigen analysis (as described in Section 2.3.1.3) demonstrated that HLA-DR expression was induced by all concentrations tested in four experiments (Fig 3.3-1).
The time course showed that the effect of each dose after 24h incubation was greater than the corresponding dose after 48 h incubation in each of three experiments. One typical result is showed in Fig 3.3-2. It demonstrated that the median fluorescence was 73, 179, 235, and 245 at the concentrations of IFNγ 0, 100, 300, and 500 μ/ml respectively after 24h, and 65, 139, 201, and 215 after 48 h. Hence, the cells were treated with 500 μ/ml of IFNγ after 24 h in subsequent experiments.

3.3.2. INHIBITION OF THE PROLIFERATION OF STIMULATORS

Mitomycin C is an anti-metabolic agent. It affects cells in exponential growth by preventing their division. Thus, it can be used to inhibit the proliferation of stimulator cells used in the MLR, thereby ensuring that any measure of cell proliferation may be attributed to the responder cells.

Conventionally, appropriate cells have been incubated with 50 μg/ml of mitomycin C (sigma) for 25 min (Knight, 1987) or 25 μg/ml for 60 min (Goto et al., 1990). However, since U937 cells are a transformed tumour cell line, they may respond differently to mitomycin C when compared with non-transformed cells. Thus, in order to determine the concentration of mitomycin C required to effectively inhibit the stimulator cell growth, IFNγ -treated U937 cells (10^6 /ml) were exposed to a range of concentrations of mitomycin C (5-50 μg/ml in
Fig. 3.3-1. Dose response of IFNγ on the induction of HLA-DR expression on U937 cells.

U937 cells (5x10⁵/ml) were treated with 0, 100, 300, and 500 μ/ml of IFNγ for 24 h and 48 h respectively. The expression of HLA-DR was detected with FACScan. (control; ...100 μ/ml; ..........300 μ/ml; _ _ _ 500 μ/ml. A. 24 h; B. 48 h ).
Fig. 3.3-2. Time course of IFNγ on the induction of HLA-DR antigen expression on U937 cells.

U937 cells (5x10⁵ /ml) were treated with 0, 100, 300, 500 u/ml of IFNγ for 24 and 48 h respectively. The mean of fluorescence was detected with the FACScan.
RPMI in the dark for 1 h at room temperature. After washing three times, the cells (5x10^6/ml) were incubated for three days. A dose dependent inhibition of cellular proliferation was observed in all experiments. The lowest concentration of mitomycin C which totally prevented the growth of the cells in two experiments and caused 60% inhibition in another experiment was 20 μg/ml. (see Fig. 3.3-3). Also, this concentration was the least toxic. It caused less than 50% cell death after treatment for three days, whereas higher concentrations caused greater than 60% cell death.

The dose response of mitomycin C on the growth of U937 cells was confirmed by cell cycle distribution analysis (see Section 2.3.1.2 for methodology). The cells were treated with or without various concentrations of mitomycin C (5-30 μg/ml) for 24 h in three experiments. One typical result is shown in Fig 3.3-4a. It demonstrated that maximal inhibition was observed at a concentration of 20 μg/ml. The percentage of cells in S phase increased from 25%, in the untreated, to 60% in the treated, cells. This change was reflected in a decrease of cells in G0/G1 from 68% in the untreated, to 28% in the treated, cells. A graph of the inhibition of DNA synthesis at the optimal concentration is shown in Fig. 3.3-4b.

In view of the effect of mitomycin C on both cellular proliferation and cytotoxicity, 20 μg/ml of mitomycin C was chosen and used in subsequent experiments.
Fig. 3.3-3. Titration of mitomycin C concentration on the growth of IFN\(\gamma\) treated U937 cells.

The cells were incubated with various concentrations of mitomycin C in the dark for 1 h at room temperature. After washing three times, the cells (5x10^5/ml) were transferred into 24 well tissue culture plates and incubated for 3 days. Each point is the mean of duplicates count from three experiments.
**Fig. 3.3-4(a).** Dose response of mitomycin C on the cell cycle distribution of U937 cells. IFNγ-pretreated U937 cells were incubated with various concentrations of mitomycin C for 24. After washing three times, the cells were stained with propidium iodide and analysed with the FACScan. The figure is a typical result from three separate experiments.

**Fig. 3.3-4(b).** Effect of mitomycin C (20 μg/ml) on the cell cycle distribution of U937 cells (...... control; . . . . IFNγ only treated; _____ IFNγ and mitomycin C treated).
3.3.3. USE OF U937 CELLS AS STIMULATORS IN AN MLR

Freshly isolated PBMNC were incubated at various concentrations with 0.6x10^5 U937 cells per well pre-treated with IFNγ and mitomycin C for 5 days. (500 u/ml) (20 µg/ml) Tritiated thymidine incorporation (See Section 2.3.3 for methodology) was found to be directly proportional to the number of responder cells added in three experiments. Maximal response was seen with 1.75x10^5/ well responders (ratio of S to R was 1: 3; see Fig. 3.3-5).

When the number of stimulator cells was varied and the responder cells maintained at a constant level (1.5x10^5 /well), [³H]-thymidine incorporation after 5 days was highly variable. In five experiments, two showed an MLR, and three did not. The results are shown in Fig. 3.3-6.

To exclude the possibility that manipulation of the cell harvesting may have contributed to the variability observed, 10^6 U937 cells per well were incubated with tritiated thymidine (1 µCi/well) for 4 h. The mean and standard deviation of CPM (x10^3) in 27 wells was 163 ±14. This is consistent with the data described by the manufacturer's instruction manual (ILACON). This suggests that the variable results obtained were not the result of improper manipulation in cell harvesting.
Fig. 3.3-5. Effect of amount of PBMNC on the one-way MLR.

IFNγ and mitomycin C-treated U937 cells (0.6x10^4/well), along with various concentrations of PBMNC were incubated for 5 days. Each point above represents the mean CPM from quadruplicate cultures.
Fig. 3.3-6. Effect of amount of U937 cells on one-way MLR.

PBMNC (1.5x10^5/well), along with various concentrations of IFNγ and mitomycin C treated U937 cells, were incubated at 37 °C for 5 days. The cellular proliferation was measured with [³H]-thymidine uptake. Each point above is the mean of quadruplicate cultures from five separate experiments.
3.4. MLR USING MACS-SEPARATED T LYMPHOCYTES AND NON T CELLS

Conventionally, when PBMNC are employed in an MLR, they may be used either unfractionated, or fractionated into stimulator and responder population. The former has low sensitivity owing to the low density of alloreactive T lymphocytes and MHC class II antigen bearing cells. The latter requires other separation procedures, which are either time-consuming, or result in the partial loss of certain populations and therefore require relatively large volumes of fresh blood from two individuals. Thus, it is inconvenient to use these procedures routinely.

The MACS is a magnetic cell sorter used for the separation of a large number of cells according to their specific cell surface markers. Cells labelled with monoclonal antibody conjugated with magnetic microbeads are loaded on a column containing a steelwool-fibre matrix held in a powerful magnetic field. The negatively labelled cell populations flow through the column and are collected. Whilst the positive population is retained in the column. These cells are harvested by removing the column from the magnetic field and flushing it with buffer (Miltenyi et al, 1990).

Owing to the problem encountered using U937 cells as stimulators in an MLR, human PBMNC were separated into non-T cells (which include monocytes, B lymphocytes and dendritic cells) and T lymphocytes using the
MACS and used as stimulators and responders respectively in a one-way MLR. The advantage of this system is that both the cells expressing MHC class II antigens and CD4 molecules may be collected for use.

3.4.1. ASSESSMENT OF THE EFFICIENCY OF CELL SEPARATION USING THE MACS

To prepare PBMNC for magnetic cell sorting, PBS was used throughout the separation procedure instead of RPMI to prevent the biotin in the medium affecting the procedure. The PBMNC \((10^7)\) were incubated with 20 \(\mu l\) anti-CD4 and 20 \(\mu l\) anti-CD8 microbeads suspended in 80 \(\mu l\) cold buffer (PBS/5 mM EDTA/1% FCS) and separated through an A2 column (the methodology was described in Section 2.4.2).

After the sorting, portions of the magnetic fraction (positive cells), and the non-magnetic fraction (negative cells), as well as unsorted cells were stained with FITC-conjugated anti-human CD3 MoAb and analysed using the FACScan. This showed that before separation, the mean of CD3 positive cells accounted for approximately 55% of the isolated PBMNC. After sorting, this increased to 91% in the magnetic fraction; whilst only 8% of the non-magnetic fraction were positive for CD3. Fig 3.4-1 shows the effect of the separation.
Fig. 3.4-1. T cells enrichment of $7.5 \times 10^6$ PBMNC in a column of type A2.
The cells was sorted with CD4 and CD8 microbeads, and then stained with FITC-CD3 mAb. (____ nonseparated; . . . negative fraction; ........ positive fraction).
3.4.2. STIMULATORY EFFECT OF DIFFERENT RATIOS OF STIMULATOR: RESPONDER CELLS

According to the standard procedure (Goto et al., 1990), the MACS-separated non-T cells from two unrelated donors were treated with 25 μg/ml (final concentration) of mitomycin C at 37 °C for 1 h. At the end of the incubation period, the cells were washed three times with RPMI and then adjusted to a concentration of 1x10⁶ /ml in RPMIc. T lymphocytes were washed with RPMI, and resuspended in RPMIc to concentrations of 1x10⁶ /ml, 1.5x10⁶ /ml, and 2x10⁶ /ml.

Twenty-five microlitres of the stimulator cell suspension from individual A was dispersed into the wells of a round-bottomed microtitre plate. To each well was added 25 μl of a responder cell suspension from individual B. The ratios of stimulator to responder was 1:1, 1:1.5, and 1:2 respectively. A second plate was set-up with stimulators and responders from the alternate individual. Wells containing only stimulators or responders and culture medium were used as background controls.

After 5 days culture, DNA synthesis was significantly higher than controls at all ratios of stimulator to responder cells (p<0.01, using Student's T test; n=4). No significant difference was observed between the cultures containing different ratios of stimulators to responders (Fig 3.4-2).
3.4.3. STIMULATION TIME COURSE

Stimulators (non-T cells; 2.5x10^4 cells / well) and responders were cultured in different ratios for 4, 5, or 6 days. The [³H]-thymidine uptake showed that the MLR increased with length of culture. However, this increase only reached significance when stimulators and responders were cultured at a ratio of 1:1. At higher ratios (1:1.5 and 1:2) there was no significant difference in the proliferation between day 4 to day 6 of culture (Fig 3.4-3). This may be due to the limitation in nutrients at the higher cell densities.

3.5. ONE-WAY MLR USING Mono Mac 6 CELLS AS STIMULATORS

Mono Mac 6 is a well-characterised phagocytic cell line with many of the characteristics of mature peripheral blood monocytes and tissue macrophages, including the ability to produce cytokines upon stimulation, and the expression of CD14 and HLA-DR (Ziegler-Heitbrook et al., 1988). It provides another target to which CD4-positive T lymphocytes may react. In the later stages of the study this cell line became available and was used as a stimulator in the one-way MLR.
Fig. 3.4-2. Effect of ratios of stimulator to responder on one-way MLR.
The cells were cultured in the different ratios of S to R (indicated above) with 2.5x10⁴/well stimulator in a total volume of 50 µl for 5 days, including 18 h pulse with [³H]-thymidine. The results are the means and standard deviations of triplicate cultures from four experiments.
Fig. 3.4-3. Time course of the one-way MLR.
MACS-separated non T cells (stimulators) and T lymphocytes (responders) were cultured in the different ratios for 4, 5, or 6 days. Results are shown as the mean and standard deviation of tritiated thymidine incorporation expressed as counts per minutes (n=3).
3.5.1. EXPRESSION OF HLA-DR ON MONO MAC 6 CELLS.

The expression of MHC class II antigens on Mono Mac 6 cells was examined using a phycoerythrin (PE)-conjugated anti-HLA-DR MoAb and flow cytometry. Fifty to sixty percent of the Mono Mac 6 cell line were positive for HLA-DR expression (See Fig 3.5-1).

3.5.2. STIMULATING EFFECT OF MONO MAC 6 CELLS IN AN MLR

Mono Mac 6 cells were pre-treated with mitomycin C (25 μg/ml) at 37 °C for 1 h, and resuspended in RPMIc (1x10⁶/ml). Freshly isolated PBMNC (100 μl of 1.5x10⁵ cell / ml) were mixed with the stimulators (60 μl) at a ratio of stimulators (1) to responders (2.5) in a total volume of 160 μl per well for 5 days. The results showed that Mono Mac 6 cells clearly stimulated an MLR. The proliferative response was significantly higher in wells containing both stimulators and responders than control wells containing either of the population alone (p<0.05 in one, p<0.01 in three experiments, using Student's T test; n=3). However, in one experiment the responders alone showed a high background. Table 3.5-1 shows four separate experiments.
Fig.3.5-1. HLA-DR expression on Mono Mac 6 cells. The cells \((10^6)\) were stained with PE-conjugated anti HLA-DR MoAb or anti mouse IgG isotype control (\.\.\. control; \_____ anti-HLA-DR MoAb stained).
Table 3.5-1. Effect of Mono Mac 6 cells as stimulators in an MLR.

PBMNC were incubated with mitomycin C treated Mono Mac 6 cells (25 µg/ml; 37 °C; 1 h) in a ratio of stimulator to responder 1: 2.5 with 6x10⁴ stimulator per well for 5 days. The total volume per well was 160 µl. The results were the means and standard deviations from triplicate cultures.

<table>
<thead>
<tr>
<th>Expt</th>
<th>Stimulator only</th>
<th>Responder only</th>
<th>Stimulator+Responder</th>
<th>p*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2.4±/-1.4</td>
<td>25.8±/-9.4</td>
<td>47.2±/-12.2</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>2</td>
<td>1.0±/-0.6</td>
<td>4.9±/-1.6</td>
<td>9.2±/-1.7</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>3</td>
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<td>7.0±/-1.8</td>
<td>16.9±/-0.8</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>4</td>
<td>1.1±/-0.9</td>
<td>4.6±/-2.1</td>
<td>18.6±/-1.8</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

* between stimulators + responders and responders only.
SECTION 2. IDENTIFICATION AND PARTIAL CHARACTERISATION OF IMMUNOREGULATORY FACTORS FROM DECIDUA

4.1. THE IMMUNOREGULATORY EFFECT OF CRUDE DECIDUAL PROTEIN FRACTIONS (CDPF)

4.1.1. METHODOLOGY OF EXTRACTING DECIDUAL INTRACELLULAR PROTEINS

In order to extract intracellular soluble proteins from decidua prior to isolating any suppressive factors, it was necessary to disrupt the cells. Different approaches, including chemical, physical and mechanical are available for this purpose. The approach used here was homogenisation by which large quantities of decidua were disrupted in a short time (Section 2.1.2.1). To promote protein release, the homogenisation of samples was performed after freezing at -70 °C.

4.1.2. PREPARATION OF CRUDE DECIDUAL PROTEIN FRACTIONS.

After releasing intracellular proteins, cellular debris were removed by centrifugation (Section 2.1.2.1). The decidual supernatant obtained was concentrated and crudely separated with a Minidan ultrafiltration system using
molecular weight cut-off membranes with nominal exclusion rates of 10, 30, 100 and 300 KDa. Thus, the crude decidual protein fractions (CDPR) with M.w approximately <10, 10-30, 30-100, 100-300, >300 KDa were obtained (see Section 2.1.2.2). When the total volume of the fractions obtained was compared to the total volume of the unfractionated supernantant, it was found that the individual fractions were concentrated by 1-4% (Table 4.1.1), which is consistent with the leaflet of the manufacturers (Millipore Corp, 1990).

4.1.3. PROTEIN CONCENTRATIONS IN CRUDE FRACTIONS

To determine the protein concentrations in each fraction, a standard curve was constructed by measuring the optical density at 725 nm of BSA solutions of known concentration using the modified Biuret reagent of Ohnishi and Barr (1978) for the Lowry procedure (1951). It showed that the absorbance at 725 nm of the BSA solution was directly proportional to the protein concentration (r=0.98; Fig 4.1-1). The protein concentration of each decidual fraction was estimated using the standard curve (Table 4.1-1).

4.1.4. EFFECT OF CDPF ON MITOGEN-INDUCED LYMPHOCYTE PROLIFERATION

The fractionated CDPF, at a concentration of 10% (v/v), were added to PHA-stimulated lymphocyte cultures (Section 2.3.2.2). The same volume of PBS was added to controls. It showed that the fractions of 30-100 KDa and >300
Fig. 4.1-1. Standard curve of protein concentrations at 725 nm.

The known protein concentration was prepared from bovine serum albumin.
Table 4.1-1. Effect of preliminary separation and concentration of decidual supernatant with ultrafiltration system.

The decidual supernatant was initially filtered through two 10 KDa filters, generating < 10KDa fraction. The retentate was collected and filtered through two 30 KDa filters, producing a fraction with approximate molecular weight range of 10-30 KDa. In a similar manner, 30-100 KDa, 100-300 KDa, and >300 KDa fractions were collected by using appropriate membranes. The Protein concentration in individual fractions was estimated using a standard curve of protein concentrations at 725 nm.

a). First batch:

<table>
<thead>
<tr>
<th></th>
<th>Volume of sample (ml)</th>
<th>Protein concentration (mg/ml)</th>
<th>Total protein (mg)</th>
<th>Yield (%v/v)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unfractionated</td>
<td>300</td>
<td>12.2</td>
<td>3660</td>
<td>100</td>
</tr>
<tr>
<td>&lt;10 KDa</td>
<td>10</td>
<td>0.16</td>
<td>1.6</td>
<td>3.3</td>
</tr>
<tr>
<td>10-30 KDa</td>
<td>12</td>
<td>0.05</td>
<td>0.6</td>
<td>4.0</td>
</tr>
<tr>
<td>30-100 KDa</td>
<td>12</td>
<td>3.9</td>
<td>46.8</td>
<td>4.0</td>
</tr>
<tr>
<td>100-300 KDa</td>
<td>10</td>
<td>0.81</td>
<td>8.1</td>
<td>3.3</td>
</tr>
<tr>
<td>&gt;300 KDa</td>
<td>256</td>
<td>7.5</td>
<td>1921</td>
<td>85</td>
</tr>
</tbody>
</table>

a). Second batch:

<table>
<thead>
<tr>
<th></th>
<th>Volume of sample (ml)</th>
<th>Protein concentration (mg/ml)</th>
<th>Total protein (mg)</th>
<th>Yield (%v/v)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unfractionated</td>
<td>10,000</td>
<td>5.5</td>
<td>55,000</td>
<td>100</td>
</tr>
<tr>
<td>&lt;10 KDa</td>
<td>100</td>
<td>0.16</td>
<td>16</td>
<td>1</td>
</tr>
<tr>
<td>10-30 KDa</td>
<td>100</td>
<td>0.19</td>
<td>19</td>
<td>1</td>
</tr>
<tr>
<td>30-100 KDa</td>
<td>120</td>
<td>1.3</td>
<td>1560</td>
<td>1.2</td>
</tr>
<tr>
<td>100-300 KDa</td>
<td>120</td>
<td>1.7</td>
<td>2040</td>
<td>1.2</td>
</tr>
<tr>
<td>&gt;300 KDa</td>
<td>9,000</td>
<td>5.7</td>
<td>51,300</td>
<td>90</td>
</tr>
</tbody>
</table>

* The term 'first batch' and 'second batch' refer to two different decidua sample preparations. The 'second batch' was used for all the experiments using FPLC decidua fractions.
KDa did not suppress PBMNC response to PHA. While the fractions of <10 and 10-30 KDa significantly suppressed the cellular proliferation (<10 KDa culture: p< 0.01; 10-30 KDa: p<0.05; using Student’s T test). In addition, the fraction of 100-300 KDa slightly suppressed the response, but this did not reach significance (Fig. 4.1-2 A).

When the extracts were added at a concentration of a 20% (v/v), inhibition occurred with the 30-100 and 100-300 KDa fractions. The >300 KDa fraction failed to inhibit the proliferative response to PHA (Fig. 4.1-2B). Since the control contained the same volume of PBS, it is unlikely that the inhibitory effect resulted from buffer toxicity.

4.1.5. THE EFFECT OF CDPF ON LYMPHOBLASTIC CELL LINE PROLIFERATION

A lymphoblastic cell line, Jurkat E6.1, was employed as a model to test the effect of CDPF on the growth of transformed T lymphocyte. The cells (200 µl; 2x10^5 cells /ml) were cultured with CDPF fractions or PBS as control (10% v/v) for three days. Their growth was significantly suppressed in the presence of <10 KDa fraction (mean +/- SD: 0.80 +/- 0.16 in control; 0.40 +/- 0.04 in <10 KDa; p< 0.01 using Student’s T test). By contrast, the 10-30 KDa fraction had no inhibitory effect on the growth of E6.1 cells (mean +/- SD: 0.78 +/- 0.24); and the 30-100 KDa and 100-300 KDa fractions slightly enhanced the cell growth (0.95 +/- 0.26 in 30-100 KDa; 0.96 +/- 0.34 in 100-300 KDa; Fig. 4.1-3).
Fig. 4.1-2. Effects of CDPF on PHA-induced lymphocyte proliferation.

PBMNC were cultured for 72h in the presence of 1 μg/ml of PHA, with 10% (A) and 20% (B) fractionated decidua extracts. The same volume of PBS was added to control culture. Cellular proliferation was measured using an MTT assay. The results are expressed as percentage proliferation (mean +/- SE) of control from three experiments, i.e. OD 540 test culture containing CDPF / OD 540 PHA-stimulated control x 100%.
Fig. 4.1-3. Effect of CDPF on the growth of Jurkat E6.1 cells.

Jurkat E6.1 cells (2x10^5 /ml) were cultured for three days in the presence of CDPF at a concentration of 10% v/v. Cellular proliferation was assessed using an MTT assay. The results are expressed as the mean (+/-SD) of four separate experiments.
Interestingly, these fractions exerted an inhibitory effect on the mitogen-induced lymphocyte proliferation assay as described in Section 4.1.4.

4.1.6. EFFECT OF CDPF ON THE PROLIFERATION OF MYELOMONOCYTIC CELLS

A myelomonocytic cell line, THP1, was used as a model to test the effect of decidual fractions on the growth of transformed monocytic cells. The cells were cultured (3x10^5 / ml) with CDPF or PBS as control (10% v/v) in a 24-well tissue culture plate for 3 days. The cell concentration in each individual well was then counted in duplicate. The <10 KDa fraction alone significantly inhibited the growth of the cells (p < 0.01, using Student's T test). Fig 4.1-4 shows the mean result from five experiments.

4.1.7. EFFECT OF THE <10 KDa FRACTION ON CELLULAR VIABILITY

The previous experiments demonstrated that the <10 KDa fraction inhibited mitogen-induced lymphocyte proliferation and the growth of Jurkat E 6.1 and THP1 cells. In order to examine this effect further, the cell cycle distribution and viability of Jurkat E6.1 cells were examined using propidium iodide (Section 2.3.1.2).

When Jurkat E6.1 cells were incubated with the <10 KDa fraction at various concentrations for 16 hours, ostensibly there was no change in cell cycle
Fig. 4.1-4. Effect of CDPF on the proliferation of THP1 cells. The cells (3x10^5/ml; 1 ml/well) were cultured with 10% (v/v) of each of CDPF for three days. The cell growth was measured with Coulter counter. The results are expressed as percentge growth (mean +/- SD) against control from five experiments.
distribution when compared to the control (Fig 4.1-5, A), although the treated cells tended to show a lower intensity of staining with PI. However, the cell viability was greatly decreased in a dose-dependent manner in those cultures containing the <10 KDa fraction. With 2.5%, 5%, and 10% of the fraction, the viability of the cells was decreased to 87.3%, 54.7% and 52.9% respectively (Fig 4.1-5, B). Hence, this would suggest that the decreased intensity may reflect changes in cellular DNA affecting its binding capacity for PI due to the induction of cytolysis or apoptosis by the decidual fraction.

In order to investigate this further, more cell lines, including several monocytic lines (U937 and THP2), and a breast cancer (ZR75) cell line were employed to test the effect of the fraction on their growth. Using the same experimental conditions, it was found that the extent of suppression on these cell lines by this fraction was similar to that on Jurkat E6.1 and THP1 cell lines. Table 4.1-2 shows these results.

4.2. THE IMMUNOREGULATORY EFFECT OF PURIFIED DECIDUAL PROTEIN FRACTIONS

4.2.1. GEL FILTRATION CHROMATOGRAPHY

Crude decidual protein fractions with M.w. 10-30 and 30-100 KDa were found to be inhibitory to mitogen-induced lymphocyte proliferation in preliminary
Fig. 4.1-5. Cell cycle distribution and cell viability of Jurkat E6.1 cells treated with <10 KDa fraction.

Cells were incubated in the presence or absence of various concentration of <10 KDa fraction for 16 h. The cell cycle distribution (A) and cell viability (B) were analysed simultaneously using propidium iodide.
Table 4.1-2. Effect of <10 KDa fraction on monocytic (U937, THP1 and THP2), Jurkat E6.1 and breast cancer (ZR75) cell lines.

The appropriate cells were incubated with 2.5%, 5%, and 10% (v/v) of <10KDa fraction for 24h. The cellular growth was assessed using an MTT assay. The results were mean percentage growth as compared with PBS alone as control (n=3). The standard deviation is less than 15%.

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>2.5%</th>
<th>5%</th>
<th>10%</th>
</tr>
</thead>
<tbody>
<tr>
<td>THP1</td>
<td>81</td>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td>THP2</td>
<td>96</td>
<td>21</td>
<td>18</td>
</tr>
<tr>
<td>U937</td>
<td>60</td>
<td>19</td>
<td>15</td>
</tr>
<tr>
<td>Jurkat E6.1</td>
<td>69</td>
<td>20</td>
<td>16</td>
</tr>
<tr>
<td>ZR75</td>
<td>83</td>
<td>41</td>
<td>28</td>
</tr>
</tbody>
</table>
experiments (Section 4.1.4). To isolate the suppressive factors, these fractions were pooled, centrifuged, concentrated to 3-4 mg protein per ml, and applied to a Sephacryl S-100 HR column connected to an FPLC system. The gel filtration chromatogram obtained (Fig. 4.2-1) showed a relatively high protein concentration in the >30 KDa region with three peaks. According to the standard curve of protein concentration absorbance at 280 nm (Fig 4.2-2), the protein concentration was 145 µg/ml in peak 1, 140 µg/ml in peak 2, and 350 µg/ml in peak 3 respectively. Four relatively low peaks appeared in 10-30 KDa region, their protein concentrations were between 25-50 µg/ml.

4.2.2. EFFECT OF DF ON PHA-INDUCED PBMNC PROLIFERATION

The fractions obtained from FPLC (DF) were added to PHA-stimulated PBMNC at a concentration of 10% (v/v). The cellular proliferation, as measured by [³H]-thymidine incorporation (Section 2.3.2.3), showed that the greatest suppressive activity was associated with one or more molecules of > 70 KDa (large molecular weight fraction, LMWF; DF Numbers 6-11), with lesser activity associated with one or more molecules of approximately 7-20 KDa (small molecular weight fraction, SMWF; fraction Numbers 38-44). The inhibition caused by both of the fractions was significant when compared to control cultures containing elution buffer alone (p< 0.01, compared with LMWF; p<0.05 compared with SMWF; using Student's T test). The biggest inhibition achieved was almost 30% of the control (Fig 4.2-3). Other fractions did not significantly affect proliferation.
Fig. 4.2-1. Chromatogram of the decidua crude fraction with MW 10-100 KDa.

The concentrated sample was applied to a column (XK 2.6/70) packed with Sephacryl S-100 HR. PBS (pH 7.1) was used as elution buffer. The eluate was collected in 2.6 ml aliquots.
Fig 4.2-2. Standard curve of protein concentrations against OD 280 nm.

The known protein concentration was prepared from bovine serum albumin.
Fig. 4.2-3. Effects of FPLC-separated decidual fractions on PHA-stimulated lymphocyte proliferation.
PBMNC (1×10^6/ml; 200μl) were stimulated with 2 μg/ml of PHA for three days. The fractions, at a concentration of 10% (v/v), were added in triplicate at the beginning of the assay. Cellular proliferation was assessed by [³H]-thymidine incorporation. The results were calculated using the equation

\[ \frac{\text{CPM test culture containing DF}}{\text{CPM PHA-stimulated control}} \times 100\% \]

and expressed as the mean of proliferation of five separate experiments. The standard deviation is less than 15% (proliferation of PBMNC stimulated with PHA alone and containing 10% v/v elution buffer).
4.2.3. EFFECT OF DF ON ONE-WAY MIXED LYMPHOCYTE REACTION

The effect of those factors shown to have suppressive activity in the mitogen-stimulated lymphocyte proliferation assay (LMWF and SMWF) were tested in a one-way MLR. After five days incubation, cellular proliferation as measured by \[^{3}H\]-thymidine incorporation was significantly suppressed (p <0.05 using Student's T test). The mean percentage stimulation from six experiments was 84% (± 4%) of that of control cultures in the presence of LMWF and was 88% (± 10%) in the presence of SMWF (Fig. 4.2-4).

4.2.4. EFFECT OF DF ON THE PROLIFERATION OF MONO MAC 6 CELLS

A monocytic cell line, Mono Mac 6, exhibits many characteristics of mature monocytes. In this study the effect of purified DF on these cells was investigated. After three days incubation, inhibition of the growth of Mono Mac 6 cells was more pronounced in the presence of LMWF than SMWF. The former induced suppression of up to 40% of the control containing elution buffer alone (Fig 4.2-5).

4.2.5. EFFECT OF DF ON THE PROLIFERATION OF JURKAT E6.1 CELLS

In order to determine whether the effect of LMWF and SMWF on Mono Mac 6 cells was related to their transformed state or to their cell type, the human T cell line Jurkat E6.1 was incubated with these fractions. No difference was
Fig 4.2-4. Effects of LMWF and SMWF on the one-way MLR.
Mitomycin C-treated Mono Mac 6 cells and PBMNC (Ratio of stimulator and responder, 1:2.5) were cultured at 37°C for five days. Each of the fractions, at 10% concentration, was added, in triplicate, at the beginning of the assay. Proliferation was assessed by ^3H- thymidine incorporation. The data are expressed as the mean (+/-SD) of five separate experiments as compared with elution buffer as control.
Fig. 4.2-5. Effects of FPLC-separated decidual fractions on the growth of Mono Mac 6 cells.

Mono Mac 6 cells (2x10^5/ml; 200 µl) were cultured for three days. The fractions, at a concentration of 10% (v/v), were added in triplicate at the beginning of the cultures. The growth of the cells was assessed using an MTT assay. The results were calculated using the equation

\[
\frac{\text{OD} 540 \text{ in test culture containing DF}}{\text{OD} 540 \text{ in elution buffer control}} \times 100\%
\]

and expressed as the mean growth in seven separate experiments. The standard deviation is less than 18% (______ the growth of the cells with RPMIc alone and containing 10% v/v elution buffer).
found between the proliferation of control cultures and those exposed to LMWF in six experiments. Moreover, SMWF had a slight stimulatory effect on the growth of Jurkat E6.1 cells. Fig. 4.2-6 shows these results in relation to those obtained with Mono Mac 6 cells.

4.3. EFFECT OF ACIDIFICATION ON THE BIOLOGICAL FUNCTIONS OF DECIDUAL FRACTIONS

4.3.1. CHROMATOGRAPHY UNDER ACID CONDITIONS

The decidua-derived factors were found to inhibit the response of PBMNC to mitogens, an MLR, and the growth of Mono Mac 6 cells, but not the growth of Jurkat E6.1 cells. These effects show some similarity to those described for TGFβ1 (Section 4.5.1). It has been reported that immunosuppressive activity from murine decidua was associated with a molecule of 60-100 KDa when isolated under neutral conditions. This activity was largely enhanced after treatment of the decidua solution with acid; the suppressive activity being associated with a molecule of 13 KDa when isolated under acid conditions. This activity could be neutralised by an anti TGFβ2 antibody and was thought to be a TGFβ2-related factor (Clark et al., 1985, 1988, 1990, 1992).

The LMWF isolated from human decidua had a molecular weight >70 KDa on gel filtration chromatography (Fig 4.2-1). To determine whether this was
Fig 4.2-6. Effects of LMWF and SMWF on the growth of Jurkat E6.1 and Mono Mac 6 cells.

Both of the cells were cultured with 10% (v/v) of the fraction of interest for 3 days (2x10^5/ml; 200 µl/well). The cellular proliferation was measured with MTT assay. The figures above are the mean from six experiments as compared elution buffer alone as percentage control (The standard deviation is < 18%).
identical to that detected in murine decidua, the concentrated crude decidua protein fraction with M.w. 10-100 KDa from the same batch was applied to the same column and separated under acid conditions (Section 2.1.3.2). The protein concentration of sample used was the same as that used under neutral conditions.

Fig 4.3-1 shows the chromatogram obtained under acid conditions. The majority of proteins of molecular weight >12 KDa was degraded. There were four protein peaks between 5 to 12 KDa. The elution volumes of the protein standards are marked on the top of the chromatogram.

4.3.2. EFFECT OF ACID FRACTIONS ON THE GROWTH OF MONO MAC 6 CELLS

Purified TGFβ1 has been found to be a potent inhibitor of the growth of Mono Mac 6 cells (Uhm et al., 1993); an effect also seen with the decidual fractions separated under neutral conditions. If the LMWF contained inactive precursors of TGFβ, the acidified fractions should exhibit a much greater level of suppression. In order to investigate this, acid fractions were added to the cultures of Mono Mac 6 cells with four volumes of the neutralizing agent (3.5 ml of 7.5% NaHCO₃, 3.5 ml of DH₂O, in 100 ml of RPMIc). In order to determine if dilution of the fractions by neutralisation had affected the results, the acidic elution buffer was used as control in each experiments. When 10% (v/v) of the individual fractions was added to Mono Mac 6 cultures, there was
4.3-1. Chromatogram of the decidua crude fractions with MW 10-100 KDa under acid conditions.

The concentrated solution was applied to a column (XK 2.6/70) packed with Sephacryl S-100 HR. 0.58% acetic acid (v/v), 0.1 M KCl (pH 2.9) was used as elution buffer. The eluate was collected in 3 ml aliquots.
a slight reduction in cell proliferation in the presence of fractions numbers 34 to 40 (M.w. approximately 12 KDa), which caused 5% to 12% inhibition. However, none of these fractions achieved significant inhibition (p>0.05). Other fractions did not affect the cell growth. This would suggest that the suppression observed with LMWF and SMWF isolated under neutral conditions was not due to the presence of acid labile precursors of TGFβ.

4.4. INVESTIGATION OF THE MECHANISMS UNDERLYING THE REGULATORY ACTIVITY OF DECIDUA FRACTIONS

Since both LMWF and SMWF have shown the ability to suppress the growth of Mono Mac 6 cells, the mechanisms underlying this activity were studied further by investigating the role of decidua fractions on cell cycle distribution, apoptosis, and MHC class II expression.

4.4.1. EFFECT ON CELL CYCLE DISTRIBUTION

The effect of both SMWF and LMWF on the cell cycle distribution of Mono Mac 6 cells was examined using a PI staining procedure (Section 2.3.1.2). As shown in Table 4.4-1, both fractions caused significant suppression of the growth of Mono Mac 6 cells after three days incubation. However, only the LMWF significantly affected cell cycle. By contrast, no obvious change in cell cycle distribution was observed in the presence of SMWF. These results
Table 4.4-1. Effect of LMWF and SMWF on the cell cycle, viability, as well as the growth of Mono Mac 6 cells.

The cells (2x10^5/ml) were cultured in the presence of 10% (v/v) of PBS (as control), LMWF or SMWF. The cell cycle distribution and viability was analysed with PI staining. The growth of the cells was monitored with MTT assay. These data are the means (+/-SD) of six experiments.

<table>
<thead>
<tr>
<th>Sample</th>
<th>% Cell cycle distribution</th>
<th>% Viability (% Survived)</th>
<th>% Proliferation Index</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>G0/G1</td>
<td>S</td>
<td>G2/M</td>
</tr>
<tr>
<td>Control</td>
<td>55(2)</td>
<td>21(2)</td>
<td>25(4)</td>
</tr>
<tr>
<td>LMWF</td>
<td>64(4)**</td>
<td>20(2)</td>
<td>17(4)*</td>
</tr>
<tr>
<td>SMWF</td>
<td>57(5)</td>
<td>22(2)</td>
<td>22(5)</td>
</tr>
</tbody>
</table>

** Significance p<0.01; * p<0.05 when compared with control using Student's T test.
indicate that the two decidua-derived fractions may affect the growth of Mono Mac 6 cells by different mechanisms.

Neither of the LMWF and SMWF affected cell viability (Table 4.4-1) suggesting that the observed decrease in cellular proliferation was not due to cytotoxicity.

4.4.2. EFFECT ON APOPTOSIS OF MONO MAC 6 CELLS

Apoptosis, also termed programmed cell death, represent a common mechanism involved in the regulation of the growth of cells and tissues. It is characterised by condensation of the chromatin, DNA fragmentation, and eventually cell disruption. The apoptosis-associated DNA fragmentation can be assessed by flow cytometry. When cells are stained with PI for cell cycle analysis, a distinct subpopulation of cells can be observed which express a hypodiploid level of DNA (below G0/G1), denoted as A0 (Tetford et al., 1991; Zamai et al., 1993).

In order to elucidate whether or not the decidual fractions inhibited the growth of Mono Mac 6 cells by inducing apoptosis, and eventually cell death, the cells were incubated with 10% of PBS, LMWF, or SMWF for 24h and 48h. No obvious change in A0 phase was seen in the presence of decidual fractions after 24h incubation. After 48h incubation, SMWF-treated cells were not obviously different from the control [median (range): 3.5(1.70-5.96) in control;
5.2 (2.41-6.70) in SMWF, n=8]) while LMWF-treated cells showed a significant increase in the AO region [6.8(1.79-16.59) in LMWF, n=8; p<0.05; statistical analysis was performed using the Wilcoxon signed rank test). Fig 4.4-1 shows these results.

4.4.3. EFFECT ON MHC CLASS II MOLECULAR EXPRESSION

In order to understand the effect of decidual suppressive factors on the expression of MHC class II molecules, Mono Mac 6 cells (which are MHC class II positive), were preincubated with the fractions (10%; v/v) for 24 h. A PE-conjugated MoAb raised against HLA-DR was employed to assess the expression. In general the expression in the treated cells did not differ from that of the untreated cells with either LMWF or SMWF [median (range) of FL: control, 611 (607-615); LMWF, 609 (578-664); SMWF, 594 (574-616); n=8]. This suggests that decidual suppressive factors do not down-regulate the expression of MHC class II molecules.

4.5. COMPARISON OF THE EFFECT OF TGFβ AND DECIDUA FRACTIONS

Previous work in mice has demonstrated that an immunosuppressive factor derived from decidua was in fact TGFβ (Clark et al., 1990; Altman et al., 1990). In order to determine whether or not the suppression caused by the
Fig 4.4-1. Effect of LMWF on the percentage cells in A0 region of Mono Mac 6 cells.

The cells (2x10^5/ml) were incubated with 10% of PBS (as control), or LMWF for 48h. The cells were then stained with propidium iodide and analysed with FACScan. The percentage of cells in the A0 region was obtained from DNA histogram.
decidua-derived factors in this study are caused by TGFβ, its biological functions were compared those of the decidua-derived immunoregulatory factors.

4.5.1. EFFECT OF TGFβ ON IMMUNOREGULATION

Human platelet-derived TGFβ1 was purchased from Becton Dickinson (USA, lot No. 900503). According to the manufacturer's direction, 1 μg/ml of hTGFβ1 was diluted to 200 ng/ml in 36% acetonitrile (methycyanide, Fisons, Leicestershire, UK) / 0.1% trifluoacetic acid (TFA; Sigma), dispersed into polypropylene tubes and stored at -70 °C (stock solution). Immediately before use, the stock solution was diluted to the required concentration with culture medium.

Human TGFβ2 was obtained from Crystal Chem, USA (Lot No. CR 7-042). It was diluted to 400 ng/ml in 5 mM HCl. Prior to test, it was diluted with RPMIc to give the required concentration.

4.5.1.1. Effect of hTGFβ1 on the one-way MLR

MACS-separated human peripheral blood T lymphocytes (Section 2.4.2) were cultured for 5 days with mitomycin C -treated, non-T cells from an unrelated individual at a ratio of 1:1.5 (stimulator: responder) with 2.5x10⁴ stimulators /well in a total volume of 50 μl. The addition of various concentrations of TGFβ1 (range 0.02 ng/ml to 20 ng/ml) to the culture resulted in a
dose-dependent inhibition of proliferation as determined by the uptake of 
$[^3]$H-thymidine. As shown in Fig 4.5-1, the maximal inhibition occurred at the 
concentration of 2 ng/ml (p< 0.01; using Student's T test). The optimal 
concentration is in keeping with Kondo's work (1993).

4.5.1.2 Effect of hTGFβ1 on Mitogen-induced cell proliferation

When human PBMNC were stimulated for 3 days with 1 μg/ml of PHA in the 
absence or presence of various concentrations of TGFβ1, a variable effect 
was observed at the lower concentrations (0.02 - 0.2 ng/ml). As the dose 
increased (>2 ng/ml), a moderate suppression was observed in all 
experiments performed, this was most evident at a concentration of 20 ng/ml. 
In terms of percentage inhibition this corresponds to a median inhibition of 
9% to 17% (see Table 4.5-1). Owing to the variability between experiments, 
the results did not reach significance. However, all cultures containing 
TGFβ1 showed a response when compared to the untreated control.

4.5.1.3. Effect of hTGFβ1 on Human monocytic and T lymphoblastoid cell 
lines.
THP1, a human monocytic cell line, expresses a range of antigens, including 
Fcy1 (CD64), FcγII (CD32) and C3b receptor. These cells are phagocytic and 
can differentiate into macrophage-like cells in response to appropriate 
stimuli.

In this assay, two human monocytic cell lines: THP1, and Mono Mac 6, and 
one T lymphoblastoid cell line (Jurkat E6-1), were incubated in the presence
**Fig. 4.5-1.** Effect of TGFβ1 on the one-way MLR.

MACS-separated human peripheral blood T lymphocytes were cultured with mitomycin C-treated non-T PBMNC from an unrelated individual for 5 days. hTGFβ1 at varying concentrations (as indicated above) was added at the initiation of the reaction. The results are expressed as the mean and standard deviation of stimulation index (%) from four separate experiments.

** Significance p<0.01; * p<0.05 when compared with control using Student's T test.
Table 4.5-1. Effect of hTGFβ1 on PHA-induced PBMNC proliferation.

Human PBMNC (10^5 cells; 100 μl) were challenged with 1 μg/ml of PHA. hTGFβ1 at varying concentrations was added at the initiation of the culture. Each number is the median of quadruplicate cultures expressed as counts per minute.

<table>
<thead>
<tr>
<th>hTGFβ1 (ng/ml)</th>
<th>0.00</th>
<th>0.02</th>
<th>0.2</th>
<th>2</th>
<th>20</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exp 1</td>
<td>31.2</td>
<td>35.7</td>
<td>32.2</td>
<td>30.8</td>
<td>25.8</td>
</tr>
<tr>
<td>Exp 2</td>
<td>42.8</td>
<td>35.9</td>
<td>34.4</td>
<td>34.6</td>
<td>35.9</td>
</tr>
<tr>
<td>Exp 3</td>
<td>57.0</td>
<td>56.5</td>
<td>66.2</td>
<td>53.2</td>
<td>51.7</td>
</tr>
<tr>
<td>Exp 4</td>
<td>37.8</td>
<td>43.0</td>
<td>39.6</td>
<td>34.7</td>
<td>30.0</td>
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<td>median</td>
<td>40.3</td>
<td>39.4</td>
<td>37.0</td>
<td>34.6</td>
<td>33.0</td>
</tr>
<tr>
<td>range</td>
<td>31.2-57.0</td>
<td>35.7-56.5</td>
<td>32.2-66.2</td>
<td>30.8-53</td>
<td>25.8-51.7</td>
</tr>
<tr>
<td>p</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

NS. no significant difference.
of different concentrations of TGFβ1 (0.02-20 ng/ml). No obvious change in the growth of Jurkat E6.1 cells was observed in the presence of TGFβ1. In contrast to this, a slight inhibition of the growth of THP1 cells was observed (which did not reach significance) at the higher concentrations of hTGFβ1 (2-20 ng/ml). At lower concentration, its effect was variable and difficult to reproduce. However, hTGFβ1 clearly inhibited the growth of Mono Mac 6 cells at a concentration as low as 0.2 ng/ml (p<0.05). Concentrations of 2-20 ng/ml reduced cellular proliferation by 40% when compared with control cells. Fig 4.5-2 illustrates these results.

4.5.1.4. Effect of hTGFβ2 on the growth of Mono Mac 6

Under the same experimental condition, Mono Mac 6 cells were exposed to hTGFβ2 at various concentration (0.02-20 ng/ml) for three days. The growth of all the treated cells did not differ from that of control untreated cells in six experiments, although the magnitude of the optical density varied between one experiment and another. Table 4.5-2 shows these results.

4.5.2. PROLIFERATION OF MONO MAC 6 CELLS AND NEUTRALISATION WITH ANTI-TGFβ ANTIBODY

Rabbit anti hTGFβ1 polyclonal neutralising MoAb (Becton Dickinson; Lot No. J792), goat anti hTGFβ2 (R&D system; Lot No. EE212) and rabbit pan anti hTGFβ (anti TGFβ1 and β3; R&D system; Lot No. E0033091) were diluted to
Fig. 4.5-2. Effect of hTGFβ1 on the growth of Mono Mac 6, THP1, and Jurkat E6.1 cell lines.

Each of the cells (2x10^5/ml; 200 µl) was dispensed into a microplate and incubated for 3 days. hTGFβ1 at varying concentrations (ng/ml) was added to the start of the culture. Cellular proliferation was assessed by an MTT assay, and results were expressed as % proliferation i.e., OD 550nm from cultures containing hTGFβ1/ control cultures x 100%. Each column represents the mean and standard deviation of four experiments. ** Significance p<0.01; * p<0.05 when compared with control using Student's T test.
Table 4.5-2. Effect of hTGFβ2 on the growth of Mono Mac 6 cells.
The cells (2x10^5/ml; 200 μl) were cultured for three days with various concentrations of hTGFβ2. Cellular proliferation was assessed with an MTT assay. Each number was the mean of triplicated cultures.

<table>
<thead>
<tr>
<th>TGFβ2 (ng/ml)</th>
<th>0</th>
<th>0.02</th>
<th>0.2</th>
<th>2</th>
<th>20</th>
</tr>
</thead>
<tbody>
<tr>
<td>Expt 1</td>
<td>1.034</td>
<td>0.869</td>
<td>0.972</td>
<td>0.996</td>
<td>1.082</td>
</tr>
<tr>
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<td>1.102</td>
<td>1.038</td>
<td>0.999</td>
<td>0.941</td>
</tr>
<tr>
<td>Expt 3</td>
<td>1.111</td>
<td>1.360</td>
<td>1.117</td>
<td>1.155</td>
<td>1.035</td>
</tr>
<tr>
<td>Expt 4</td>
<td>1.423</td>
<td>1.515</td>
<td>1.953</td>
<td>2.152</td>
<td>1.959</td>
</tr>
<tr>
<td>Expt 5</td>
<td>2.139</td>
<td>2.207</td>
<td>1.949</td>
<td>2.204</td>
<td>1.933</td>
</tr>
<tr>
<td>Expt 6</td>
<td>2.109</td>
<td>2.078</td>
<td>2.076</td>
<td>2.267</td>
<td>2.252</td>
</tr>
<tr>
<td>mean</td>
<td>1.475</td>
<td>1.522</td>
<td>1.518</td>
<td>1.573</td>
<td>1.589</td>
</tr>
<tr>
<td>+/-SD</td>
<td>+/-0.523</td>
<td>+/-0.531</td>
<td>+/-0.523</td>
<td>+/-0.560</td>
<td>+/-0.546</td>
</tr>
<tr>
<td>p</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

NS. no significant difference.
200 μg/ml with RPMI, aliquotted, and stored at -20 °C

Cultures of Mono Mac 6 cells (2x10⁵/ml; 200 μl) were supplemented with 10% (v/v) PBS, LMWF, SMWF or 2 ng/ml of TGFβ1 and incubated for three days. Appropriate antibody was added at the start of the culture (final concentration 10 μg/ml). Inhibition of the growth of Mono Mac 6 cells by hTGFβ1 treatment was reversed (85%+-17%) by the addition of anti-hTGFβ1. By contrast, this concentration of antibody did not reverse the inhibition caused by either of the DF (Fig. 4.5-3 A). Adding the pan anti hTGFβ antibody achieved the same results. In three separate experiments, anti-hTGFβ2 polyclonal neutralizing antibody was added into the culture instead of anti-TGFβ1. There was no reversal of the suppression caused by either of the decidual fractions or TGFβ1 (Fig 4.5-3 B).

4.5.3. CELL CYCLE DISTRIBUTION OF MONO MAC 6 CELLS AND NEUTRALIZATION WITH ANTI TGFβ

The cells (2x10⁵/ml) were cultured for 48h in the presence of 10% (v/v) of PBS, LMWF, SMWF or 2 ng/ml of TGFβ1. Cell cycle analysis using a PI staining procedure (Section 2.3.1.2) showed that after 48 h incubation, TGFβ1-treated cells tended to show a lower intensity of staining with PI. The number of cells in the G1/G0 phase, including in A0 region were remarkably increased (mean %+/-SD: 55+/-2 in control; 72+/-4 in TGFβ1; p<0.01); and
**Fig. 4.5-3. Neutralization of hTGFβ1 biologic activity.**

Mono Mac 6 cells (2x10^5 /ml) were cultured, in the presence or absence of 10 μg/ml of anti TGFβ1(A) or TGFβ2 (B) neutralizing antibodies, with 2 ng/ml of hTGFβ1, or 10% v/v of LMWF, or SMWF respectively for 3 days. The growth of cells was measured with an MTT assay. The results are the mean from five separated experiments (________ the base line represents the growth of the cells in the presence of appropriate agents without adding the Abs).
decreased the number of cells in the G2/M phase (25+/−4 in control; 8+/−4 in TGFβ; p<0.01). Fig 4.5-4 shows the result.

To further investigate whether the suppressive activity caused by the LMWF was TGFβ1 related or not, rabbit, polyclonal, pan anti-TGFβ1 neutralising antibody (final concentration, 10 μg/ml) was added to the cell suspension at the beginning of the culture. Analysis of the cell cycle showed that the addition of the anti-hTGFβ1 antibody prevented the accumulation of cells in the G0/G1 phase and in A0 region in those cultures treated with purified hTGFβ1, but not those treated with LMWF (Fig 4.5-4). The histograms in Fig 4.5-5 shows a typical result.

Anti-TGFβ2 antibody (10 μg/ml) was added into the cells treated with LMWF in two separate experiments, it did not reduce the cell distribution in G0/G1 phase either (Table 4.5-3).

4.5.4. EXPRESSION OF HLA-DR MOLECULES AND NEUTRALIZATION WITH ANTI TGFβ1 ANTIBODY

It has been shown that LMWF and SMWF did not affect the expression of MHC class II molecules (Section 4.4-3). In this experiment the effect of purified TGFβ1 on HLA-DR was investigated, and compared with that of decidual fractions.
**Fig 4.5-4.** Effect of anti-hTGFβ1 neutralizing antibody on the cell cycle distribution of LMWF- and TGFβ1-treated Mono Mac 6 cells. The cells were incubated with 10% (v/v) of LMWF or 2 ng/ml of hTGFβ1 for 48h. The cell cycle distribution was analysed with PI staining. The results were expressed as mean (+/-SD) of percentage distribution from four experiments.
Fig 4.5-5a. Neutralisation of hTGFβ1 activity on the cell cycle distribution and apoptosis of Mono Mac 6 cells.

Mono Mac 6 cells were incubated with 10% v/v of the LMWF or 2 ng/ml of purified TGFβ1 for 48 h, with and without 10 μg/ml of anti TGFβ1 neutralising antibody. The cell cycle analysis was performed with PI staining and FACScan (controls ; A. TGFβ1...; B. TGFβ1 & antibody....; C. LMWF ......; D. LMWF & antibody....).
Fig 4.5-5b. Neutralisation of hTGFβ1 activity on the cell cycle distribution and apoptosis of Mono Mac 6 cells.

Mono Mac 6 cells were incubated with 10% v/v of the LMWF or 2 ng/ml of purified TGFβ1 for 48 h, with and without 10 μg/ml of anti TGFβ1 neutralising antibody. The cell cycle analysis was performed with PI staining and FACScan (controls _____; A. TGFβ1....; B. TGFβ1 & antibody....; C. LMWF .....; D. LMWF & antibody....).
Table 4.5-3. Effect of anti-hTGFβ2 neutralising antibody on the cell cycle distribution of LMWF-treated Mono Mac 6 cells.

The cells were treated with 10% v/v of LMWF with and without 10 μg/ml of the antibody for 48h. The cell cycle distribution was analysed using PI staining.

<table>
<thead>
<tr>
<th>Expt</th>
<th>Ab</th>
<th>G0/G1</th>
<th>S</th>
<th>G2/M</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>-</td>
<td>64</td>
<td>23</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>62</td>
<td>24</td>
<td>13</td>
</tr>
<tr>
<td>2</td>
<td>-</td>
<td>64</td>
<td>23</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>64</td>
<td>24</td>
<td>12</td>
</tr>
</tbody>
</table>
When Mono Mac 6 cells were treated with 2 ng/ml of purified TGFβ1, a significant suppression of HLA-DR expression was found after 24 hour incubation [median (range) of median fluorescence: control, 611 (607-615); TGFβ, 576 (561-587); p<0.01, n=7]. Treatment with 10 µg/ml of neutralizing anti TGFβ1 antibody significantly reduced its suppression [TGFβ1, 576 (561-587); plus antibody, 596 (564-650); n=9; p<0.01. Statistical analysis was performed using the Wilcoxon signed rank test]. However, this antibody did not have an obvious effect on the expression of HLA-DR on LMWF and SMWF treated cells [LMWF, 609 (582-664); plus antibody, 600 (562-658); n=9. SMWF, 584 (574-616); plus antibody, 597 (570-642)]. Fig 4.5-6 illustrates these results.

4.6. CHARACTERISATION OF DECIDUAL FRACTIONS

In this study, SDS-PAGE analysis was employed to estimate the molecular weight of the decidual fractions of interest (Section 2.2.1). To further study the relationship between decidual fractions and TGFβ, immunodetection of TGFβ from the decidua fractions of interest was performed using Western blotting (Section 2.2.2).
Fig 4.5-6. Effects of anti-hTGFβ1 neutralising antibody on HLA-DR expression of DF-treated Mono Mac 6 cells.

The cells were incubated with 10% of PBS (as control), LMWF, SMWF and 2 ng/ml of TGFβ1 for 24 h respectively with or without 10 μg/ml of anti-hTGFβ1. The expression was measured using an anti-HLA-DR MoAb (PE) and the FACScan. Results are shown paired with the median for each group.
4.6.1. SDS-PAGE ANALYSIS

4.6.1.1. Analysis of LMWF

On a 12% polyacrylamide gel, a major band with a M.w. greater than 140 KDa (the highest M.w marker in the protein standard range), and a minor band were detected under non-reducing condition. With 20 mM DTT to reduce the disulphide bonds, two bands were observed one with M.w. similar to the minor band seen under non-reducing conditions, one with a very low M.w. Fig 4.6-1 (a) shows the results.

Using a logarithmic plot of the M.w. of the protein standards versus their relative electrophoretic mobility (Rf) and a regression formula (Fig 4.6-1 b), the M.w of each band was calibrated as follows: the minor band without DTT and that with DTT were 70 KDa, and the tiny band with DTT was 29 KDa.

In order to estimate the molecular weight of the major band more accurately, a 7.5% gel and large M.w. standard were employed. Under these conditions, the major band appeared below 205 KDa (the highest M.w of the standard, see Fig 4.6-2). From the regression analysis of the standard curve, the M.w of the band was found to be 183 KDa (Fig 4.6-2, b).
4.6.1.2. Analysis of SMWF

Due to the low protein concentration in the SMWF, it could not be detected with Coomassie brilliant blue staining. To enhance the effect of visualisation, the SMWF was concentrated 16 times with Microconcentrators before analysis. From Fig 4.6-3, it was showed that four bands were detected in the SMWF, having M.ws of 7, 9, 11, 14 KDa.

4.6.2. OPTIMISATION OF IMMUNODETECTION OF TGFβ

Preliminary experiments showed that the LMWF was detected by both polyclonal rabbit pan anti- hTGF and an goat polyclonal Ab to TGFβ2 following staining with an HRP-conjugated secondary antibody. The SMWF did not bind either of these two primary antibodies. However, in control experiments, using Ab dilution buffer without the primary antibody, the "positive band" derived from the LMWF still existed. Thus, the following experiments were performed to minimise the background, non-specific binding and optimise the conditions of immunodetection.

4.6.2.1. Blocking non-specific binding sites

After protein transfer, non-specific binding sites on the blot were blocked before further processing. The blocking buffer used was TTBS recommended by the manufacturer of the primary antibody (R/D). In preliminary experiments 5% dried, low fat milk was used as a blocking solution. However, it did not achieve a satisfactory effect. Increasing the concentration to 8%, or
Fig 4.6-1 (a, above). SDS-PAGE analysis of LMWF (12% gel).
Tracks from left to right: 1, 2, 3, LMWF run under reducing conditions; 4, Standard of M.w. (bands from top to bottom: 140, 87, 48, 33, 29, and 21 KDa respectively); 5, 6, 7, LMWF run under non-reducing conditions.

Fig 4.6-1 (b, below). Calibration of M.w. of LMWF under reducing conditions by the plot of relative electrophoretic mobilities versus the logarithm of M.w for known protein standards.

\[
y = 121.70 \times 10^{(-1.0732x)} \quad R^2 = 0.899
\]
Fig 4.6-2 (a, above). SDS-PAGE analysis of LMWF with 7.5% gel. Tracks from left to right: 1, Standard of large M.w. (bands from top to bottom: 205, 116, 97, 66, 45, 29 KDa respectively); 2,4, LMWF run under non-reducing conditions; 3, buffer control; 5, Standard of M.w. (bands from top to bottom: 140, 87, 48, 33, 29, and 21 KDa respectively).

Fig 4.6-2 (b, below). Calibration of Mw. of LMWF under non-reduced condition by the plot of relative electrophoretic mobilities versus the logarithm of M.w for known protein standards.

\[ y = 240.50 \times 10^{(-0.99456x)} \quad R^2 = 0.979 \]
Fig 4.6-3 (a, above). SDS-PAGE analysis of SMWF with 12% gel under non-reducing conditions. Tracks from right to left: 1, Standard of M.w. (bands from top to bottom: 140, 87, 48, 33, 29, and 21 KDa respectively); 2, hTGFβ1 (30 ng) in 1% BSA; 3, hTGFβ2 (30 ng) in 5mM HCl; 4. LMWF; 5. SMWF.

Fig 4.6-3 (b, below). Calibration of Mw. of SMWF by the plot of relative electrophoretic mobilities versus the logarithm of M.w for known protein standards.

\[
y = 166.18 \times 10^{-1.5456x} \quad R^2 = 0.935
\]
alternatively using 3% BSA, in addition to the 3% BSA contained in the antibody preparation, improved the blocking effect.

4.6.2.2. Titration of secondary antibodies

A goat anti-rabbit antibody (HRP-conjugated) was employed as the secondary antibody in the detection of hTGFβ1 and β3. In order to determine the lowest concentration of the secondary Ab needed whilst minimizing non-specific binding, the LMWF was incubated with various concentrations of the secondary Ab. A dot blot showed that in the control, without secondary antibody, the LMWF did not react with the ECL reagent. A 1:5,000 dilution did not prevent the non-specific reaction. With a dilution of 1:10,000 only minimal non-specific binding was observed with the LMWF (Fig 4.6.4 A).

A rabbit anti-goat antibody (HRP-conjugated) was used as secondary antibody for detection of hTGFβ2. A dot blot showed a similar result to that described above, and a 1:10,000 dilution was found to be optimal for minimising the non-specific binding. (Fig 4.6.4 B).

4.6.2.3. Titration of the primary antibodies

To optimise the concentration of TGFβ1 antibody, the effect of 1 μg/ml and 10 μg/ml of the antibody with various concentrations of secondary antibody was studied using a dot blot. It was found that at a concentration of 1μg/ml of the antibody with a 1:10,000 dilution of the secondary antibody, minimized background, non-specific staining and detected 10 ng of purified TGFβ1. As
the concentrations of the primary and secondary antibody were increased,
the background increased (Fig. 4.6-5 A).

Using 2.5, 5, 10 ng of hTGFβ2, the highest concentration (10 µg/ml) of anti-
hTGFβ2 antibody used detected 10 ng of TGFβ2 following a 1:3000 dilution of
secondary antibody (Fig 4.6-5 B).

4.6.2.4. Enhancement of signal with avidin-biotin amplification
A difficulty encountered was that when blots were transferred from SDS-
polyacrylamide gel, the band of the purified TGFβ control was very weak. To
increase the intensity of staining, a biotinylated secondary antibody with
extravidin (HRP-conjugated) were employed. Due to four binding sites with
avidin for biotin, the signal from TGFβ control was greatly improved. Fig 4.6-6
summarised the conditions of these optimisation experiments.

4.6.3. DETECTION OF TGFβ IN THE DECIDUAL FRACTIONS

Using the optimal conditions described above, it was found that the LMWF
under both reducing and non-reducing conditions was still detected by
antibody to TGFβ1 following secondary Ab staining, the bands of the SMWF
and TGFβ2 were not detected by this procedure. Purified TGFβ1 showed a
band of M.w 25 KDa (Fig 4.6-7, a). Despite efforts to reduce the non-specific
binding, parallel control experiments using antibody-dilution buffer only
(without primary antibody), the LMWF still showed a strong band. By
contrast, the 25 KDa TGFβ band did not appear (Fig 4.6-7. b). Using anti-TGFβ2 antibody as primary antibody, a similar result was obtained.

To exclude the possibility that the non-specific binding seen in the LMWF was due to the relatively high concentration of protein, another decidua fraction (No.16) was used. This fraction had a greater protein concentration than LMWF but did not exhibit immunosuppressive activity. Fig 4.6-8(a) shows that fraction 16 had three bands on SDS-PAGE analysis. Densitometric analysis showed the bands to be 9829, 2415, 48260 arbitrary units at absorbance 550 nm. That of the LMWF was 6016 under non-reducing conditions; and 31778 under reducing conditions. When they were analysed by Western blotting, the band from DF 16 was negative, whilst that of the LMWF was “positive”.

In order to determine the cause of the apparently non-specific staining of the LMWF, the primary Ab was omitted from the following experiments. In one experiment both the primary and secondary Abs were omitted and no signal was detected. This indicates that the LMWF did not possess inherent peroxidase activity. A second run which included the secondary antibody, produced a positive band as seen in the original experiments. In order to block potential non-specific binding sites on the LMWF, blots were pre-incubated for 2h with 1:1000 or 1:500 dilutions of the secondary Ab (not conjugated to HRP) before being processed as before. Following detection with ECL, it was found that even the highest concentration of Ab used did not
Fig 4.6-4. Titration concentrations of secondary antibodies.

Each pair (from left to right) containing 10 μl of 3, 6, 12 μg/ml of LMWF in each sample. The blots were blocked with 8% milk in TTBS, and then incubated with various concentrations of appropriate secondary antibodies:

A: Goat anti rabbit IgG (HRP-conjugated). A1, 1:20,000; A2, 1:10,000; A3, 1:5,000; A4, w/o antibody.
B: Rabbit anti goat IgG (HRP-conjugated). B1, 1:5,000; B2, 1:10,000; B3, 1:20,000; B4, w/o antibody.
4.6-5. Titration concentrations of primary antibodies.

A. Polyclonal rabbit anti-TGFβ1 antibody: A1, 10 μg/ml; A2, 1 μg/ml.
   Secondary Ab: 1:1,000(I); 1:3,000(II); 1:5,000(III); 1:10,000(IV).
   On each blot, the samples from the left are: LMWF, 5ng and 10 ng of TGFβ1.

B. Polyclonal goat anti-TGFβ2 antibody: The concentrations: B1, 0; B2, 0.25; B3, 0.5; B4, 1;
   B5, 10 μg/ml. Secondary Ab, 1:3,000.
   On each blot, the samples from the left are: 2.5ng, 5ng, and 10 ng of hTGFβ2.
Samples boiled in sample buffer for 5 min

SDS-PAGE

Transfer the blot onto a nitrocellular membrane

Blotting non-specific binding sites (3% BSA; 1 h)

Primary antibody (1 µg/ml in 3% BSA; 2 h)

Washing

Bioninylated secondary antibody (1:10,000 in 3% BSA; 1 h)

Washing

HRP-conjugated Extravidin (1:10,000 in 3% BSA; 1 h)

Washing

Detected by ECL

Fig 4.6-6. Optimised conditions for detection of TGFβs by Western blotting.
Fig 4.6-7. Western blotting analysis of TGFβ1.
The blots were blocked with 3% BSA, incubated with 1 μg/ml of rabbit anti TGFβ1 for 2 h, and followed by biotinylated goat anti rabbit antibody and Extravidin (HRP). The right side one is control without the primary Abs. Tracks from left to right: 1. standard M.w (bands from top to left: 140, 87, 48, 33, 29 and 21 KDa respectively); 2. hTGFβ1 30 ng/ml in 1% BSA; 3. hTGFβ2 (30 ng) in 5 mM HCl; 4. LMWF; 5. SMWF. The SDS-PAGE analysis performed was shown in Fig. 4.6-3.
Fig 4.6-8. SDS-PAGE and immunodetection of TGFβ1 from decidual fractions of interest.

(A). SDS-PAGE; (B). Immunodetection of TGFβ1; Tracks from left to right: 1, Standard M.w (bands from top to bottom: 140, 87, 48, 33, 29 and 21 KDa); 2. hTGFβ1 (20 ng/ml); 3. LMWF (w/o DTT); 4. LMWF (with DTT), 5. Decidual fraction No. 16.
Fig. 4.6-9. Assessment of the binding of LMWF to secondary antibody (HRP).

Each blot contained 10 μl of undiluted decidual fraction No. 16 (left) and LMWF (right). The blots were blocked with 8% milk, and then 1) directly detected with ECL reagents; 2) incubated with goat anti rabbit (HRP) 1:10,000 for 1 h, and then detected with ECL; incubated with non-conjugated goat anti rabbit 1:1000) (3) and 1:500(4) for 2h before being incubated with HRP-conjugated the antibody following detection with ECL.
block the non-specific binding (Fig. 4.6-9). This would suggest that the positive reactions seen may have been due to some interaction between LMWF and HRP itself. Or that LMWF contains a molecule which acts like a rheumatoid factor of hDP200.

4.6.4. IMMUNODETECTION OF IMMUNOGLOBULINS

Kitano et al (1990) reported a decidua-derived immunoregulatory factor with a M.w. of approximately 158 KDa was related to IgG. It was not clear whether or not the LMWF was identical to this factor. In this experiment 5μl of LMWF was subjected to Ouchterlony double immunodiffusion, using anti-human IgG (γ chain specific), anti-human Ig A (α chain specific), and anti-human Ig M (μ specific) Abs to detect. The concentrations of the Abs used were from 1:16 to 1:64 dilution. Normal human serum was used as a control in each case. It was found that no precipitin bands were observed with either anti human Ig G, Ig A or Ig M. By contrast, the human serum control formed precipitin bands with each of the Abs used. (see Fig 4.6-10). To exclude the possibility that the negative result was due to a low protein concentration in LMWF, the fraction was concentrated 4 times in a microcentrifugator (the protein concentration was about 1 mg/ml). No precipitin bands were observed with the concentrated fraction either.
4.6-10. Detection of human Ig G, Ig A, and Ig M from LMWF by Ouchterlony Radial immunodiffusion.

In the centre: (A) 5 μl of Ig G, (B) 5 μl of Ig A; (C) 5 μl of Ig M. In each circle the top left is human serum control; others are the LMWF (four time concentrated, protein concentration approximately 1 mg/ml).
SECTION 3. PRELIMINARY INVESTIGATION OF THE ROLE OF
NK CELLS IN THE IMMUNOREGULATION

In this study it has been shown that factors derived from first-trimester human
decidua suppressed mitogen-induced lymphocyte proliferation, a one-way
mixed lymphocyte reaction, and the growth of the human monocytic cell line,
Mono Mac 6. By contrast, they did not inhibit the growth of the human T
lymphoblastoid cell line, Jurkat E6.1. In order to determine which cells within
the decidua produce these immunoregulatory factors, experiments were
designed in which the supernatant from short term cultures of non-
fractionated and fractionated decidual mononuclear cells (decidua cell
supernatant, DCS) was used to modulate the growth of Mono Mac 6 and
Jurkat E6.1 cells.

Human first-trimester decidua contains many different cell subpopulations,
any of which could be the source of the decidua-derived suppressive factors.
Since the most abundant cells are CD56+ NK cells which have been reported
to have immunosuppressive activity in vitro (Daya, et al, 1985; Nakayama, et
al, 1985; Starkey, et al, 1988; Ferry, et al, 1990), the present study
concentrated on the role of NK cells in the production of decidua-derived
suppressive factors.
5.1. METHODOLOGY OF EXTRACTING DMNC

In the last decade, the study of human decidual cells has become more commonplace and techniques have been developed in order to extract DMNC from decidual tissue. These methods may be divided into two categories. One is enzymatic dispersal using enzymes, such as collagenase (Ritson, et al, 1987; Parhar, et al, 1988), collagenase plus dispase (Ferry, et al, 1990), or hyaluronidase (Starkey, et al, 1988). The advantage of this approach is that greater cell yields may be obtained. However, the treatment may alter the immunological properties of cells (Wood, et al, 1988), or may result in the selective loss of some cell types, such as CD4+ or CD8+ cells (Vince, et al, 1990). The alternative approach is mechanical dispersal (Saito, et al, 1992, 1993; Burrows, et al, 1993). The disadvantage of this technique is low cell yields, which may not be representative of the whole tissue. However, the cells are maintained in a more physiological environment, and the technique avoids alteration of the immunological properties of cells (Wood, et al, 1988). Thus, in the current study, it was decided to use mechanical dispersal.

In early experiments, decidual cells were extracted by straining the tissue through a stainless steel mesh. Quite a few clotted clumps were seen macroscopically in the cell suspension. A large proportion of dead cells, as detected by trypan blue exclusion, and large non-lymphoid cells (glandular epithelial and stromal cells) were also seen in the cell suspension (Fig 5.1-1,
a). After isolating mononuclear cells by a conventional density gradient centrifugation procedure, a few clotted clumps still appeared in the interface and the proportion of dead and large cells remained high (Fig. 5.1-1,b). In order to remove the clumps, a fine cotton gauze was used to filter the cell suspension, before it was layered over Ficoll-hypaque. A two-step percoll gradient method (see Section 2.4) was adopted in order to get rid of the dead and large cells (Starkey, et al 1988; Norwitz, et al 1991). Using these methods, the proportion of immunocompetent cells from DMNC was increased (see below Section 5.2). The cell viability was > 97% as determined by trypan blue exclusion.

5.2. CHARACTERISATION OF IMMUNOCOMPETENT DECIDUAL CELLS

Decidual mononuclear cells (DMNC) were isolated from first-trimester human tissue by mechanical dispersion and two-step percoll density-gradient centrifugation (Section 2.4). The immunocompetent cells of DMNC were studied by flow cytometry after labelling with appropriate monoclonal antibodies. The results of ten separate experiments, each containing more than four pooled decidual samples, showed that the mononuclear cells comprised 64% (median, range 37-73%) of the isolated DMNC. Among the mononuclear cells, CD14+ macrophages were found to be the most abundant cell type, accounting for 23% (range 12-43%) of the total population (Fig. 5.2-1).
Fig 5.1-1. Mechanically-extracted decidual cells (a), and interface cells after Ficoll-hypaque separation (b).

The cell preparation was mixed with a equal volume of 0.2% trypan blue (Magnified 320x). The smaller cells are leukocytes (L), and large nuclear cells (stained blue) are non-leukocyte cells (N) or dead cells (D).
CD56+ cells comprised 14% (range 6-61%); CD3+ T lymphocytes, 13% (range 6-23%) and CD16+ cells comprised 5% (range 3-17%). By double antibody labelling, a small proportion of CD56+ NK cells also expressed the CD16 antigen (3%; range 1-9%). These results were consistent with the findings by others (Bulmer, 1989; Dietl, et al, 1992).

5.3. SUPPRESSIVE ACTIVITY IN THE DMNC CULTURE SUPERNATANTS

5.3.1. INFLUENCE OF CON A AND IL-2 ON THE GROWTH OF MONO MAC 6 CELLS

The lymphocyte stimulators Con A and IL-2 were added to the cultures of DMNC to stimulate the release of immunomodulatory factors. However, these may influence the growth of Mono Mac 6 cells when the DCS are added to them. In order to eliminate this variable, the same doses of Con A (10 μg/ml), and IL-2 (25 μ/ml) as those used in the culture medium of DMNC were added to Mono Mac 6 cells and their effect compared to that of TGFβ1 (2 ng/ml). After incubation for three days, it was found that neither Con A nor IL-2 exhibited suppressive or stimulatory effects on the growth of Mono Mac 6 cells (Table 5.3-1), while TGFβ1 significantly inhibited it (p<0.05).
Fig. 5.2-1. Flow cytometric analysis of mononuclear cells from freshly isolated DMNC.

The cells (5×10^5) were labelled with FITC-conjugated anti-CD3 or CD16, or PE-conjugated CD14 or CD56, and CD16 and CD56 respectively. The number of cells positive for a given antibody was expressed as a percentage of total processed DMNC. The data are the results of ten experiments, each containing at least four pooled decidua samples. The bars represent the medians of each population.
Table 5.3-1. Effect of Con A, IL-2 on the growth of Mono Mac 6 cells as compared with that of TGFβ1.

The cells (2x10^5/well; 200 µl) were incubated with Con A (10 µg/ml), IL-2 (25 µg/ml), and TGF β1 (2 ng/ml) respectively for three days. The cellular growth was monitored with MTT assay. The results are the mean from triplicated cultures.

<table>
<thead>
<tr>
<th>Expt</th>
<th>OD 540 nm</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>TGFβ1</td>
<td>Con A</td>
<td>IL-2</td>
</tr>
<tr>
<td>1</td>
<td>1.266</td>
<td>0.931</td>
<td>1.292</td>
<td>1.246</td>
</tr>
<tr>
<td>2</td>
<td>1.111</td>
<td>0.818</td>
<td>1.101</td>
<td>1.174</td>
</tr>
<tr>
<td>3</td>
<td>1.110</td>
<td>0.660</td>
<td>1.076</td>
<td>1.120</td>
</tr>
<tr>
<td>mean</td>
<td>1.162</td>
<td>0.803*</td>
<td>1.156</td>
<td>1.180</td>
</tr>
<tr>
<td>+/-SD</td>
<td>0.090</td>
<td>0.136</td>
<td>0.118</td>
<td>0.063</td>
</tr>
</tbody>
</table>

* p<0.05 when compared to control using Student T test.
5.3.2. SUPPRESSIVE ACTIVITY OF DCS

In this study DMNC, or subpopulations thereof (3x10^6/ml in RPMIc), were divided into three fractions, one with 10 µg/ml of Con A, one with 20 µg/ml of IL-2, and one with medium only as control. The cells were then incubated at 37 °C for 3 days. At the end of the incubation time, the cell suspensions were transferred into Eppendorf tubes and centrifuged to pellet the cells. The supernatants were harvested, pooled and stored at -20 °C prior to use. The supernatants were added (in different concentrations) to Mono Mac 6 cells. After incubation for three days, a dose-dependent inhibition of Mono Mac 6 growth was observed with all supernatants. DCS caused 17%, 38%, and 45% suppression (compared to control cultures) at concentrations of 12.5%, 25%, and 50% respectively (Fig 5.3-1). All these suppression are significant (p<0.05).

Comparing the cell growth in the absence and presence of the stimulators, it was found that supernatant from IL-2 treated cells did not increase the inhibition induced by untreated DCS. However, supernatant from Con A-stimulated cells enhanced the mean inhibition to 38%, 50%, and 61% at the concentrations of 12.5%, 25%, and 50% respectively, although it was not significantly different from that without simulators (p>0.05; see Fig 5.3-1).
Fig. 5.3-1. Suppressive activity of the culture supernatants from DMNC on the growth of Mono Mac 6 cells.

The cells were cultured at a concentration of 3x10⁶/ml in RPMIc, in the presence or absence of Con A (10 µg/ml); or IL-2 (25 µg/ml), for three days. The culture supernatants were harvested, and added into the appropriate microculture of Mono Mac 6 cells (2x10⁵/ml; 200 µl) at different percentage of culture medium for three days. The growth of the cells was assessed with MTT assay. The results are the mean (+/-SD) of percentage growth against control from five experiments.
5.3.3. COMPARISON OF DCS FROM ADHERENT AND NON-ADHERENT DMNC

Monocytes easily adhere to plastic surfaces, a property often used to isolate them from other PBMNC. It has been reported that macrophages are responsible for the production of prostaglandins by enzymatically extracted DMNC (Wood et al., 1988). In an attempt to get an NK cell-enriched subpopulation, DMNC (at a concentration of 3x10⁵/ml in RPMIα) were incubated in a plastic tissue culture dish at 37 °C for 1h to allow the adherence of monocytes. The non-adherent cells were harvested and after washing, their concentration was adjusted to 2.5x10⁶/ml. The adherent cells were removed by vigorous washing (following cooling of the dish for 3-5 min at -20 °C) and their concentration adjusted to 5x10⁵/ml. The culture supernatants harvested from both adherent and non-adherent cells showed similar levels of inhibition of Mono Mac 6 cell proliferation. Addition of Con A or IL-2 did not affect the potency of the resulting supernatant (Fig 5.3-2). This suggests that the adherent cells may be capable of releasing more of the regulatory factors than the non-adherent cells since far fewer of the former were used to prepare the supernatant.

Surface antigen expression of the separated cells was analysed using the FACScan. The adherent fraction, which should have contained mostly macrophages, contained a large proportion of CD3+ and CD56+ cells. However, the total recovery of CD56+ cells was decreased compared to the
unfractionated cells (Fig. 5.3-2). This indicates that the procedure used was not efficient at separating monocytes from other cells.

5.3.4. EFFECT OF DCS ON THE JURKAT E6.1 CELLS

The current project has demonstrated that both LMWF and SMWF inhibited the growth of Mono Mac 6 cells, but not that of Jurkat E6.1 cells (Section 4.2). In order to determine whether or not the factors released from DMNC during short term incubation were responsible for this suppression, the supernatant from DMNC (adhesive, non-adhesive and non-separated) was added to Mono Mac 6 and Jurkat E6.1 cells for 72 hours. Compared with the medium control, the supernatant did not markedly suppress the growth of Jurkat E6.1 cells at a concentration of 25% (p>0.05). Compared with the effect on Jurkat E6.1, however, the supernatants from unfractionated DMNC, and Con A-treated non-adherent cells significantly inhibited the growth of Mono Mac 6 cells (p<0.05). At a higher concentration (50%), the growth of Jurkat E6.1 cells was markedly inhibited. However, in each case it was less than that observed for Mono Mac 6 cells (Table 5.3-2). The supernatants from unfractionated DMNC, non-adherent, and IL-2-treated non-adherent decidual cells inhibited Jurkat E6.1 cells significantly less than that of Mono Mac 6 cells (p<0.05).
Fig. 5.3-2. Effect of culture supernatant from adherent and non-adherent DMNC on Mono Mac 6 cells.
DMNC (3x10⁶/ml) were incubated in 24-well plate at 37 °C for 1 hour; the adherent and non adherent cells were harvested. The appropriate DCS harvested after three days culture were added into microculture of Mono Mac 6 cells at a concentration of 50%. The cell growth was monitored by MTT assay. These results are the mean of percentage growth (+/- SD) against control from six separate experiments. Below is the subpopulation of adherent and non-adherent DMNC analysed with Flow cytometry. Each figure is the mean of two individual experiments.

<table>
<thead>
<tr>
<th>Labelled Ab</th>
<th>positive cells (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before Sorting</td>
</tr>
<tr>
<td></td>
<td>Adherent</td>
</tr>
<tr>
<td>CD3</td>
<td>16</td>
</tr>
<tr>
<td>CD14</td>
<td>26</td>
</tr>
<tr>
<td>CD56</td>
<td>22</td>
</tr>
</tbody>
</table>

160
Table 5.3-2. Effect of culture supernatants of DMNC on the growth of Mono Mac 6 and Jurkat E6.1 cells.

Appropriate DMNC (3x10⁶/ml; non separated, plastic adherent or non-adherent) were cultured with or without Con A (10 μg/ml) or IL-2 (25 μg/ml), for three days. The culture supernatants were added into the culture of Mono Mac 6 and Jurkat E6.1 cells at various concentrations. The cell growth was monitored using an MTT assay. Results are expressed as the growth index of decidua treated samples (calculated using the equation:

\[
\text{Growth Index} = \frac{\text{abs of decidua supernatant treated cells}}{\text{abs of control cells}}
\]

The data are represented as the mean growth index of six separate experiments.

<table>
<thead>
<tr>
<th>Supernatants from</th>
<th>25% DCS added</th>
<th>50% DCS added</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MM6</td>
<td>E6.1</td>
</tr>
<tr>
<td>DMNC</td>
<td>0.61±0.06</td>
<td>0.90±0.05*</td>
</tr>
<tr>
<td>Non-adherent</td>
<td>0.92±0.04</td>
<td>0.98±0.01</td>
</tr>
<tr>
<td>+Con A</td>
<td>0.58±0.10</td>
<td>0.92±0.02*</td>
</tr>
<tr>
<td>+IL-2</td>
<td>0.87±0.11</td>
<td>0.95±0.13</td>
</tr>
<tr>
<td>Adherent</td>
<td>0.83±0.23</td>
<td>0.90±0.15</td>
</tr>
</tbody>
</table>

* p<0.05 showing a significant difference between the results obtained from Mono Mac 6 and Jurkat E6.1 cells cultures in the precent of the same volume of DCS.
5.4. ROLE OF PROSTAGLANDINS IN SUPPRESSION

Prostaglandins have been shown to be responsible for the non-specific immunoregulatory activity associated with murine early pregnancy decidua. Macrophages and glandular epithelium were reported to be the cellular source (Matthews, et al, 1987; Wood, et al, 1988, Smith, et al, 1987). We investigated the role of prostaglandins in the suppression associated with DCS.

Indomethacin (an inhibitor of prostaglandin synthesis) was dissolved in ethanol (10 mM), and diluted in RPMIc into 100 μM. The final concentration used was 5 μM or 10 μM according to the literature (Matthews, et al, 1987; Wood, et al, 1988), and added at the start of DMNC culture. After three days, the supernatants were harvested and added to culture of Mono Mac 6 cells later. It was found that indomethacin-treatment did not eliminate the suppression caused by DCS from either the non-stimulated, or stimulated, adherent or non-adherent fractions (Table 5.4-1). As indomethacin alone did not affect the growth and viability of Mono Mac 6 cells in previous experiments, these results indicate that the suppressive activity derived from DCS is independent of the production of prostaglandins.
Table 5.4-1. Effect of indomethacin-treated DMNC on the growth of Mono Mac 6 cells.
DMNC (3x10^6/ml; unseparated, plastic adherent and non-adherent) were cultured with or without Con A or IL-2 for three days. Indomethacin (final concentration 10 µM) was added into parts of these culture at the beginning. The appropriate supernatants harvested from these cultures were added into the culture of Mono Mac 6 cells at a concentration of 50%. The cell growth was assessed with MTT assay. The results are the mean (+/-SD) of percentage growth against control from three experiments.

<table>
<thead>
<tr>
<th>Indomethacin</th>
<th>Non-separated</th>
<th>Non-adherent</th>
<th>Adherent</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

Non-stimulated: 57+/-10 78+/-13 69+/-13 78+/-4 84+/-46 76+/-20

+ Con A: 59+/-1 57+/-12 45+/-13 50+/-27 86+/-13 86+/-1

+ IL2: 82+/-17 81+/-4 68+/-12 67+/-19 95+/-1 95+/-8
5.5. THE ROLE OF NK CELLS IN SUPPRESSION

5.5.1. DEPLETION OF T CELLS AND MACROPHAGES FROM DMNC

The role of NK cells was studied by immunomagnetic depletion of cells bearing CD3, CD4, CD19, CD33 and CD14 (The protocol was outlined in Section 2.4.2.2.). The viability of > 95% was observed in both magnetic and non-magnetic fractions. The cell component in each fraction was analysed by flow cytometry using MoAb against CD3, CD14, and CD56 respectively. It was found that CD56+ NK cells were only slightly enriched in the non-magnetic fraction (from 17% to 19%), despite a marked depletion of CD3+ lymphocytes and CD14+ macrophages. These latter two populations were enriched in the magnetic fraction (Fig. 5.5-1). Interestingly, the proportion of CD56+ cells was also enriched in this (Table 5.5-1). This is possibly due to the aggregation of cells, as a result of cell clumping.

5.5.2. EFFECT OF T CELL AND MACROPHAGE DEPLETION ON SUPPRESSION

The cells from the magnetic and non-magnetic fractions were adjusted to a concentration of 1.5x10⁶/ml, and incubated with or without stimulators for three days. The supernatant was added to cultures of Mono Mac 6 cells at different concentrations. It was found that supernatant from the magnetic
fraction strongly suppressed the growth of Mono Mac 6 cells from a concentration of 30% (Fig. 5.5-2). The mean growth index of the cells exposed to supernatant from the magnetic fraction at a concentration of 50% was: without stimulator, 52% (+/-20%); stimulated with Con A, 44% (+/-24%); stimulated with IL-2, 42% (+/-4%). While in the presence of the same concentration of supernatant from non-magnetic fraction, the mean growth index was 97% (+/-10%), 86% (+/-11%), and 93% (+/-20%) respectively. Hence, the suppressive activity from T cell and macrophage depleted decidual cell population (non-magnetic fraction) was remarkably decreased. However, since the magnetic fraction also contained higher proportions of CD56+ cells than the non-magnetic fraction, it is difficult to conclude which cells are the source of the suppressive factors.
Fig 5.5-1. Depletion of CD3⁺ lymphocytes and CD14⁺ macrophages in a column of B2 with MACS.

The DMNC were separated with NK negative enrichment kit plus CD14 microbeads, and analysed with FACSscan (A, anti CD3 MoAb staining; B, anti CD14 MoAb staining; _____ control; . . . magnetic positive fraction, ......magnetic negative fraction).
Table 5.5-1. Effect of the separation of DMNC by using NK-negative enrichment kit plus CD14 microbeads. The cells were labelled with appropriate Abs before sorting, and after sorting. The No. of cells positive for a given Ab was expressed as a percentage of total processed DMNC.

<table>
<thead>
<tr>
<th>Labelled Ab</th>
<th>% of positive cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before Sorting</td>
</tr>
<tr>
<td></td>
<td>Magnetic</td>
</tr>
<tr>
<td>CD3</td>
<td>10</td>
</tr>
<tr>
<td>CD14</td>
<td>12</td>
</tr>
<tr>
<td>CD56</td>
<td>17</td>
</tr>
</tbody>
</table>
Fig. 5.5-2. Regulatory effect of culture supernatants of NK and non-NK cells on the growth of Mono Mac 6 cells.

DMNC were labelled with NK-negative enrichment kit plus CD14 microbeads and separated with MACS. The cells from non-magnetic and magnetic fractions were adjusted to 1.5x10^6/ml and incubated separately for three days. The culture supernatants were harvested and added to cultures of Mono Mac 6 cells at different concentration for three days. These results are the mean (+/-SD) of growth index against control from four separate experiments.
During normal pregnancy, the fetus is not rejected by the immune system of the maternal body despite bearing paternal MHC antigen. It has been proposed that this is due to the local production of immunoregulatory factors by the maternal decidua. Previous research showed that the cells from human decidua can suppress immune responses (Golander et al., 1981; Nakayama et al., 1985; Matsui et al., 1989a). However, the factors responsible for maintenance of the fetus during early pregnancy and the way in which they mediate their regulation in humans have not been characterised. The present study was designed to investigate potential immunosuppressive factors from first trimester human decidua, to identify and characterise the factors which showed suppressive activity, and to identify the cells which produce them.

6.1. IDENTIFICATION AND PARTIAL CHARACTERISATION OF DECIDUAL FACTORS

In order to identify the suppressive factors, it was necessary to extract decidual intracellular proteins. Several approaches can be used for this purpose. Repeated freezing and thawing of the cells is a simple and gentle method, which results in extensive membrane lesions. It is thought that intracellular ice formation appears to be the major cause of membrane leakage. The "split" or "holed" cells easily release their intracellular component (Harris E et al, 1990). One disadvantage is that proteinases may be activated and degrade the relevant proteins during thawing at 37 °C; and some
exogenous proteinases derived from infection by microorganisms may also play a part. During the early stages of the study, this procedure was performed. However, it was found to be very time consuming and difficult to disperse the cells from large quantities of decidual tissue by the mechanical method prior to freezing and thawing the cells. Homogenisation was then used to promote intracellular protein release. The shearing pressure may cause denaturation of relevant proteins, but it is thought to be reversible (Creighton, 1989). In order to minimise the protein inactivation, the decidua samples were maintained at a low temperature (4 °C), and diluted with physiological buffer (PBS). Processing time was kept short to prevent DNA release and cell wall break up.

In order to concentrate the extracts an ultrafiltration system was used. It offers several advantages over alternative methods. For example, precipitation results in poor recovery; dialysis and freeze-drying needs a longer processing time (Harris et al., 1990). In addition, ultrafiltration can crudely fractionate the proteins according to their M.w. allowing prescreening of the filtrate to identify which molecular weight ranges contain the factors of interest and means that smaller volumes need to be used to purify the active molecules.

To investigate the suppressive activity of decidual extracts, it was necessary to establish immunoassays. The mixed lymphocyte culture response is an assay which is frequently used as a measure of T lymphocyte reactivity.
Although a number of different techniques have been described, the most commonly used employs peripheral blood as a convenient source of cells. However, one of the drawbacks of the assay is that it requires a relatively large amount of blood from at least two individuals for each assay. An alternative approach is to use an MHC class II expressing cell line as stimulator. In the early stage of this study, none of them was available. But IFNγ has been found to have the capacity to induce the expression of MHC class II antigens in several cells, including monocytic cell lines (Piacibello et al., 1983; Harris P. et al., 1985). U937 cells have been reported to have a differential response to IFNγ: HLA-DQ and DP expression being enhanced, whilst HLA-DR expression is unaffected (Shaked et al., 1992). In the present study, although the modulation of DQ and DP expression by IFNγ was not directly investigated, the expression of HLA-DR was found to be significantly enhanced (Fig 3.3-1; 3.3-2). This difference with Shaked's group may be due to the use of different antibodies which recognise different epitopes on the antigen, or the use of IFNγ which was obtained from a different source.

The study of IFNγ-treated U937 cells as stimulators in an MLR showed equivocal results. The proliferation of responder cells increased as their number was increased (Fig 3.3-5). However, when the responder population was kept constant and the stimulators increased, the results were very variable (Fig 3.3-6). Pulsing U937 cells in exponential growth phase with tritiated thymidine showed uniform uptake of [³H]-thymidine, eliminating the
possibility that the observed variability was due to manipulation during the harvesting process.

The alloreactive immune response is dependent partly on the presence of T lymphocytes capable of recognising and responding to allogeneic MHC class II antigens, and partly on the quantitative and qualitative expression of MHC molecules. Since it is reasonable to believe that T lymphocytes from healthy donors would function normally as responder cells, it is likely that the deficient MLR responses were due to the density of MHC class II antigens on U937 cells induced with IFNγ not being sufficient to stimulate the reaction under the experimental conditions. It is also possible that IFNγ activated cells may be secreting immunosuppressive factors eg PGs or cytokines, which subsequently inhibit the MLR. In addition, some MHC identity between the responder and stimulator cells may have existed, resulting in a reduced MLR. This inconsistency necessitated the use of alternative cells as stimulators.

Mono Mac 6 is one of a few human monocytic cell lines which naturally express high levels of MHC class II antigens. Hence, the cells may act as a target for alloreactive T lymphocytes. In the later stage of this study, this cell line was used as a stimulator in the MLR. Although the role of Mono Mac 6 cells as stimulators in an MLR has not been documented previously, the results obtained from this study clearly show that the cells are very effective stimulators for alloreactive T lymphocytes, and the results are reproducible (Table 3.5-1).
Obviously, the Mono Mac 6 cell line achieved better stimulation than the U937 cell line. This may be due to the level of expression of MHC class II molecule in the population as a whole, or on a per cell basis.

Non-T cells (comprising mainly monocytes and B lymphocytes) and T lymphocytes from human PBMNC were separated using the MACS and used in an allogeneic MLR. Purification was not very efficient (8% T cells were found in the non-T fraction) but a high recovery meant that only 10-15 ml of blood was required from each donor. The microbeads used did not affect the cell viability or functionality. This supports previous work showing that the cellular functions of natural killer cells and B lymphocytes are preserved during the separation (Abts et al., 1989; Pflueger et al., 1990).

The contamination of the non-T population by 8% CD3+ T cells (Fig 3.4-1) was probably due to γδ T cells which express CD3, but may not express CD4 or CD8 (CD3+, CD4-, CD8- or +). Improved purity could be obtained, but at the expense of cell recovery. Since their presence did not dramatically affect the MLR, cell yield was given highest priority.

The effect of the total volume of the cell suspension on the reproducibility of the MLR was examined. It was found that, provided evaporation was prevented, as little as 50 μl per well could give a sensitive and reproducible reaction. This allows the use of smaller volumes of blood making the assay
quicker. Actually, 20 µl hanging drop cultures have been shown to give meaningful results (Knight, 1987).

In summary, the use of either the Mono Mac 6 cell line, or MACS separation of peripheral blood gave a high response, without the requirement for a large amount of blood.

Having established separation techniques and standardised the assays the effect of decidual fractions on them was investigated. In preliminary experiments, crude decidual protein fractions of 10-30, 30-100, and 100-300 KDa were found to significantly inhibit mitogen-stimulated lymphocyte responses (p<0.05, 10% of 10-30 KDa; 20% of 30-100 KDa, and 100-300 KDa respectively, v/v). Further separation of the fractions of 10-30 and 30-100 KDa by chromatography showed that both LMWF (183 KDa) and SMWF (7-14 KDa) suppressed not only PHA-induced proliferation of PBMNC (p<0.01, LMWF; p< 0.05, SMWF; Fig 4.2-3), but also the one-way mixed lymphocyte reaction (p<0.05; Fig 4.2-4). Since control cultures contained the same volume of elution buffer as those treated with decidual fractions, the observed suppression was unlikely to be due to adverse effects of this buffer.

This study uses the method of direct separation of the suppressive factors from decidua homogenates. It differs from previous work using human decidua cell culture supernatants (Golander et al., 1981; Nakayama et al., 1985; Matsui et al., 1989 a). However, this result is in agreement with
previous work which showed that decidual cell supernatants inhibited
mitogen-induced lymphocyte proliferation and the mixed lymphocyte reaction
(Golander et al., 1981; Nakayama et al., 1985; Matsui et al., 1989 a).

The mitogen and MLR studies showed an effect of decidual extracts on T cell
proliferation. However, when fractions were cultured with a transformed T cell
line (Jurkat E6.1), inhibition of proliferation was not observed. By contrast,
the transformed human monocytic cell line, Mono Mac 6, was inhibited by the
fraction suggesting that the differences observed with primary and
immortalised T cells were not due to the transformed state of the cells but
possibly to a cell specific effect which, in the case of monocytes, would affect
their ability to promote an immune response. This would also explain the lack
of T cell proliferation in the mitogen and MLR studies.

As mentioned before, Mono Mac 6 cells have many of the characteristics of
mature peripheral blood monocytes and tissue macrophages, including the
expression of CD14 and HLA-DR. It is possible that these decidual factors
may have a capacity of inhibiting the function of antigen presenting cells. This
need to be further investigated. However, this cell line provides a model for
studying the mode of action of the suppressive factors further. In order to do
so, the effect of these factors on the cell cycle distribution of Mono Mac 6
cells, and the expression of MHC class II molecules by them were
investigated.
When Mono Mac 6 cells were treated with the decidual fractions, it was found that the LMWF caused the cells to accumulate in the G1/G0 phase (Table 4.4-1). However, despite the ability of SMWF to suppress the growth of Mono Mac 6 cells, no change in the cell cycle distribution was observed in treated cells. This indicates that these two factors are distinct.

Additionally, the LMWF-treated cells showed a significant increase in the number of cells showing a level of DNA less than that observed in cells in G0/G1 after 48 h incubation (Fig 4.4-1). It has been suggested that this may represent cells undergoing apoptosis (Telford et al., 1991; Zamai et al., 1993). Cell death by apoptosis (i.e. programmed cell death) is a major regulator of growth in normal tissues (Williams, 1991; Cohen, 1993). However, whether the induction of apoptosis by the LMWF was independent of the stage of cell cycle or was the result of the accumulation of cells in the G0/G1 phase of the cell cycle, is not known. It has been suggested that there are two major control points in the cell cycle, one in late G1 (called START), where the cells becomes committed to replicate DNA, and one at the onset of mitosis or the G2/M boundary. A single gene, CDC 28 appears to be implicated in both the START and G2/M transitions (Hunter, 1992). It is possible that the LMWF may inhibit the proliferation of Mono Mac 6 cells by modulating either CDC 28 gene expression or the functionality of its product.

During pregnancy, decidua-specific proteins and peptides are produced in the uterine endometrium. More than ten proteins with diverse functions have
been identified from early pregnancy decidua (reviewed by Halperin et al., 1990). Some of these factors are thought to be associated with immunoregulation, such as TGFβ, and PP14. The work presented here shows that decidua-derived factors, one with M.w. 183 KDa and four bands with M.w. 7-14 KDa, were inhibitory to mitogen-induced lymphocyte proliferation, the MLR, and the growth of the monocytic cell line, Mono Mac 6, but not the lymphoblastic T-cell line, Jurkat E6.1.

The molecular weight of the isolated proteins did not appear to compare exactly to those of known decidua factors. However, the potential similarities between these factors and TGFβ, the LMWF and hDP200, and the SMWF and TGFβ stimulated studies to compare these factors.

Purified human TGFβ1 was added to MLR cultures and was found to inhibit the proliferation observed in untreated cultures. This agrees with observations made in the murine system by Kondo et al (1993). In addition, TGFβ was found to exert moderate inhibition of cellular proliferation in mitogen stimulated culture of PBMNC. This is agreement with the work of Stoeck et al (1989) and Uhm et al (1993). Although the inhibition observed in the current study was less pronounced than that reported in the literature, this may be due to the lower dose of TGFβ employed (2 ng/ml) compared to that reported to induce significant inhibition (25 ng/ml; Uhm et al., 1993).
Jurkat E6-1 is an IL-2 producing T cell line. Since TGFβ inhibited the growth of primary T cells, its effect on the Jurkat cells was examined. Interestingly, TGFβ failed to effect the growth of Jurkat cells. By contrast, at a higher concentration, it slightly inhibited the growth of the human monocytic cell line THP1. However, the growth of another monocytic cell line, Mono Mac 6, was markedly inhibited (Fig 4.5-2). This confirms that the effect of TGFβ on cellular growth is cell line-dependent (Rizzino, 1987). The mechanism of the different response is far from clear yet. However, the Mono Mac-6 cell line was considered to be a useful model for studying the suppressive effect of TGFβ further.

The majority of studies of the activity of TGFβ were carried out using TGFβ1, and only a few using TGFβ2 or β3. Previous studies have shown that they share very similar activity (Lyons et al, 1990). However, in the present study, in contrast to TGFβ1, TGFβ2 did not suppress the growth of Mono Mac 6 cells (Table 4.5-2). This would support the work of Cheifetz and colleagues (1987), who demonstrated that TGFβ1 and TGFβ2 show distinct interactions with a family of receptors on target cells. They share a 280 KDa receptor, but only TGFβ1, not TGFβ2, interacts with both the 65 KDa and 85 KDa receptors. This would explain the distinct effect of TGFβ1 and TGFβ2 and may explain the different responses to TGFβ1 observed with Mono Mac 6 cells and Jurkat E6.1 cells, since the cell lines may express different receptors.
TGFβ is a well-characterised, immunosuppressive cytokine. Previous work has demonstrated that it may be involved in the survival of fetus allografts during early pregnancy. A TGF-β2-like immunosuppressive factor was detected in murine amniotic fluid (Altman et al., 1990). Moreover, both TGFβ1 and β2 activity were detected in human amniotic fluid (Lang & Searle, 1994). Although the precise cellular source of these factors has not been defined, they are thought to play an important role in regulating maternal immunity during pregnancy.

In murine decidua, Clark and his colleagues have demonstrated an immunosuppressive molecule related to TGFβ2 (Clark et al., 1988, 1990; Lea et al., 1992). Recently, CD56+ NK cells were reported to be the source of TGFβ2 production (Clark et al., 1994). Another group (Temada et al., 1990) found that TGFβ1 was expressed in mouse decidua, and epithelia cells were found to be the primary sites of TGFβ1 synthesis. In humans, TGFβ (β1 and β2)-specific immunostaining was demonstrated in situ in the glands and stroma (Selick et al., 1994), and in the extracellular matrix of first trimester decidua (Graham et al., 1992). However, this study did not try to demonstrate the biological activity of TGFβ in these tissue.

The decidua-derived factors observed in the present study showed some similarity in their biological activity to TGFβ1. In murine decidua, immunosuppressive activity was shown to be associated with a molecule of 60-100 KDa when chromatographed under neutral conditions (Clark et al., 1994).
1988), but was associated with a fraction of M.w. 13 KDa under acid separation conditions. In order to determine whether the LMWF presented here might be a precursor of TGFβ, the fractions were isolated under acid conditions, only slight inhibitory activity was demonstrated after the acid treatment (5-12%); much lower than that exhibited by the LMWF separated under neutral conditions (Section 4.3-2). Previous work has shown that acidification activates latent TGFβ (reviewed by Lyons et al., 1990), and when murine decidua fractions were treated this way, the immunosuppressive activity of the fraction was greatly increased (Clark et al., 1988). This would suggest that the LMWF isolated in the present study is not a precursor of TGFβ. Further work using a rabbit anti-TGFβ antibody (against TGFβ1 and TGFβ3) abolished the effect of purified TGFβ on the growth and cell cycle distribution of Mono Mac 6 cells, but did not affect the activity of the LMWF at all (Fig 4.5-3, 4.5-4, & 4.5-5). It is possible that the ultrafiltration separation procedure used in the current study eliminated TGFβ (due to its noted ability to be absorbed on membranes and plastic surfaces), allowing other immunosuppressive molecules to be identified.

Since the SMWF had a molecular weight of 7-14 KDa under neutral conditions (Fig 4.6-3). It was possible that it might be a TGFβ-related factor. However, neutralising anti-TGFβ1 and β3 antibodies did not affect its suppressive activity (Fig 4.5-3A) and Western blot analysis did not detect any TGFβ1 in the SMWF (Fig 4.6-7). Thus, it is unlikely that the factor is TGFβ1. In addition, since purified TGFβ2 did not inhibit the growth of Mono Mac 6
cells (Table 4.5-2), as did the decidua-derived factors and this inhibition could not be reversed by the use of neutralising anti-TGFβ2 antibody (Fig 4.5-3B), it is unlikely that either the SMWF or LMWF are TGFβ2.

In 1990, Halperin and his colleagues (1990) reported a new human decidua associated protein. Under non-reducing SDS-PAGE analysis, it appeared as a single protein band with molecular weight of approximately 200 KDa (hDP200). Under reducing conditions it formed two polypeptide chains with molecular weight of 55 and 25 KDa. When analysed by SDS-PAGE, the LMWF appeared to have similar M.w bands: under non-reducing conditions, 183 KDa; under reducing conditions, 70 and 29 KDa; raising the possibility that the immunosuppressive LMWF is hDP200. Recently, Halperin et al (1994) demonstrated that the human-decidua associated protein hDP200 was a rheumatoid factor. Amino acid sequencing of the N-terminal region suggested that this factor is IgG. Western blot analysis demonstrated heavy chains of IgA, IgG and IgM associated with hDP200. However, Western blot analysis in the present study suggested that although the LMWF non-specifically binds to horseradish peroxidase (HRP) conjugated antibodies, this could not be blocked by preincubation of the blot with high concentrations of the unlabelled antibody (Fig 4.6-9).

The amino acid sequencing performed by Halperin et al does suggest that hDP200 is an IgG-like molecule. Moreover, another group has shown that human decidua extracts inhibited mitogen induced proliferation of PBMNC,
and the causative factor (M.w 158 KDa) is thought to be IgG or a substance copurified with IgG as demonstrated by immunoelectrophoresis and double immunodiffusion (Kitano et al., 1990). In the present study, the Ouchterlony double diffusion technique did not demonstrate the presence of heavy chains of IgG, IgA, or IgM in LMWF (Fig 4.6-10) even after concentration of the LMWF to four times that used in the Western blot analysis. This suggests that the LMWF may not be identical to hDP200 or the 158 KDa factor, Ig G.

The SMWF identified from this study has a similar molecular weight to placental protein 14 (PP14) which was initially isolated from human term placenta with subsequent studies indicating that its main site of synthesis during pregnancy is the decidua (Julkunen et al., 1988). Glandular epithelial cells in human decidua are the cellular source of PP14 (Reviewed by Seppala et al., 1994) which was found to have immunosuppressive properties; it inhibited allogeneic (Bolton et al., 1987), mitogen or IL-2 induced lymphoproliferation and NK activity (Pockley et al., 1988; Okamoto et al., 1991). It has been suggested that its inhibition is mediated by decreasing the production of IL-1 (Pockley et al., 1990), and IL-2 (Pockley et al., 1989). Thus, PP14 shares biological activities with SMWF. However, purified PP14 is a 42 KDa glycoprotein (reviewed in Pockley et al., 1990), migrating as a 28 KDa band on SDS-PAGE (Julkunen et al., 1988), whereas SMWF appears as four bands between 7-14 KDa. Thus, the SMWF is unlikely to be PP14.
Other low molecular weight substances have been associated with immunosuppression and decidua. Prostaglandin E2 has been well documented as a non-specific immunoregulatory factor from murine early pregnancy decidua. It inhibited antigen- and mitogen-induced lymphocyte proliferation, MLR, CTL (Mattews & Searle, 1987; Wood et al., 1988) and NK cell activity (Malygin et al., 1993). Recently, an endogenous inhibitor of prostaglandin synthesis has also been found in human decidua (Sun et al., 1994). However, since the M.w. of prostaglandins is less than 1 KDa, the immunosuppressive activity observed in the present study could not be caused by these chemicals.

6.2. INVESTIGATION OF THE ROLE OF NK CELLS IN DECIDUA-ASSOCIATED IMMUNOREGULATION

In order to study the role of immunological cells in the immunosuppression associated with human decidua, it was first necessary to develop a method for their separation. Mechanical, rather than enzymatic, dispersal was used to dissociate decidual cells in order to avoid alteration of the immunological properties of the cells. Others have shown that cells obtained mechanically are generally smaller than those derived enzymatically (Wood et al., 1988); implying that cells obtained mechanically contain a relatively high proportion of immunocompetent cells, since decidual glandular epithelial and stroma cells are large. However, in the present study, mechanically dissociated cells were mostly dead and contained a high proportion of large cells. Thus, a
two-step percoll gradient separation (62%/31%/PBS) was modified from a previously reported method (Starkey et al., 1988) to eliminate the dead and large cells. Using this method, the yield of immunocompetent decidual cells was higher (on average 65% of DMNC) with greater viability (>97%).

When cells isolated in this way were analysed by flow cytometry, it was found that an average of 65% of DMNC were immunocompetent cells (including macrophages, NK cells, and T lymphocytes). This is slightly different from previous work: Starkey et al (1988) showed a higher proportion (75%); whilst Vince et al (1990) showed a lower proportion (47%). This may be due to the different methodologies employed in extracting DMNC.

Among the immunocompetent cells, CD14+ macrophages were found to be the most abundant cell type (23% of DMNC), followed by CD56+ NK cells (14%), and CD3+ T lymphocytes 13% (Fig. 5.2-1). This is opposite to previous reports (Starkey et al., 1988) which suggested LGL were the most abundant cell type. One reason for this discrepancy may be that the overnight incubation during enzymatic digestion allowed the loss of macrophages due to adherence. On the other hand, the process of enzymatic digestion may cause the selective loss of specific cell types, or membrane marker antigens.

The majority of NK cells were found to be CD56+ CD16- with only a small proportion being CD56+ and CD16+. This is consistent with the observation of others (Starkey et al., 1988).
To determine whether or not NK cells were responsible for the production of the immunosuppressive factors, the cells had to be purified further. This was done by the use of an NK-negative enrichment kit and the MACS magnetic cell separator. The contamination of the non-magnetic fraction by T lymphocytes and macrophages was very low (Fig 5.5-1 & Table 5.5-1). This is consistent with the observation of others (Pflueger et al, 1990) who have demonstrated a 2-9% contamination of CD3+ cells following the negative separation of NK cells from peripheral blood. However, in the present study, this negative selection procedure did not enrich the NK cells in the non-magnetic fraction which still contained a large proportion of decidual stromal cells. Also, many NK cells were retained in the magnetic fraction. This may be due to the expression of adhesion molecules on the NK cells, such as fibronectin receptors, and β2 integrins (Burrows et al., 1993), or due to cell clumping.

Both separated and non-separated cell fractions were cultured for short periods of time and their supernatants assessed for immunosuppressive activity. The cells from human decidual tissue, or the mediators released by them in short-term culture, have been found to suppress mitogen-induced lymphocyte proliferation and MLR (Golander, et al., 1981; Parhar, et al, 1988; Matsui, et al, 1989 a & b). In the present study, the supernatant from short-term cultures of DMNC (non-separated and separated) was similar to that observed with the decidua extract. They suppressed the growth of Mono Mac
6 cells in a dose-dependent manner. However, they did not affect the growth of the T lymphoblastoid cell line Jurkat E 6.1 at a lower concentration. At higher concentrations they inhibited the cells but the effect was much less than that observed with Mono Mac 6 cells (Table 5.3-2).

The treatment of DMNC with IL-2 did not increase the observed suppression (Fig 5.3-1). It is possible that the cells which produce the suppressive factors are IL-2-independent. Alternatively, since pooled decidua cells were used to produce the supernatants, mixed lymphocyte responses may have resulted in the production of the immunosuppressive activity which could not be further increased by the addition of exogenous IL-2. Nevertheless, addition of Con A enhanced the suppressive activity of DCS (Fig. 5.3-1 &Table 5.3-2). This suggests that the suppressive factors are produced by Con A-sensitive cells or that they respond to stimuli produced by them.

The suppressive activity of the non-magnetic fraction of DCS was markedly decreased (Fig 5.5-2), despite approximately 25% of the cells being immunocompetent (Table 5.5-1). The majority of the cells in this fraction were non-immunocompetent and therefore unlikely to be the source of suppressive factors.

In terms of absolute numbers of NK cells, the suppressive activity of the non-magnetic fraction was far less than that of the magnetic fraction, suggesting that other cells, rather than CD56+ NK cells, are likely to be responsible for
producing the suppressive factors. However, the suppressive factor may need to reach a critical number before its effect is evident. Thus, NK cells cannot be excluded as the source of the suppressive factors. Recently, Clark et al (1994) have reported that TGFβ2-related immunosuppressive factors were produced by CD56+ NK cells in human first trimester decidua.

In summary, this study has identified 4-5 bands on SDS-PAGE which are associated with suppressive activity on cellular proliferation. Both of the LMWF and SMWF are unlikely TGFβ-like factors. The LMWF shows distinct differences to hDP200 and the SMWF appear to be distinct from PP14. Clearly the identities of these factors need to be established as do their cellular source(s).

6.3. FUTURE WORK

In order to clarify some of the findings there is a need to further purify the factors. Thus, the production of specific monoclonal antibodies to the factors observed by SDS-PAGE analysis would be the first step. These would be used to further purify the suppressive factors by affinity chromatography and to detect precisely which cell was producing them. Following isolation, the amino acid sequences of the purified factors could be determined and compared with other known immunoregulatory factors. Ultimately, probes could be developed to identify the genes responsible for the suppressing factors. A study could then be made of the possible role of these factors in
recurrent abortion and other immunologically based diseases, especially those of an autoimmune nature, along with our increased understanding to the fetal-maternal immune relationship.
7.1. SODIUM DODECYL SULPHATE - POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

30% acrylamide mix (with 0.8% bisacrylamide, 37.5: 1.
from National Diagnostics)
Ammouium persulphate (10%, freshly prepared, from BDH)
Butanol saturated with water
Dithiothreitol (DTT, from Sigma)
Molecular weight standard (prestained, from Biorad)
SDS (Lauryl sulfate, from Sigma)
TEMED (N,N,N',N'-Tetramethyl-ethylenediamide, from Sigma)
Tris (Tris[hydroxymethyl] aminomethane, from Sigma)

Resolving buffer x4

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris-HCl PH8.8</td>
<td>1.5M</td>
</tr>
<tr>
<td>SDS</td>
<td>0.4% (w/v)</td>
</tr>
</tbody>
</table>

Stacking buffer x4

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris-HCl PH6.8</td>
<td>4% (w/v)</td>
</tr>
</tbody>
</table>

Resolving gel

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>water</td>
<td>20 ml</td>
</tr>
<tr>
<td>30% acrylamide mix</td>
<td>24 ml</td>
</tr>
<tr>
<td>4x resolving buffer</td>
<td>15 ml</td>
</tr>
<tr>
<td>*10% ammonium persulphate</td>
<td>300 µl</td>
</tr>
<tr>
<td>*TEMED</td>
<td>30 µl</td>
</tr>
</tbody>
</table>
5% stacking gel

- Water: 13.9 ml
- 30% acrylamide mix: 4.0 ml
- 4x stacking buffer: 6.0 ml
- *10% ammonium persulphate: 200 μl
- *TEMED: 20 μl

Sample buffer x4

- Stacking buffer x4: 2.4 ml
- 20% SDS: 4 ml
- Glycerol: 4 ml
- Bromophenol blue: 0.01%
- DTT (optional): 20 mM

Tank buffer x10

- SDS: 1%
- Glycine: 0.55 M
- Tris: 0.5 M

* Added immediately before pouring.

7.2. DETECTION OF PROTEINS BY COOMASSIE BRILLIANT BLUE

Coomassie brilliant blue stain

- Methanol: Acetic acid: Water: 5:2:5
- Coomassie blue: 0.1%

Fix/Destain

- Methanol: 30%
- Acetic acid: 10%
- in MQ water
7.3. PROTEIN TRANSFER

Western blot transfer buffer

<table>
<thead>
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<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris</td>
<td>25 mM</td>
</tr>
<tr>
<td>Glycine</td>
<td>190 mM</td>
</tr>
<tr>
<td>Methanol</td>
<td>200 ml</td>
</tr>
</tbody>
</table>

Water to 1 L

7.4. IMMUNOSTAIN

Primary antibody: diluted in TTBS with 3% BSA (Sigma).

Secondary antibodies (HRP-conjugated): diluted in TTBS with 3% BSA.

(Alternatively biotinylated secondary antibodies plus Extravidin (HRP-conjugated) were used).

Enhanced chemiluminescence (ECL) detection reagents 1 and 2 (Amersham):

**TTBS X5**

<table>
<thead>
<tr>
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<td>Tris</td>
<td>250 mM</td>
</tr>
<tr>
<td>NaCl</td>
<td>2.5 M</td>
</tr>
<tr>
<td>PH</td>
<td>7.4</td>
</tr>
<tr>
<td></td>
<td>autoclaved</td>
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</tbody>
</table>

**TTBS**

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>TTBSx5</td>
<td>200 ml</td>
</tr>
<tr>
<td>MQ water</td>
<td>800 ml</td>
</tr>
<tr>
<td>Tween 20</td>
<td>0.5 ml</td>
</tr>
</tbody>
</table>

**Western blot blocking solution**

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dried low fat milk</td>
<td>8% in TTBS</td>
</tr>
<tr>
<td>or BSA</td>
<td>3% in TTBS</td>
</tr>
</tbody>
</table>
7.7. IMMUNOLOGICAL REAGENTS

Conjugated MoAb against CD3(FITC), CD56(PE), CD16(PE), CD14(FITC) and HLA-DR(PE) (all from Becton Dickinson, Cowley, Oxford, UK).
Conjugated MoAb against CD16(FITC) (Eurogenetics, Teddington, Middx).
Extravidin (HRP-conjugated; Signal Chemical Ltd, Poole, UK).
Goat anti hTGFβ2 polyclonal neutralising antibody (R&D system; Abingdon, UK; Lot No. EE212).
Goat anti-rabbit IgG (Biotin conjugated, Signal Chemical Ltd, Poole, UK).
Goat anti-rabbit IgG (HRP-conjugated; Signal Chemical Ltd, Poole, UK).
hTGFβ1 (Becton Dickinson; Cowley, Oxford, UK. lot No. 900503 and 911593).
Immunobeads (including CD3, CD4, CD8, CD14, CD16, CD56, and negative NK cell isolation kit; all from Miltenyl Biotel GmbH, Germany).
Interleukin 2 (R&D System, Abingdon, UK).
Rabbit anti hTGFβ1 polyclonal neutralising antibody (Becton Dickinson; Cowley, Oxford, UK. Lot No. J792).
Rabbit anti-goat IgG (HRP-conjugated; Signal Chemical Ltd, Poole, UK).
Rabbit pan anti hTGFβ neutralising antibody (anti TGFβ1 and β3; R&D system; Abingdon, UK; Lot No.E0033091).
Recombinant hTGFβ2 (Crystal Chem, Chicago, USA; Lot No. CR 7-042).
7.5. RADIAL IMMUNODIFFUSION

0.3M Phosphate buffer:

\[
\begin{align*}
\text{KH}_2\text{PO}_4 & \quad 2.24 \text{ g} \\
\text{K}_2\text{HPO}_4\cdot3\text{H}_2\text{O} & \quad 64.7 \text{ g} \\
\text{Distilled H}_2\text{O} & \quad 1.0 \text{ L} \\
\text{pH} & \quad 8.0
\end{align*}
\]

1.5% Noble Agar in 0.3 M Phosphate buffer

Antibodies (Sigma)

Goat anti-human IgG (γ-chain specific; Lot 022H8808)
Goat anti-human IgA (α-chain specific; Lot 50H8836)
Goat anti-human IgM (μ-chain specific; Lot 28F-8920)

Human serum (HS; Sigma; lot No. 53H0780)

7.6. PREPARATION OF PERCOLL

Percoll, 62.5%
Diluent, 37.5%

\[
\begin{align*}
(45 \text{ ml } & 10x \text{ PBS} \\
3 \text{ ml } & 0.6 \text{ M HCl} \\
132 \text{ ml } & \text{dH}_2\text{O}
\end{align*}
\]

Sterilise through a 0.2 μm filter

36% Percoll:

50% of 62.5% Percoll
50% of RPMIc
7.8. OTHER REAGENTS

Complete RPMI: RPMI supplemented with 10% FCS, 2mM L-glutamine, and 100 μg/ml kanamycin (all from Signal Chemical Ltd, Poole, UK).

Concanavalin A (Sigma).

3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide (Sigma).

Lymphoprep (Nycomed, Birmingham, UK).

Mitomycin C (Sigma).

Phenol red-free RPMI medium (Gibco, Paisley, UK).

Phytohemagglutinin (ICN, High Wycombe, UK).

Propidium iodide (Sigma).

Prestained M.w. markers for PAGE analysis (Bio-Rad, California, USA).

Protein M.w standards for chromatographic study (Sigma).

Sephacryl S-100 HR (Sigma).

Tritiated thymidine (specific activity 25 Ci/mmol; Amersham, Buckinghamshire, UK).

7.9. EQUIPMENT

Cell harvester (ILACON, Tonbridge, Kent, UK).

FACScan flow cytometer (Becton Dickinson, Cowley, Oxford, UK).

High performance liquid chromatography (FPLC; Pharmacia, Sweden).

Magnetic activated cell sorter (MACS; Miltenyl Biotel GMBH, Germany).

Minitan ultrafiltration system (Millipore, Bedford, USA).

Ultracentrifuge (Beckman, UK).
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