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STUDIES ON THE CELLS FROM THE BASAL AND CHORIONIC PLATES OF HUMAN
PLACENTA

A thesis submitted for the degree of
Doctor of Philosophy at the
University of Glasgow

by

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DECLARATION

This Thesis is dedicated to my mother , and all my family and friends
with grateful thanks for their love and support. The work is my own
except where specifically acknowledged.

John Arthur
John Arthur

ACKNOWLEDGEMENT

It is with gratitude and thanks that I acknowledge the tremendous effort and help of my supervisor Dr. Roger Sutcliffe, who encouraged, guided and enthused me throughout the course of this work.

I would like to record my debt to the Head of this Department, Professor David Scarratt for allowing me to pursue my studies here. My sincere thanks to Mrs Susan Henderson for her invaluable assistance and help. Thanks are due to Mrs Flory Rodger, Rane Hussain, Craig Lyle, John Holley, Debra Nickson and all the rest of the

DECLARATION

academic and technical staff in the institute for their help, friendship

and assistance. I would also like to thank the staff of the Institute for their help and assistance. I am also grateful to the Arab Student Aid International, New Jersey, USA, for their financial support.

Finally, I gratefully acknowledge the assistance of my husband and his family who supported me morally and financially throughout my study.



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S U M M A R Y

Throughout the post-implantation stages of pregnancy, the trophoblasts provide the major site of direct interaction between the mother and her genetically alien fetus. The survival of this fetal-allograft appears to be ensured by more than one mechanism. A review of the literature revealed the importance of the placental trophoblast populations especially at the materno-fetal interfaces. These cells were shown to express class I MHC antigens which appear to be different from the classical class I transplantation antigens. Additionally, they were shown to display TLX-type antigens which may be related to the MHC antigens and seem to elicit a non-destructive immune response as well as the production of immunoregulatory factors.

The purpose of this investigation was to localize the trophoblastic cells in different placental regions, to isolate them and to characterize them for different antigenic determinants in particular the HLA antigens. The following results were obtained. Several studies were carried out on different regions in human term placenta. These regions include the chorionic plate, the placental villi and the uteroplacental interface (Nitabuch's layer). ^{35}S -met labelled short term organ tissue culture experiments revealed the synthesis of several polypeptides. A heavy ^{35}S -met incorporation into a 230k supernatant polypeptide was detected after 24-48 hrs. of culture. This polypeptide had the properties of fibronectin. It was cold-insoluble, being detected in the supernatant only when the cultured tissue was incubated at 37°C , not after incubation at 4°C . Analysis of the culture supernatants using anti-fibronectin and gelatin Sepharose columns, revealed the binding of this 230k polypeptide to these columns.

Another polypeptide with a molecular weight corresponding to that of PAPP-A (187k) was also detected in the culture supernatants analysed on SDS PAGE. The antibody-antigen crossed electrophoresis (AACE) yielded a radioactive immunoprecipitin line. However, the attempt to detect the molecular weight of that precipitated line using SDS gels failed; no radiolabelled band could be detected. Accordingly, these results did not provide ample evidence for PAPP-A synthesis in the above mentioned areas.

The immunohistological study using the indirect immunoperoxidase staining technique revealed similar distribution for both Fibronectin and PAPP-A in the uteroplacental interface, the placental septae, cytotrophoblast islands and the chorionic plate.

These results incited me to explore the uteroplacental interface and the chorionic plate, in order to isolate their cells and to do further identification and immunological studies including monoclonal antibody preparation.

Accordingly, the cells from these layers were isolated using the trypsinization method. These cells showed variation in their viability from one placenta to another. They also were highly heterogeneous in size and number of nuclei. These cells grow on different substrates including plastic, glass coverslips coated with Fibronectin or gelatin, and human plasma clot. The cultured Nitabuch's layer cells were active in digesting the plasma clot compared to those from the chorionic plate. The clot digestion was inhibited using 3AB and EACA. No fibroblast overgrowth was detected. These placental cells were cultured for a maximum of 2-4 months.

Indirect immunoperoxidase staining was performed on both frozen sections and cytospin preparations from both Nitabuch's layer and the

chorionic plate. Different antigenic determinants were studied using several monoclonal antibodies. These included cytokeratin (TR1 and PKK1), placental proteins (using anti-SP1, -hPL, -PAPP-A, -Fibronectin and -hCG alpha and beta subunits), placental antigenic determinants (H315, H316 and 7A11) as well as other markers for the endothelial cells (anti-UEA I lectin), leucocytes (anti-LCA) and fibroblasts (PK-V). The staining for these specificities was very clear in both layers. However, no staining was detected for the endothelial cells, leucocytes or fibroblasts, using anti-UEA I lectin, anti-LCA and PK-V antibodies respectively.

In the cytopsin preparations, a substantial number of cells from both Nitabuch's layer and the chorionic plate displayed positive staining for cytokeratin, the placental proteins and the placental antigenic determinants. Few of these highly heterogeneous isolated cells stained with anti-UEA-I lectin, anti-LCA and PK-V. This indicates low cellular contamination with endothelial cells, leucocytes, and fibroblasts respectively.

Additionally, the antigenic determinants for the MHC class I and class II were studied using the monoclonal antibodies DA6.231, 19/9 for class II, and the monoclonal antibodies W6/32 and beta-2-microglobulin (B₂M) for class I. Both the frozen sections and the cytopsin preparations revealed positive staining for class I antigens. Only a few cells in both preparations showed positive reaction for class II.

These results encouraged me to subject the isolated chorionic plate cells in particular, because of their location away from the maternal decidua cells, to the ordinary tissue typing assay. The aim for this was to study the HLA phenotypes expressed by these cells. The results obtained from such study indicated that these cells are relatively

resistant to the antibody and complement attack, although complement and antibody binding was detected using the indirect immunofluorescent staining method. In these experiments, cellular viability was assessed using both eosin dye exclusion and ^{35}S -met incorporation. Furthermore, when these cells were mixed with lymphocytes at the ratios 1:4, 1:10 and 1:20, several polypeptide bands on the SDS autoradiographic films were observed. Among these proteins was a 51k polypeptide which distinguished the trophoblasts from the lymphocytes. Because the molecular weight of this polypeptide lies within the cytokeratin range (40-58k), several attempts were carried out to try and purify this protein hoping to raise monoclonal antibodies against it in order to use it as a marker for the cytotrophoblasts. These purification and extraction attempts failed due to the presence of several protein bands in the extract.

ABBREVIATIONS

AACE	Antibody-antigen crossed electrophoresis
3AB	3-diaminobenzamide
ACT	Ammonium chloride tris
CM	Complete media
CP	Chorionic plate
DTT	Dithiothreitol
EACA	E-aminocaproic acid
FCS	Fetal calf serum
FN	Fibronectin
hCG	Human chorionic gonadotrophin
hPL	Human placental lactogen
NL	Nitabuch's layer
O/N	Over night
PBL	Peripheral blood lymphocytes
PBS	Phosphate-buffered saline: 0.16M NaCl, 5mM potassium phosphate, pH 7.5
RT	Room temperature
SDS	Sodium dodecyl sulphate
³⁵ S-met	³⁵ S-methionine
SP1	Pregnancy specific beta-1-glycoprotein

CHAPTER 1

INTRODUCTION

1:1 I N T R O D U C T I O N

1:1 The Human Placenta.

The human placenta, which is of the haemo-chorial type, can be defined broadly as a union of maternal and fetal tissues. This unit possesses unique functional characteristics including full growth and development within a limited life span (9 months), during which it serves a number of purposes including the physiological exchange of substances between the fetal and maternal circulations as well as elaboration of steroid and protein hormones (Wynn, 1975) in order to satisfy the requirements of the fetus throughout the successive stages of pregnancy.

1:1:1 Preparation for Blastocyst Implantation

Implantation and subsequent development of the human placenta depend on certain changes in the endometrium that culminate in the decidual cell formation. Extensive studies were concerned with the endocrine control of the generation of uterine sensitivity to decidual stimuli and the extent of decidualization (Glasser, 1972; O'Grady and Bell, 1977; Bell, 1983). It was shown that decidualization is induced spontaneously after hormonal preparation during the late secretory phase of the menstrual cycle and does not proceed further unless pregnancy ensures a maintained production of progesterone. The decidua directly beneath the site of implantation forms the decidua basalis. Surrounding the fertilized ovum and separating it from the rest of the uterine cavity in the early months of gestation is the decidua capsularis which forms as a result of deep implantation of the human fertilized ovum. The remainder of the pregnant uterus is lined by decidual parietalis. Since the fertilized ovum does not occupy the entire uterine cavity in the early months of pregnancy, there is a space between the capsular and parietal portions

of the decidua. By the fourth month the growing fertilized ovum hits the uterine cavity leading to the fusion of both the capsularis and parietalis, obliterating the endometrical cavity (Wynn, 1975).

1:1:2 Development and Structure

After ovum fertilization and the subsequent blastocyst formation, the human blastocyst loses its surrounding zona pellucida. The outer layer of the blastocyst proliferates to form the trophoblastic cell mass, from which cells infiltrate between the endometrial epithelium, thus the blastocyst completely becomes embedded in the endometrium by the eleventh or twelfth day (Fox 1978). This is followed by the differentiation of the trophoblast into a peripheral layer of primitive syncytiotrophoblast and an inner layer of cytotrophoblast which starts proliferating to form the precursors of the primary villi (Boyd and Hamilton, 1970). Later, after mesodermal cores growth leads to the secondary and tertiary villi, the cellular trophoblast at the tips of the villi forms the cytotrophoblastic cell columns, which are not invaded by mesenchyme and not vascularized but are anchored to the decidua of the basal plate. These cells, subsequently, proliferate and spread laterally to form a continuous cytotrophoblastic shell which divides the syncytiotrophoblast into two layers, the definitive syncytium on the fetal aspect of the shell and the peripheral syncytium between the shell and the decidua. The definitive syncytium persists as the limiting layer of the intervillous space, whereas the peripheral syncytium gives rise to masses of syncytium-like giant cells that extend through the decidua basalis into the myometrium (Boyd and Hamilton, 1970; Fox, 1978; Pijenburg et al, 1981a; Wynn, 1975) which together form the placental bed. Furthermore, the cytotrophoblastic cells invade and partially replace the endothelium of the decidual

portion of the decidual spiral arteries; these trophoblasts cause considerable disruption of the arterial wall, with the deposition and formation of fibrinoid material (Pijnenborg et al, 1981b).

The establishment of the trophoblastic shell would allow rapid growth of the developing placenta. This, subsequently, leads to an expansion of the intervillous space, into which sprouts of syncytiotrophoblasts extend from the primary villous stems. These villi, as the placental development proceeds, become orientated towards the uterine cavity, degenerate and form the chorion laeve which come into contact with the parietal decidua of the opposite wall of the uterus. Moreover, those villi on the side of the chorion towards the decidua basalis proliferate to form the chorion frondosum, which develops into the definitive placenta. During this period, some regression of the cytotrophoblastic elements in the chorionic plate and in the trophoblastic shell would be taking place, where the cytotrophoblastic cell columns degenerate and become largely replaced by fibrinoid material (Rohr's Layer); clumps of cells remain, to form the 'cytotrophoblastic cell islands' (Fox, 1978; Boyd and Hamilton, 1970).

The placental septa appear during the third gestational month; those structures protrude into the intervillous space from the basal plate and divide the maternal surface of the placenta into 15 to 20 lobes. As the basal plate is formed principally by the remnants of the trophoblastic shell embedded in fibrinoid (Fox, 1978) it follows that these septa are similarly formed; some decidual cells may also be present and the relative proportions of fetal and maternal elements may vary not only from septum to septum but also in different areas of individual septa (Boyd and Hamilton, 1970). The cytotrophoblastic cells which predominate in both the basal plate and the septa, have been referred to

as 'X-cells', however, their fetal origin has been clearly confirmed both by electron microscopy and by studies which have utilized the quinacrine fluorescence technique to show that they always contain a Y-chromosome if the fetus is male (Fox, 1978).

By the end of the fourth month of pregnancy, the placenta has attained its definitive form and undergoes no further anatomical modifications. It is composed of the chorionic plate which is on one side covered with the amnion, and on the other attached to the fetal cotyledons. These project towards the decidua basalis which together with the cytotrophoblastic shell forms the basal plate. The smallest fetal cotyledons form groups of villi near the chorionic plate. The terminal villi are formed from the side branches of the anchoring villi which extend from the chorionic plate to the basal plate. The terminal villi float in the intervillous space, some of which are united by syncytial bridges, or by deposits of fibrinoid substances on the surface of the adjacent villi.

These terminal villi which are the functional units of the placenta are composed of an outer syncytiotrophoblastic layer, an inner cytotrophoblastic layer (Langhans cells) and the villous stroma which is composed of fibroblasts, Hofbauer cells (which appear to be macrophages) (Wynn, 1975; Fox, 1978), endothelial cells of the fetal capillaries and other mesenchymal elements. As the placenta ages, these form numerous subdivisions. The more obvious histologic changes that are consistent with increased efficiency of transfer include an increase in the ratio of the villous surface to volume, a decrease in thickness of the syncytium, discontinuity of the Langhans layer, and reduction in the proportion of villous connective tissue relative to the trophoblast (Boyd and Hamilton, 1970; Wynn, 1975).

1:2 Cellular and Protein Composition of the Basal and Chorionic Plates

Several studies from early pregnancy showed the deposition of fibrin and the related substance fibrinoid in the utero-placental interface (Nitabuch's Layer), and in relationship to the syncytium in the villi (Boyd and Hamilton, 1970; Sutcliffe et al, 1982). Fetal tissues come into direct contact with maternal blood, lymph and interstitial tissue fluids, all of which contain fibrinogen. Accordingly, it is not surprising that fibrin comes to be laid down in those areas. This deposition of fibrin might be significant during the implantation and trophoblast invasion for the establishment of the placenta. It is surprising to see such amounts of fibrin deposition, since it has been shown that trophoblastic cells at the implantation site produce fibrinolytic enzyme (plasminogen activator), (Glass et al, 1983; Sheppard and Bonnar, 1978). Accordingly, it could be argued that this phenomenon is due to the local synthesis of fibrinolysis inhibition by the trophoblasts themselves. For, depending on their situation and the age of the conceptus, they seem to show considerable variations in shape and perhaps in function and protein synthesis (Boyd and Hamilton, 1970).

The absence of coagulation in the intervillous space where villus trophoblastic cells - the syncytiotrophoblasts - are in direct contact with the maternal blood could be attributed to the production of anti-coagulant protein by this layer. Recent reports (Grudzinskas et al, 1979) suggested the production specifically by the syncytiotrophoblast of a protein called placental protein 5 (PP5), which could be involved in the maintenance of the intervillous blood flow by locally influencing the coagulation system. Furthermore, Salem et al (1980), proposed the possibility that PP5 might represent a placental analogue of antithrombin III due to its interaction with both heparin and thrombin

in vitro. Another placental protein PAPP-A, is also thought to be involved in the coagulation process. Bischof, (1984), claimed that pure PAPP-A is capable of inhibiting blood coagulation in vitro. It was claimed that this protein does not inhibit fibrin polymerization directly, but through a factor which is thought to be factor XIII. This factor which is a transglutaminase, is able to cross-link proteins, particularly soluble fibrin to produce insoluble polymerized fibrin (Bischof, 1984).

1:2:1 Basal Plate

The placental basal plate has been defined as the area of fusion between the chorion and the decidua basalis (Pijnenborg, Robertson and Brosens, 1982).

In the course of development of human placenta, trophoblastic cells invade the maternal decidua from the proliferating tips of the anchoring villi and the cytotrophoblastic shell. Most of these proliferating and migrating cells are mononuclear cytotrophoblasts which represent a cell type different from the relatively inactive Langhan's cells of the placental villi (Pijnenborg et al, 1981b). During the invasion process bi, tri- or multinuclear trophoblastic cells make their appearance which could be due to the cytotrophoblast fusion. These cytotrophoblasts during the implantation process would tend to concentrate around the decidual spiral arteries after the displacement and the occupation of the space vacated by the uterine epithelium and decidual cells (Glass et al, 1983; Sheppard and Bonnar, 1978; Pijnenborg, Robertson and Brosens, 1982). During this time these cytotrophoblasts deeply invade the superficial myometrium. Having infiltrated the interstitium of the myometrium, they provoke a sequence of structural changes in the walls of the spiral arteries to prepare them for subsequent colonization by

the endovascular trophoblast (Pijnenborg et al, 1983). The obliquely oriented decidual spiral arteries seem to offer an easier substrate for trophoblast invasion than the decidual stroma. Accordingly, local breaching of the arterial walls with the result of multiple openings of the vessels into the intervillous space takes place. This leads to the replacement of the vessel endothelium by these trophoblasts forming the endovascular key to trophoblasts (Pijnenborg et al, 1981a; Pijnenborg et al, 1983; Pijnenborg et al, 1980). This phenomenon could be due to the synthesis of proteolytic enzymes such as plasminogen activator which has been identified in the postimplantation mouse embryo (Kubo et al, 1982; Strickland, Reich and Sherman, 1976) and guinea pig and rat blastocyst (Owers and Blandau, 1971).

Subsequent to these events, Rohr's and Nitabuch's fibrinoid layers will be formed. It has been shown by Sutcliffe et al, (1982), that the uteroplacental fibrinoid is composed of several polypeptides including fibrin protein as well as others with molecular weight over 105,000. The origin of these proteins is not clear yet. A possibility which could be put forward to theoretically account for the formation of fibrinoid layer is that as a result of endovascular trophoblast invasion to the spiral arteries and their subsequent opening into the intervillous space, the maternal blood might escape to the nearby regions where it coagulates, and thus embeds the cytotrophoblasts in the superficial decidua. From this it can be concluded that cytotrophoblasts in the interstitial myometrium might be different from those in the superficial decidua in their protein secretion products. Therefore, those cells embedded in the fibrinoid layer might synthesise a proteolytic inhibitor leading to the clot coagulation with the help of maternal blood coagulating factors which seem to play an important role.

Previous studies showed that women with Factor XIII deficiency suffer from recurrent abortion that can be remedied by plasma transfusion (Ikkala, Myllyla and Nevanlinna, 1964). In addition, it is possible that fibrinoid formation might play an important role in the regulation of the amount of blood entering the placenta. Brosens, Robertson and Dixon, (1967), suggested that the endovascular trophoblast, because of its incorporation into the vessel wall with its associated physiological changes, probably has a vital role in regulating maternal blood flow to the placenta. Furthermore, the formation of this layer might mark the site of partition at the end of pregnancy.

Besides the trophoblastic cells, two major cell populations have been distinguished in early pregnancy in the decidua. These are the large glycogen-rich decidual cells and the smaller endometrial granulocytes (Pijnenborg et al, 1980, 1982; Bulmer and Sunderland, 1984) which has been reported to accumulate in areas where trophoblast invasion is prominent, particularly around spiral arteries and endometrial glands (Pijnenborg et al, 1980, 1981a). From the distribution of these cells it was concluded by Bulmer and Sunderland (1984) that a high proportion of these endometrial granulocytes are T-lineage cells which carry the E-rosette receptor and do not express peripheral T cell markers. Additionally, these latter cells were found to express OKT 10; a marker of immature myeloid and lymphoid precursors and activated T and B lymphocytes. Preliminary studies of term placental bed reveal the absence of these endometrial granulocytes (Bulmer and Sunderland, 1984). In addition to these cells, a large population of macrophages has been identified within the decidualized endometrium in both term and first trimester tissues (Sutton, Mason and Redman, 1983; Bulmer and Johnson, 1984). The significance of these different cells and their proposed

functions will be discussed later.

Cellular components of the chorionic plate and the amniochorion seem to be comparable. Several immunohistological studies for the detection of surface antigens on the amniochorion manifest the presence of mainly four layers (Hsi, Yeh and Faulk, 1982; Nehemiah, et al, 1981; Kurman, Main and Chen, 1984). Amniotic epithelium layer, subepithelium layer with mesenchymal cells embedded in a collagen-rich connective tissue, cytotrophoblasts and finally decidua. Kurman and co-workers described an intermediate type of trophoblasts in the amniochorion which are morphologically distinctive from the other placental trophoblasts. These are termed "Intermediate Trophoblasts". In addition, several studies revealed the presence of a large population of macrophages in the subepithelial layer of the amniochorion (Sutton, Mason and Redman, 1983; Bulmer and Sunderland, 1984). As far as the chorionic plate is concerned its general structure seems comparable to that of the amniochorion, however, it might be possible that this area no longer contains decidual cells due to its inevitable distance from the uterus. Nevertheless, it is probable that the chorionic plate is composed of different types of trophoblasts each displaying different surface antigenic determinants and perhaps synthesize different proteins.

1:3 Proteins and Markers of Trophoblasts

1:3:1 Placental Proteins

Localization studies for most of the placental proteins were carried out using immunohistological methods. The localization of some of these proteins will be mentioned in this section.

Several studies were carried out to localize human chorionic gonadotrophin (hCG). Precise localization of undissociated hCG in the trophoblast was controversial. Earlier work (reviewed by Diczfalusy and

Troer, 1961) was based on the assumption that hCG production by the trophoblast was directly related to the number of cytotrophoblastic cells present in the intact or cultured villi, suggesting its synthesis by these cells. Others with the aid of immunofluorescent staining (Midgley and Pierce, 1962) and immunoperoxidase method (Dreskin, Spicer and Greene, 1970; de Ikonicoff and Cedard, 1973) observed an exclusive localization of undissociated hCG in the syncytiotrophoblast. In contrast, Thiede and Choate (1963) and Fox and Karkhongor (1970), using immunofluorescent antibody staining, found that hCG was localized in both cyto- and syncytiotrophoblast and in cytotrophoblastic cells tissue culture (Fox and Karkhongor, 1970). In addition, Tabarelli et al (1983), localized hCG in giant cells within the placental septa and cytotrophoblastic islands of the intervillous space. Other studies (Gaspard et al, 1980; Tabarelli, Kofler and Wick, 1983) demonstrated the localization of undissociated hCG and of beta hCG in the syncytiotrophoblastic cells with some cytotrophoblastic cells being weakly positive (Gaspard et al, 1980). The localisation of hCG alpha subunit was strikingly different; in immature placentae it was weak and localised in the syncytium, while in mature placentae it was more conspicuous in cytotrophoblasts (Gaspard et al, 1980). This study did not exclude the synthesis or storage of alpha hCG in the cytotrophoblasts, due to its localization in these cells under in vitro conditions after three days of culture (Gaspard et al, 1980). More recently, hCG was found to be widely distributed in the syncytiotrophoblast while small amounts were identified in the intermediate trophoblasts which were located in the placental bed, cytotrophoblastic columns, overlying the chorionic villi and in the amniochorion (Kurman, Main and Chen, 1984).

In addition to this protein, others were studied including PAPP-A, SP1 and hPL. From immunofluorescent studies it was shown that PAPP-A (Lin and Halbert, 1976; McIntyre *et al*, 1981), SP1 (Bohn, 1972; Horne *et al*, 1976; Lin and Halbert, 1976) and hPL (Osada, 1976; Gaspard *et al*, 1980; Tabarelli, Kofler and Wick, 1983; and Tabarelli *et al*, 1983) to be localized and probably synthesized in the villous syncytiotrophoblasts. However, more recently hPL was found to be localised also in giant cells within the placental septa and cytotrophoblastic cell islands of the intervillous space (Tabarelli *et al*, 1983). Furthermore Kurman, Main and Chen (1984) using the immunoperoxidase staining method revealed the localisation of SP1 and hPL in the intermediate trophoblasts until term. The staining for these proteins was less in the syncytiotrophoblast. According to this group, they failed to localize any of these proteins in the cytotrophoblasts.

1:3:2 Trophoblast Surface Markers

Different studies have been directed towards the trophoblasts, for these cells subsequently cover the surface of the placenta and extraembryonic membranes, coming into direct contact with maternal tissues over a wide area during gestation. Most attention has been focussed on the syncytiotrophoblast because it is easy to purify its microvillous brush border which is directly exposed to maternal blood throughout gestation and thus expected to play a significant role in all interactions between the fetus and its mother. IgG and transferrin receptors were identified on human syncytiotrophoblasts (Faulk and Galbraith, 1979; Faulk and Johnson, 1977; Johnson and Brown, 1980; Johnson, Brown and Slade, 1982) in addition complement component C3 and trophoblast antigen 1 (TA1) were detected in this layer (Faulk and Johnson, 1977; Faulk *et al*, 1978). Although the amniochorionic

cytotrophoblasts do not manifest transferrin receptors, they show reaction with antisera to TA1 antigens (Hsi, Yeh and Faulk, 1982). Recently, the advent of hybridoma technology has opened up new avenues of approach to the study of trophoblast - specific or trophoblast-associated molecules, and many monoclonal antibodies have now been produced which react with the fetal trophoblastic tissues and the extra-embryonic membranes (Sunderland, Redman and Stirrat, 1981b; Johnson et al, 1981; Johnson and Molloy, 1983; Brown, Molloy and Johnson, 1983; Loke et al, 1984). NDOG1 and NDOG2 are two monoclonal antibodies which reacted with the apical aspects of the syncytiotrophoblasts; no staining was observed in the villous stroma (Sunderland, Redman and Stirrat, 1981; Gatter et al, 1983). Other monoclonal antibodies react with trophoblast-specific antigenic determinants (H315 and H317), as well as human tumor cells (Johnson et al, 1981; Brown, Molloy and Johnson, 1983). The H317 mAb has been found to react specifically with the heat-stable, L-phenylalanine-inhibitable, placental type alkaline phosphatase isoenzyme (McLaughlin, Gee and Johnson, 1983). It has been shown by Johnson and Molloy (1983) that both H315 and H317 mAbs clearly stained the cytotrophoblastic layer of amniochorionic membranes, all villous trophoblast in placental bed tissue as well as occasional remaining trophoblastic sprouts proliferating from villous structures. Interstitial mononuclear cytotrophoblast were strongly stained as well as mononuclear and occasional multinuclear giant cells adjacent to the positively-stained interstitial cytotrophoblast clusters, which are found in a perivascular distribution. However, giant cells throughout other sites in maternal decidual stroma were not significantly stained (Johnson and Molloy, 1983). In addition, H310 and H316 recognize an antigenic epitope restricted to trophoblast and lymphocytes (Brown,

Molloy and Johnson, 1983) designated as trophoblast-lymphocyte cross-reactive (TLX) antigens (McIntyre et al, 1983). More recently, it was shown by Bulmer, Billington and Johnson (1984) that H315 reacted strongly with villous syncytiotrophoblast, the microvillous membrane being especially prominent, with weaker staining of the underlying cytotrophoblastic layer. In contrast, H316 showed cytoplasmic staining for both syn- and cytotrophoblasts of the chorionic villi. H315, H316 and NDOG1 were shown to strongly stain the syncytial sprouts and cytotrophoblastic cell columns associated with the chorionic villi. Studies on the placental bed of 8-10 weeks gestation specimens showed clear staining of all mononuclear trophoblast within the maternal uterine tissues including interstitial and scattered individual cytotrophoblasts. Prominent staining of large numbers of perivascular cytotrophoblastic cells around spiral arteries as well as endovascular cytotrophoblast was detected. Moreover, marked staining for H315 and H316 was demonstrated in the columnar epithelium of the endometrial glands (Bulmer, Billington and Johnson, 1984).

In addition to these antibodies, OKT9 a monoclonal antibody that is known to identify the cell surface receptors for transferrin, strongly stained the syncytiotrophoblast of term placental chorionic cells including all villous trophoblast within the placental bed immediately adjacent to the basal plate of decidual tissue. However, this mAb did not stain any non-villous trophoblasts within the maternal decidual tissue in the marginal area of the placental bed or any cells within the reflected amniochorionic membranes including the chorionic cytotrophoblast layer.

Another monoclonal antibody designated 18B/A5 has been generated against human first trimester trophoblast membranes (Loke and Day,

1984). This mAb has been reported to react discretely with the cytotrophoblast of the chorionic villi. The staining was demarkated from the syncytial layer on the outside and from the villous mesenchyme on the inside. In addition, cytotrophoblastic cell columns were strongly stained. This mAb was also found to react with certain fetal epithelial and endothelial surfaces, however, it did not react with villous mesenchymal cells, decidua, and uterine glandular epithelium (Loke and Day, 1984; Loke et al, 1984).

Because no antibody as yet is available which reacts with all non-villous trophoblast and no other cell types, monoclonal antibodies to the intermediate filaments were used as a marker for these cells. TROMA 1, LE61 and JMB2 were used for this purpose. These bound to all villous and extra-villous trophoblast populations throughout pregnancy (Oshima et al, 1981; Kemler et al, 1981; Contractor, Routledge and Sooranna, 1984; Redman et al, 1984). Redman et al, (1984) revealed that JMB2 reacted with other cell types beside the trophoblastic cells. On the fetal side, spindle shaped cells in the connective tissue around large branches of the umbilical arteries, and in the fetal membranes in the layer between the amniotic epithelium and the chorion. In maternal tissues, the reactivity of anti-JMB2 in particular was confined to epithelial cells of the endometrial glands in the uterus. Apparently, these antibodies proved to be excellent markers for the identification of all the trophoblast subpopulations in the placental tissue.

1:4 Immunological Aspects of Human Placenta

1:4:1 Transplantation Antigens

The replacement of severely injured tissues and organs does not always end in success. Grafts from an individual to himself

(autografts) and between genetically identical individuals (syngeneic grafts) almost always succeed. However, grafts between two genetically dissimilar individuals of the same species (allogeneic grafts) or between individuals of different species (Xenogeneic grafts) do not normally succeed (Bencerraf and Unanue, 1979; Playfair, 1982). The major reason for such failure is an immune response to the cell-surface antigens that distinguish donor from host. The tissue antigens that induce such response are called histocompatibility antigens coded by histocompatibility genes. These genes are divided into two main categories, genes of major histocompatibility complex (MHC), present on human chromosome 6 and mouse chromosome 17. These genes specify antigens that induce rapid graft rejection. The other category is of minor histocompatibility genes that specify antigens which cause a slower graft rejection. The major histocompatibility complex encodes three families of genes denoted class I, class II and class III. The minor histocompatibility antigens, H-Y and blood-group antigens represent polymorphism but to a much lesser extent than that of the major histocompatibility antigens within a species. The class I gene products in the mouse include the transplantation antigens K, D and L, which are responsible for graft rejection and the hematolymphoid differentiation antigens Qa-1, Qa-2,3 and TL (encoded by the T1a gene). These antigens are found on virtually all nucleated somatic cells and they provide the essential context of self in which foreign cell-surface antigens (virally infected cells) can be recognized by cytotoxic T cells which then destroy the antigen bearing cells (Hood et al, 1984; Steinmetz, 1984).

Class II genes - A alpha , A beta, E alpha and E beta - are located in the I region. These products are found on macrophages, T cells and B,

cells. They provide a self-recognition element which allows these cells to interact in the presence of foreign antigen in specific combinations to produce antibody-secreting plasma cells, to stimulate proliferation of cytotoxic T cells, or to generate suppressor T cells (Hood et al, 1984; Steinmetz, 1984). The class III genes encode several components in the activation portion of the complement cascade. The HLA complex in human appears remarkably homologous to that of the mouse (H-2) and it encodes the same three classes of gene. The three class I transplantation antigens are designated A, B and C; the three class II antigens are called DR, DQ and DP (DR, DC and SB), and the class III genes include those encoding the C2, C4 and BF complement components. Antigens homologous to those encoded by the T1a complex of the mouse are just beginning to be described in humans (Hood et al, 1984, Bodmer and Bodmer, 1984).

Accordingly, for an organ graft success, matching donor and recipient MHC antigens as far as possible and suppressing the residual immune response are required (Playfair, 1982; Hood et al, 1984).

1:4:2 Survival of the Feto-Placental Allograft

After implantation, it is the fetally derived cells of the placenta and the fetal membranes, rather than the embryo or the fetus, which constitute the graft. This graft is considered to be unique in the sense it is fed by its own vessels of fetal origin. Nevertheless, genetically alien trophoblastic cells make direct physical contact with cells of the maternal immune system remaining either within the maternal blood or the decidua (Lala et al, 1983). Moreover, trophoblastic cells may occasionally be deported from the placenta to appear in the maternal circulation (Goodfellow and Taylor, 1982) or the pulmonary bed during human pregnancy (Attwood and Park, 1961).

These placental and fetal membranes which are endowed with immunological properties essential for the successful outcome of mammalian viviparity, appear to play a significant role in the protection of the semi-allogeneic fetus. However, the survival of this semi-allogeneic conceptus in that potentially hostile maternal environment remains an enigma (Chaouat and Monnot, 1984; Chaouat and Chaffaux, 1984; Gupta et al, 1984). Many hypotheses have been proposed to explain the immunologic paradox of the fetal allograft. These include 1) an "immunologically privileged" uterus, 2) separation of maternal and fetal circulations, 3) "placental barrier", 4) specific "enhancing" or "blocking antibodies" in the maternal circulation (Mathur et al, 1982; Lala et al, 1983).

1:4:2:1 Distribution of Major Histocompatibility Complex (MHC) Antigens

Several immunohistological studies were carried out on early and term placentae to study the distribution of class I and class II major histocompatibility complex antigens. It has been manifested that human villous trophoblast does not express class I or class II major histocompatibility complex (MHC) antigens (Faulk and Temple, 1976; Goodfellow et al, 1976; Sunderland et al, 1981; Sunderland, Redman and Stirrat, 1981a). Nevertheless, staining was observed in the stroma of the chorionic villi. However, the trophoblast is not only confined to the chorionic villi; other forms are found in several extra-villous locations. These include cytotrophoblastic columns, cytotrophoblastic shell, cytotrophoblast cell islands and cytotrophoblasts in the reflected amniochorion and chorionic plate. Recent studies revealed the expression of HLA class I antigens on non-villous trophoblasts (Sunderland, Redman and Stirrat, 1981a; Redman et al, 1984). In these studies using the monomorphic mAb W6/32, cells in the tips of the

cytotrophoblast cell columns and in the infiltrating trophoblast of an early placental bed including interstitial and endovascular cells were clearly reactive. The same was observed with the cytotrophoblasts of the chorion. The latter observation contradicts an earlier finding by Hsi, Yeh and Faulk, (1982). The staining pattern for both W6/32 and B₂M was consistent.

Accordingly, it was apparent that the extra-villous trophoblast which reacted with antibodies (W6/32) to monomorphic class I MHC products, failed to react with HLA-A or HLA-B antibodies specific for the fetal phenotype (Redman et al, 1984). This was accounted for theoretically by putting forward the possibility that the class I antigens are qualitatively different, either belonging to the HLA-C series or to another MHC class. Recently, studies on the murine MHC system revealed the presence of about 36 distinct class I MHC genes which were isolated from a BALB/c genomic cosmid library, of which more than 10 genes are uncharacterized (Goodenow et al, 1982; Steinmetz, 1984). Because of the homology between the human and murine MHC, this might add relevance to the latter possibility (Redman, 1984).

As far as class II MHC antigens are concerned, it was shown that none of the trophoblasts displayed HLA-DR (Redman et al, 1984; Sunderland et al, 1981; Sunderland, Redman and Stirrat, 1981a). However, cells within the villous stroma (Sunderland, Redman and Stirrat, 1981b), the subepithelial layer of the amnion and in the decidua (Sutton, Mason and Redman, 1983) were shown to express class II HLA-DR antigens. Several studies were carried out to study the origin and identity of these cells. Sutton, Mason and Redman (1983) identified a population of heterogeneous HLA-DR positive cells using immunochemical and histochemical methods. These cells were shown to express Leucocyte-

common antigen indicating their origin from bone marrow precursors (Bulmer and Sunderland, 1983). In addition, it was shown by Bulmer and Johnson (1984) that apart from a small population of stellate intensely HLA-DR positive cells in villous stroma, the extra-embryonic HLA-DR positive cells consistently express Leu-M3 tissue macrophage antigen and lysosomal activities, suggesting that these HLA-DR positive cells seen in the uteroplacental tissue are macrophages rather than dendritic type cells as suggested by Sutton, Mason and Redman (1983) and Redman (1983). Moreover, by using monoclonal antibodies specific for fetal or maternal HLA-A or B allotypes, it has been shown that HLA-DR positive cells in the chorionic villi and the amnion are fetal in origin, whereas those in the decidua are maternal; a few adjacent to the basal plate are fetal (Sutton, Mason and Redman, 1983). It was suggested by Bulmer and Johnson (1984), that the various macrophage populations might perform vital phagocytic and degradative functions within utero-placental tissues and amniochorion.

In culture, Brami and co-workers revealed the expression of HLA-DR on cells from 9-12 week abortuses, which also were found to secrete hCG, suggesting their trophoblast identity. In addition, HLA-DR was found to be expressed on the epitheloid trophoblast cells in monolayer cultures. These cells using double immunofluorescence staining were shown to display positive reaction with anti-HCG, hence indicating their trophoblast origin (Brami et al, 1983). However, there remain uncertainties concerning the latter finding which can only be resolved by more detailed investigation. So far it is still agreed that HLA-DR is not detectable on trophoblasts (Redman, 1983; Redman et al, 1984; Bulmer and Johnson, 1985b). However, it would be of great interest to know whether the trophoblasts express HLA-DR on their surface, and if

so, why are they not rejected by the immune system of the mother, for such antigens are known to stimulate the strongest allograft rejection reactions?

As shown above, the placental interface is clearly antigenic, expressing class I MHC products, that are accessible to monoclonal antibodies. Nevertheless, it is not rejected by the maternal immune system. Usually for allograft rejection, MHC antigens of both class I and class II need to be recognized by the alloreactive T cells of the host for a maximal allograft rejection response. Precursors of the cytotoxic T cells (Tc) bearing receptors for the class I MHC molecules respond to them and undergo clonal expansion and maturation into Tc with the help provided by T helper (Th) cells in an MHC-restricted manner. Precursors of Th recognize class II MHC molecules and undergo clonal expansion, leading to the generation of Th which provide the help by way of elaborating interleukin 2. Finally, the mature Tc destroys the target cells of the graft by virtue of recognizing class I MHC antigens. From this it is apparent that the MHC antigens serve as the principal allo recognition molecules on the allografted cells. Thus the alloantigenic status of the trophoblasts is a key determinant of immune interactions between the mother and the fetus (Chatterjee-Hasrouni, Montgomery and Lala, 1983). Although these antigens are expressed on these cells, no rejection reaction is taking place. Several hypotheses were put forward to try and explain such enigma. It has been suggested that the absence of class II antigens, could possibly make these cells poorly immunogenic (Lala et al, 1983; Billington and Bell, 1983). In addition, class I antigens might be presented in a manner which is ineffective both for the cognitive as well as the effector arms of the immune system. The expression of these antigens might be very important in

eliciting the maternal immune system to respond. In addition, McIntyre and Faulk,(1982) and McIntyre et al (1983) proposed the possibility that the trophoblast antigens (TA) of the trophoblast-lymphocyte cross-reactive (TLX) type might be important in inciting the maternal recognition to mount a protective response which is important for the survival of the blastocyst. Such response might be in the form of enhancing or blocking antibodies as well as in the form of immunosuppression. The blocking antibodies possibly participate in making the antigenic sites inaccessible for the T-cells. These antibodies might form a layer of immune complexes at the maternal-fetal interface, thus the placenta at this stage will act as a barrier preventing the passage of such antibodies into the fetus, in addition, these antibodies might activate suppressor cells and inactivate CTL. The MHC-epitope configuration on the trophoblasts might be able to be recognized by the antibodies but not the alloreactive T cells (Lala et al, 1983). The presence of these antigens might also be important in protecting other non-histocompatibility antigens such as the TLX (McIntyre and Faulk,1982), that might be recognized in MHC compatible pregnancies leading to spontaneous abortions (Billington, Davies and Bell, 1984).

Pregnancy-induced hyporesponsiveness to paternal allografts has been shown to be demonstrated as early as the first pregnancy, with multiparity leading to tolerance to paternal skin graft in some combinations (Smith and Powell, 1979; Beer and Billingham, 1979). The transfer of such hyporesponsiveness by cells provided an evidence for suppressor cell involvement (Chaouat and Monnot, 1984). Such cells might block cytolytic effector generation and possibly regulate isotypic spectrum of maternal antipaternal antibodies as a safeguard mechanism,

establishing a split tolerance state against paternal alloantibodies (Chaouat and Chaffaux, 1984). Nagarkatti and Clark (1983), reported the appearance of non-T suppressor cells within the decidua and lymph nodes draining the uterus. These suppressors have been characterized as small lymphoid cells that block the generation of cytotoxic T cells (CTL) against paternal antigens and prevent the development of CTL activity in sponge-matrix allografts placed in sensitized mice. Limiting dilution studies revealed that the suppression does not affect the frequency of CTL precursors, but inhibits their development into mature CTLs. In addition, antigen-specific suppressor T cells have also been described (Nagarkatti and Clark, 1983; Kearns and Lala, 1983; Bell, 1983). Furthermore, local suppressor mechanisms of effector agents which involve suppressor cells and placenta inhibitory factors were described. Their main targets seem to be cytotoxic cells, killer cells, possibly natural killer cells, and even complement, thus inhibiting complement-dependent antibody cytotoxicity. Local immunoregulation might involve release of high concentration of immunoregulatory substances by cells at the feto-maternal interface. These might include progesterone, steroids released by the trophoblasts (Siiteri and Stites, 1982), hCG, the various SP, PP, PAPP and other placental or gestational glycoproteins (Klopper, 1982), or decidual cells; prostaglandins produced by the decidua (Williams and Downing, 1977), or other yet-undefined immunosuppressor molecules produced by the trophoblast cells, decidual cells or decidual leucocytes (Lala et al, 1983).

Furthermore, the survival of the fetal allograft might be explained by deviating the immune response into a reaction beneficial to the conceptus. According to the transplantation immunology, a complete immune reaction is made up of two interacting parts: the rejection

reaction and the facilitation reaction. The former involves cells and humoral agents. The cellular agents are cytotoxic T lymphocytes (T_c), helper T-cells (T_h) and delayed hypersensitivity T-cells (T_{dh}) activating macrophages and natural killer cells. Humoral agents are complement, complement-activating antibodies and killer-cell-activating antibodies. The regulatory facilitation reaction antagonize the rejection reaction and tends to protect the antigen-bearing target. Cells involved are the suppressor T cells and the antibodies are the enhancing antibodies which have been shown to be non-complement-fixing IgG1 in mice (Duc et al, 1975). The fate of the antigen-bearing target depends on the balance between the two sides of the immune reaction and their immune agents. During pregnancy, the balance is shifted in favour of the facilitation reaction and that the placenta plays a role in this immunodeviation. In this situation antibodies are shifted from complement fixing IgG2 class to an anaphylactic and enhancing IgG1 class. Moreover, it has been shown that placental extracts decrease mixed lymphocyte culture reactivity and cytotoxic T-lymphocyte generation and increase suppressor cell generation. They also protect against target cell destruction by CTL (Voisin, 1984).

From this it appears that the placenta contains several immunoregulatory factors, some immunosuppressive, others immunostimulant and others immunodeviatory, towards a stimulatory antigen (Voisin, 1984; Beer, 1984). The mother will reject her developing fetoplacental unit if she does not immunodeviate her responses. The trigger(s) eliciting these immunological responses are not determined in full. Trophoblast antigens, histocompatibility antigens or otherwise are responsible. These invading trophoblasts, like the invading parasites, must concentrate on regulating the immune response of the host to manipulate

it in a way which results in their survival.

1:5 Experimental objectives.

From the previous findings, it is apparent that the trophoblastic cells play very important roles at the materno-fetal interfaces. Such findings encouraged me to aim at the following:

1-Study and explore placental regions rich in cytotrophoblastic cells using immunohistological methods.

2-Isolate these trophoblastic cells.

3-Characterize them for different antigenic determinants especially the HLA antigens.

4-Although several monoclonal antibodies were raised against the trophoblastic cells, none of them was prepared from isolated cytotrophoblastic cells. For, such mAbs were prepared from the syncytiotrophoblast microvillous plasma membranes which are easy to purify. Accordingly, preparing monoclonal antibodies against specific antigenic determinants displayed by the isolated cytotrophoblastic cells, was another object to aim at.

2:1 INTRODUCTION

Biochemical studies on the uteroplacental interface by Sutcliffe et al (1982) revealed that the major insoluble polypeptides in that layer had molecular weights of 55,000 and 105,000. In addition, a variable amount of material at 48,000 was observed. Methionine-peptide mapping of these polypeptides showed that they were identical to the beta, gamma and gamma-gamma dimer polypeptides of blood fibrin and therefore confirmed that the major insoluble protein composition of the uteroplacental fibrin is mainly in the form of fibrin. In addition, this study showed a considerable amount of material of high molecular weight, over 105,000, which was distinguishable from the high molecular weight components of fibrin. **CHAPTER 2**

IDENTIFICATION AND LOCALIZATION OF PLACENTAL PROTEINS FIBRONECTIN (FN) AND PREGNANCY ASSOCIATED PLASMA PROTEIN A (PAPP-P)
revealed (Dr. Roger Sutcliffe personal communication).

Accordingly, short term culture experiments and immunohistochemical studies were carried out on the uteroplacental interface and other placental zones to investigate the origin of fibrin, and the origin and distribution of the other high molecular weight glycoproteins in placental fibrinolytic layer including PAPP-A.

MATERIALS AND METHODS

2:2:1 Materials. Culture media, fetal bovine serum and culture

2:1 I N T R O D U C T I O N

Biochemical studies on the uteroplacental interface by Sutcliffe et al (1982) revealed that the major insoluble polypeptides in that layer had molecular weights of 55,000 and 105,000. In addition, a variable amount of material at 48,000 was observed. Methionine-peptide mapping of these polypeptides showed that they were identical to the beta, gamma and gamma-gamma dimer polypeptides of blood fibrin and therefore confirmed that the major insoluble protein composition of the uteroplacental fibrinoid is mainly in the form of fibrin. In addition, this study showed a considerable amount of material of high molecular weight, over 105,000, which was distinguishable from the high molecular weight components of fibrin due to its binding to wheat germ agglutinin. Further, immunohistochemical localization of pregnancy associated plasma protein A (PAPP-A) was carried out on term placental sections. It was revealed that cells in the uteroplacental interface were positively stained (Dr. Roger Sutcliffe personal communication).

Accordingly, short term culture experiments and immunohistological studies were carried out on the uteroplacental interface and other placental zones to investigate the origin of fibrin, and the origin and distribution of the other high molecular weight glycoproteins in placental fibrinoid layer including PAPP-A.

MATERIALS AND METHODS

2:2:1 Materials. Culture media, fetal bovine serum and culture

supplements were obtained from Flow Laboratories (U.K.), Sepharose 4B was obtained from Pharmacia and protein was coupled to it by the cyanogen bromide reaction of Pornath, Axen and Ernback (1967). Scintillation counts were measured in a SL30 Liquid Scintillation Spectrometer (Intertechnique). All samples were prepared in a volume of high efficiency emulsifier cocktail (Biofluor, New England Nuclear). Gelatin from BDH, Serum fibronectin from BRL. ^{35}S -met was obtained from Amersham. Enhance, from New England Nuclear. Tris and SDS were obtained from Sigma Chemical Company.

Protein Standards: Transferrin, bovine serum albumin (BSA), ovalbumin, lactoglobulin, and RNase were purchased from the Sigma Chemical Company. Low molecular weight protein standards for SDS gel electrophoresis were also obtained from BIO-RAD Laboratories, U.S.A.

Paraffin-wax embedded blocks of human term placentae which had been fixed for 1-6 months in the neutral buffered formalin (4% formaldehyde - 4% NaH_2PO_4 - 6.5% Na_2HPO_4) were obtained from the Pathology Department in Aberdeen. Sheep anti-fibronectin antiserum, normal swine serum, rabbit anti-sheep immunoglobulins, swine anti-rabbit immunoglobulins, peroxidase-antiperoxidase (soluble complex of horseradish peroxidase and rabbit anti-horseradish peroxidase) were all obtained from DAKO (Copenhagen). Sheep anti-PAPP-A was prepared as described by Sutcliffe *et al* (1979). Purified serum fibronectin was purchased from Bethesda Research Laboratories. Pure PAPP-A was prepared as described by Sutcliffe *et al* (1980).

2:2:2 Methods

1. Tissue culture media preparation. Tissue culture media were prepared

as follows using materials obtained from Flow laboratories or Gibco Bio-Cult Ltd. Complete media: 150ml of sterile H₂O were supplemented with 18ml, 10x Eagles medium (Glasgow modification); 6.7ml, 7.5% sodium bicarbonate; 2ml, 200mM glutamine; 20ml Fetal Bovine Serum (FBS; mycoplasma and virus screened); 2ml, 100mM sodium pyruvate; 2ml, 100x non-essential amino acids; 2ml, 100x penicillin/ streptomycin. Methionine minus medium: as for complete medium, except that 10x Eagles medium (Glasgow modification) minus methionine was used and the FBS was omitted, so that in metabolic ³⁵S-met labelling experiments, the culture medium (2% FBS) is prepared by diluting complete medium (10% FBS) 1 in 5 with methionine minus medium (0% FBS), thus diluting ³⁵S-met to give activity of 10 uCi/ul.

2.Tissue culture. Tissue explants were carefully dissected from three different regions of freshly delivered placentae. These include the chorionic plate, the placental villi and the uteroplacental interface which was observed as a colorless, translucent material. These tissue pieces were then washed with PBS to remove any contaminating blood. This was followed by a wash with methionine minus medium. The tissue fragments were then cut into smaller pieces (1x2x2mm) and incubated individually in 0.5ml of culture medium at 37°C for 24-120 hrs. 50uCi of ³⁵S-met were added per well. Aliquots were analysed on SDS polyacrylamide gels (5-15%) and by autoradiography. TCA precipitation was carried out on these supernatants.

3.Identification of fibronectin. For immune precipitation, aliquots (200ul) of labelled tissue culture supernatant were added to 200ul of rabbit anti-human fibronectin coupled to Sepharose 4B. The mixture was shaken vigorously overnight, then washed three times in 0.5M NaCl, 50mM tris-HCl, pH 7.4, before a final wash in 0.5ml water. Fibronectin was

also identified through its affinity for gelatin coupled to Sepharose 4B using the method of Engvall and Roslahti (1977). Aliquots (0.2ml) of 50% slurry of Sepharose were added to 300ul of cultured supernatants. This mixture was shaken vigorously overnight, then washed with phosphate-buffered saline, pH 7.2, containing 0.01M sodium citrate. The adsorbed material was eluted using 8M urea, 0.05M tris-HCl, pH 7.5.

4. Antibody antigen crossed electrophoresis (AACE). AACE was used for the detection of PAPP-A in the supernatant of cultured Nitabuch's and the chorionic plate tissues, using the method of Laurell (1966), with the modification that 100mM tris was present in the tank and gel buffer (Sutcliffe et al, 1980). 2% anti-PAPP-A solution was used in the antibody beds.

5. Identification of PAPP-A. As described before, 200ul from the supernatants of cultured Nitabuch's layer and the chorionic plate were loaded to a Sepharose anti-PAPP-A column. Normal sheep serum was used as a control.

6. TCA precipitation of ^{35}S -labelled proteins. Duplicate aliquots (10-50ul) of labelled protein solutions were spotted onto filter paper discs (Whatman no. 1), and left to dry. Proteins were precipitated by gently stirring the discs in ice cold 5% (w/v) TCA for 30 min, then the TCA was removed by washing with 4-5 volumes of methanol. The washed and dried discs were placed in scintillation vials with 10 ml of Biofluor and the precipitated counts measured.

7. SDS/polyacrylamide gel electrophoresis. SDS-polyacrylamide gel electrophoresis was performed according to Laemmli, (1970) with 5-15% polyacrylamide gels. Sample preparation and staining was carried out using tris-buffered slab gels as described by Sutcliffe, et al (1980).

8. Immunoperoxidase staining. The method of Sternberger, et al (1970)

was used as described by Horne, et al (1982). Slides were dewaxed with two changes of xylol for 10 min each. All reactions were carried out at room temperature. Endogenous peroxidase activity was blocked by treating the sections with 1% H_2O_2 in methanol for 15 min. All dilutions of antisera were made in 0.14M NaCl, 50mM tris-HCl, pH 7.4. Each was applied to the sections for 30 min, followed by 3 washes of 10 min each with 0.14M NaCl, 50mM tris-HCl, pH 7.4. Non-specific background staining was reduced by exposing sections to a 1/5 dilution of normal swine serum before application of sheep anti-fibronectin at 1/50. The second antibody rabbit anti-sheep IgG applied to the sections at 1/100 after first being absorbed with 25mg of placental powder and normal swine serum to a total concentration of 10%. Swine anti-rabbit immunoglobulins were then applied at a dilution of 1/100, followed by the peroxidase-antiperoxidase complex diluted 1/100. Peroxidase activity was revealed by staining for 10 min with diaminobenzidine (30mg in 100ml 50mM tris-HCl, pH 7.4), and counterstaining with haematoxylin.

Negative controls were carried out as indicated in the results, using both non-immune sera and specifically adsorbed antisera. For the adsorption of anti-fibronectin: to 5ul of sheep anti-fibronectin was added 2mg of fibronectin. The adsorption proceeded for 30 min at room temperature and the mixture was then diluted to a final concentration of 1/100 and the test sections were compared with the appropriate adsorption control. For PAPP-A, to 5ul of sheep anti-PAPP-A was added 5mg of PAPP-A. The serum was diluted to 1/100 and centrifuged before use.

2:3 RESULTS

The supernatants and tissue homogenates from the cultured samples of uteroplacental interface, placental villous material and chorionic plate were reduced with 2-mercaptoethanol and analysed on SDS-polyacrylamide gels. Albumin was one of the predominant and major polypeptides seen in the supernatants (Fig.1A), due mainly to the presence of fetal calf serum in the media. A prominent polypeptide of 36K was observed in chorionic plate tissue and supernatant, but not at comparable intensity in other samples (Fig.1A).

After culture for 24 hr, ^{35}S -methionine was incorporated into many proteins in the tissue samples but into a limited number of polypeptides in the culture supernatant (Fig.1A). The number of labelled bands in the supernatant increased when the culture was exposed to ^{35}S -methionine for 120 hrs. Incorporations were inhibitable by 100ug/ml cycloheximide. No incorporation was detectable into bands of molecular weights corresponding to the fibrin polypeptides. The major protein band to be labelled in the supernatant had a molecular weight of 230,000 under reducing conditions. Protein corresponding to this molecular weight was also detected with Coomassie blue. When placental villous material was cultured for 24 hrs, no band of 230K was similarly stained or detected by autoradiography. However, this protein could be detected after 120hr culture.

In non-radioactive cultures, it was observed that the 230,000 molecular weight polypeptide was only detectable in the culture supernatant when utero-placental interface was cultured at 37°C; no protein was detectable in parallel cultures at 4°C. This apparent cold-insolubility suggested that the 230,000 molecular weight band might be fibronectin. Accordingly, the labelled supernatant of cultured uteroplacental interface layer and of chorionic plate, was applied to a

rabbit anti-fibronectin Sepharose column. Figure (1B) shows that the 230K protein bound to the antibody column and could be eluted in 0.5mM tris-HCl, pH 7.4 . Figure (1C) shows that the 230K protein bound to a column of gelatin-Sepharose and could be eluted with 8M urea, 0.05M tris-HCl, pH 7.5.

As far as PAPP-A identification was concerned, the autoradiographic films failed to show any precipitable band with molecular weight corresponding to that for PAPP-A; 187k (Fig.2B). AACE experiments showed a precipitated line which was evident on the autoradiographic films (Fig. 2A). However, this precipitate when cut and run on SDS gels did not show any band on the autoradiographic film.

Immunoperoxidase staining. Marked staining of fibronectin was observed in four regions of the term placenta. These were the uteroplacental interface (Nitabuch's layer), the placental septae, trophoblastic cell islands and the chorionic plate (Fig.4). In these regions staining for fibronectin was detected in large uninuclear cells with cytoplasm which stained with haematoxylin in all mentioned regions. In all cases adsorption and non-immune reactions showed no staining (Fig.3). No staining was observed in the syncytiotrophoblast of placental villi or in the sheet of decidual cells at the uteroplacental interface. However, clear staining for fibronectin was detected in reticular fibres surrounding the decidual cells and also in patches in the uteroplacental fibrinoid. Reticular fibres can be seen clearly staining for fibronectin in the chorionic plate and around blood vessels in trophoblastic cell islands and in placental septae. Similar distribution pattern was also observed for PAPP-A (Fig.5).

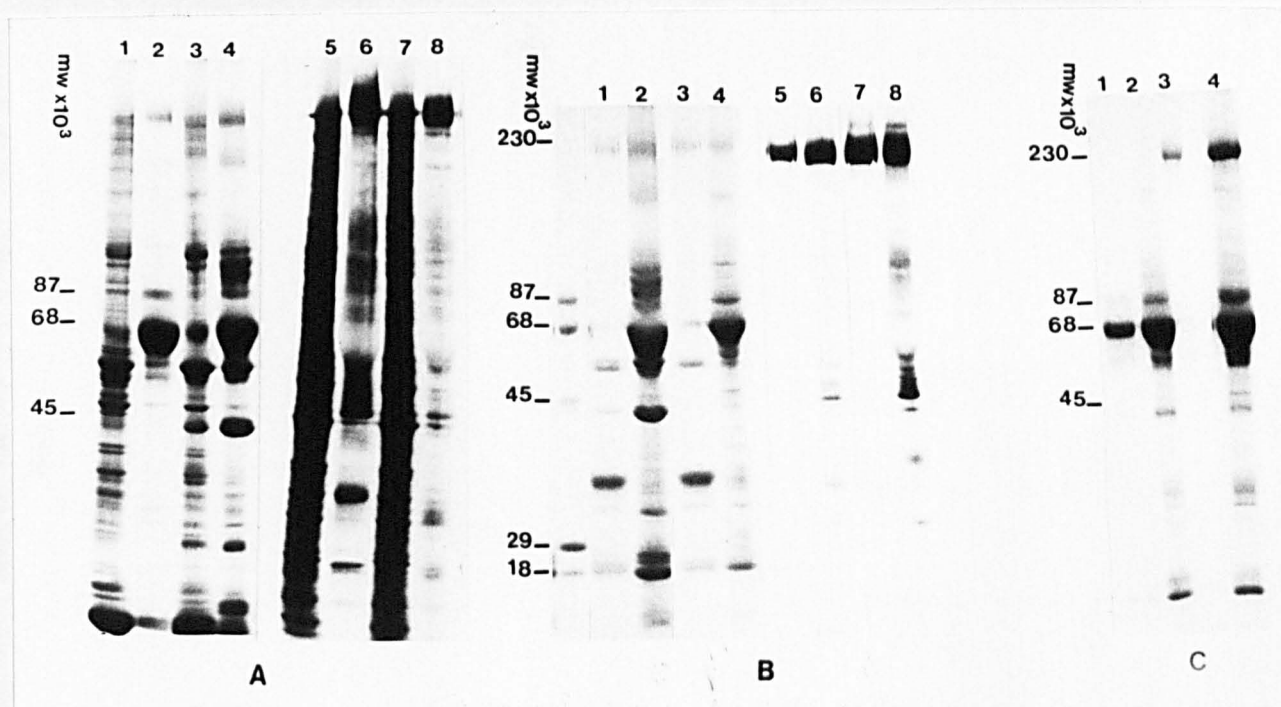


Figure 1. Analysis of ^{35}S -methionine labelled organ culture supernatants for Nitabuch's layer and the chorionic plate on 5-15% SDS polyacrylamide gels.

(A) Coomassie blue stained proteins (1-4) and the corresponding autoradiograph (5-8) of Nitabuch's layer cellular extract (track 1,5) and Nitabuch's layer culture supernatant (track 2,6); chorionic plate cellular extract (track 3,7) and its culture supernatant (track 4,8). The corresponding autoradiograph for the ^{35}S -methionine labelled proteins are shown in tracks 5,6,7 and 8 respectively.

(B) Anti-fibronectin Sepharose column used for the identification of fibronectin. Tracks 2 and 4 are the culture supernatant of Nitabuch's layer and the chorionic plate, respectively. Tracks 1 and 3 show the material eluted from the column (for details see text). The autoradiograph shows the labelled synthesized proteins in the starting material (tracks 6 and 8 respectively), and the eluted material (tracks 5 and 7 respectively).

(C) Gelatin-Sepharose column was used for further identification of FN. Track 4 shows Nitabuch's layer culture supernatant (the starting material); track 1 shows the unconjugated Sepharose material, track 2 shows the unbound material from the gelatin Sepharose column, track 3 shows the eluted material after binding to the gelatin Sepharose column.

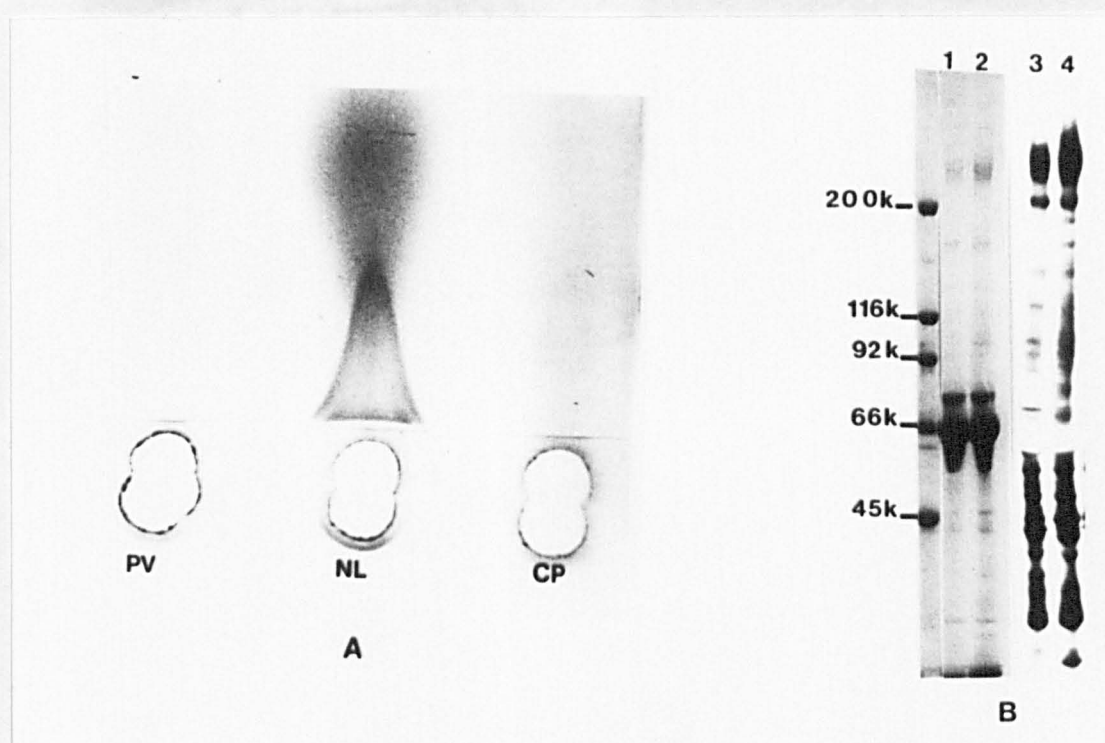


Figure 2. Studies on pregnancy associated plasma protein A (PAPP-A).
 (A) The autoradiography of the AACE analysis for the culture supernatants from the placental villi (PV), Nitabuch's layer (NL), and the chorionic plate (CP) using a 2% anti-PAPP-A bed.
 (B) Analysis for the culture supernatants of the chorionic plate and Nitabuch's layer (track 1,2 respectively) using 5-15% SDS polyacrylamide gel. Corresponding autoradiograph for the labelled polypeptides in both the chorionic plate and Nitabuch's layer are shown in tracks 3 and 4 respectively.

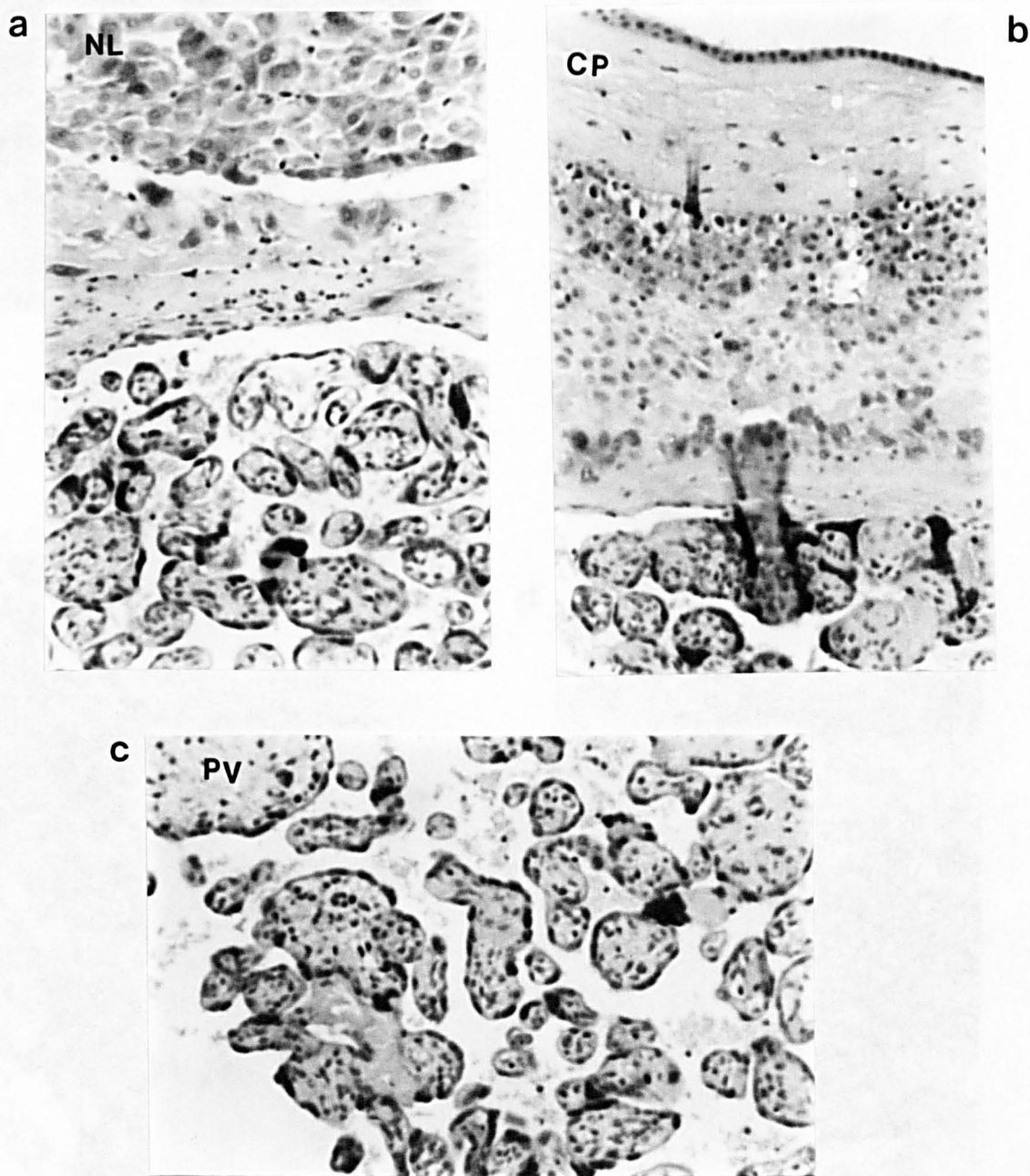


Figure 3. Wax embedded sections from term placentae. Indirect immunoperoxidase staining using normal sheep serum (negative control). (a) The uteroplacental interface (NL); (b) the chorionic plate (CP); (c) the placental villi (PV). Magnification X50.

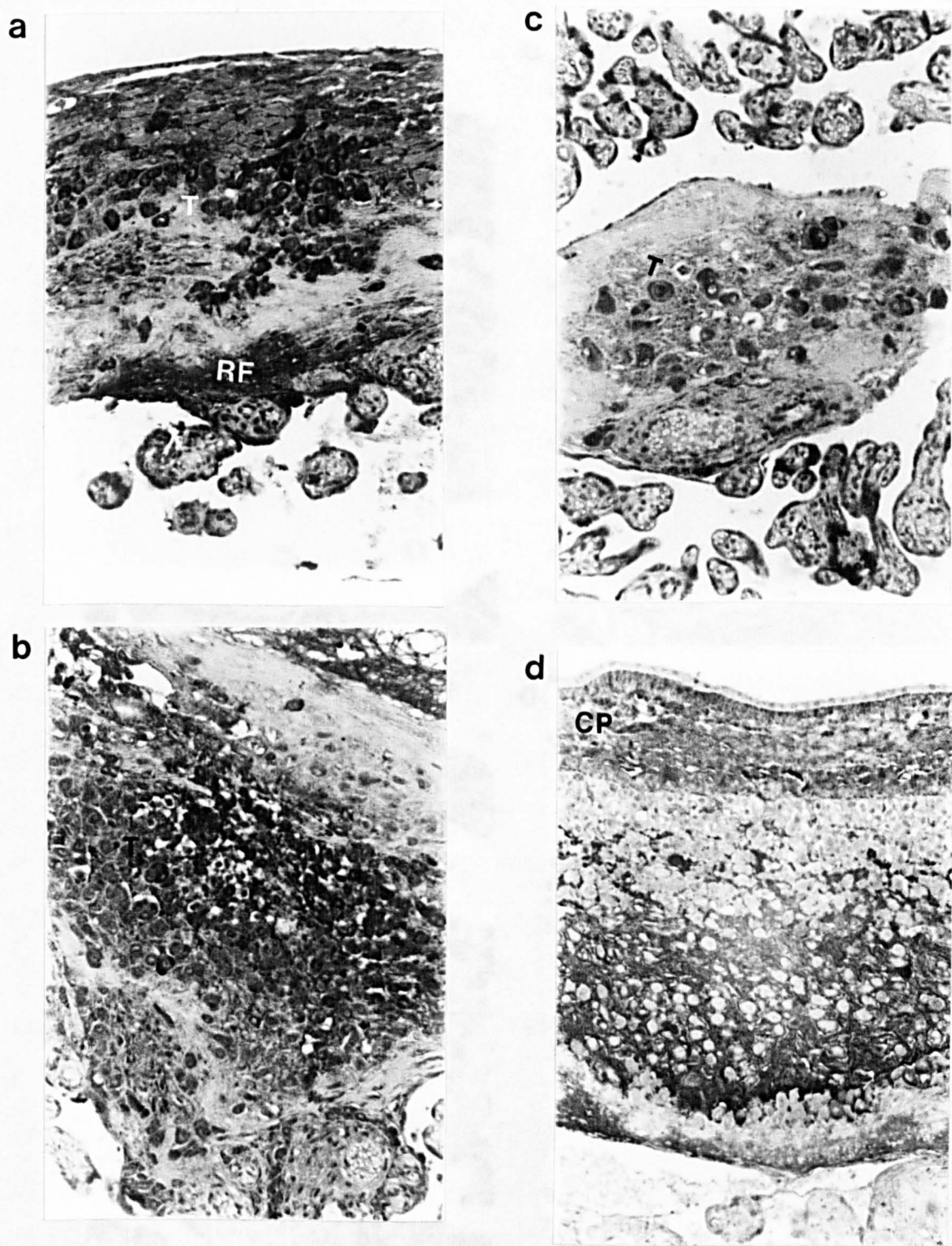


Figure 4. Wax-embedded term placental sections. Indirect immunoperoxidase staining using sheep anti-fibronectin. (a) The uteroplacental interface layer showing positively stained cells (T) and reticular fibres (RF); (b) placental septum showing positively stained cells (T); (c) cytotrophoblast cell islands with heavily stained cells (T); (d) the chorionic plate (CP). (Magnification X50).

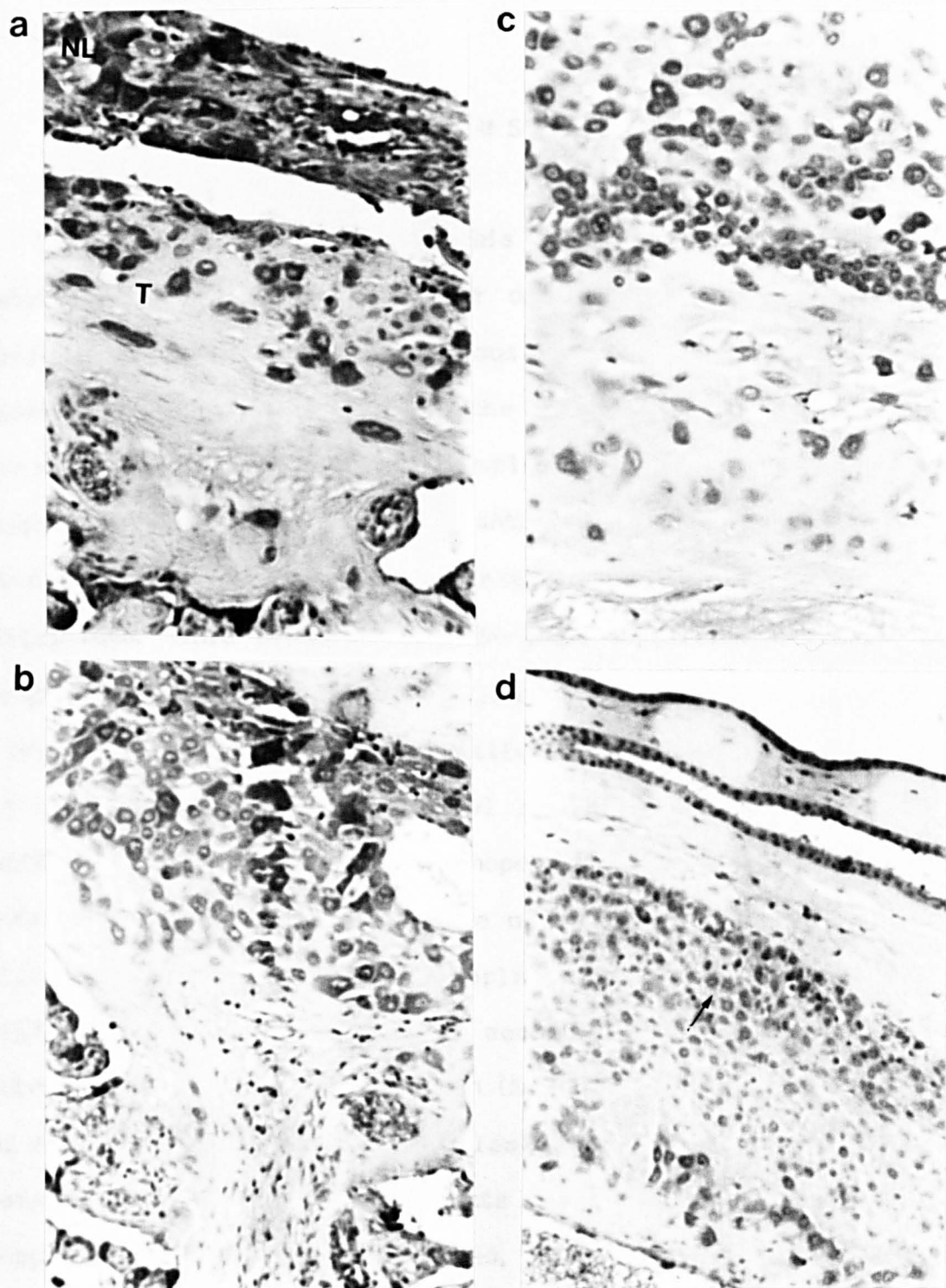


Figure 5. Wax-embedded sections from term placentae. Indirect immunoperoxidase staining using sheep anti-PAPP-A. (a) The uteroplacental interface (NL) with positively stained cells (T); (b) placental septum; (c) cytotrophoblastic cell island; (d) the chorionic plate. Magnification X50.

2:4 DISCUSSION

The observations presented in this chapter demonstrated that large numbers of proteins constitute major components of the uteroplacental fibrinoid (Nitabuch's layer), villous material and chorionic plate. Autoradiography results revealed the incorporation of ^{35}S -methionine into many proteins in the tissue samples and into a limited number of polypeptides in the culture supernatant. However, no incorporation was detected into bands of molecular weight corresponding to the fibrin polypeptides. This indicates that the local deposition of fibrin beta and gamma-gamma chains in the fibrinoid layer (Sutcliffe *et al*, 1982) is probably due to seepage and deposition of fibrinogen from the maternal circulation but not due to local synthesis of fibrinogen. In unpublished observations using immunoperoxidase staining, Sutcliffe and Horne failed to observe the presence of fibrinogen determinants inside cells in the vicinity of the uteroplacental fibrinoid. Whatever the origin of the fibrin, its local accumulation does imply a local activation of blood clotting factors (Sutcliffe, *et al*, 1982). Further, it was found that both human trophoblast and the uterus contain a pro-transglutaminase which cross-reacts antigenically with blood clotting factor XIII and which, when activated, polymerises fibrinogen alpha and gamma chains (Chung, 1972; Bohn, 1978).

A heavy incorporation of ^{35}S -methionine into the supernatant polypeptide of 230,000 molecular weight, was detected after 24-48 hr of culture. No comparable incorporation occurred into villous material in the first 24-48 hrs of culture, though it was detected in supernatants after 120 hr of culture. This 230k polypeptide had the properties of fibronectin. It was cold insoluble, being detected in the supernatant

only when the cultured tissue was incubated at 37°C; not after incubation at 4°C. The 230K polypeptide in the supernatants of the cultured tissue bound to gelatin and also to sepharose-conjugated antibodies against human plasma fibronectin. This is in accord with previous studies carried out on FNs (Engvall and Ruoslahti, 1977; Ruoslahti, et al 1982a). Such studies showed that fibronectins have an affinity for gelatin and bind to it via distinct domains. In addition, Isemura, et al (1984) who managed to isolate placental fibronectin from total placental tissue extract revealed that this isolated form when compared with the human plasma fibronectin was shown to have a higher molecular weight due to its slow migration on SDS gels.

Previously, PAPP-A protein has been demonstrated to be in the placental syncytiotrophoblastic cells (McIntyre et al , 1981). This study revealed the presence of a polypeptide with molecular weight corresponding to that of PAPP-A (187k), as shown in Fig (2B) . Immunoprecipitation experiments using Sepharose column with anti-PAPP-A antibodies did not reveal any bands corresponding to PAPP-A . Immunoprecipitation using the AACE method yielded a radioactive immunoprecipitin line (Fig.2A). However, the attempt to detect the molecular weight of that precipitated line using SDS gels failed ; no radiolabelled band could be detected. This is possibly due to low synthesis activity by the trophoblastic cells. From this it would become apparent that no ample evidence is provided to prove that PAPP-A is synthesized by the trophoblastic cells. If a PAPP-A polypeptide could be recovered from the gel, then the AACE result could be accepted as evidence for the synthesis of PAPP-A in the uteroplacental interface, placental villi and the chorionic plate.

Although it has been shown recently that in addition to fibroblastic

cells, endothelial as well as several types of epithelial cells do synthesize a tissue form FN (Ruoslahti, 1981). The immunoperoxidase localization results presented in this chapter, strongly suggest that this FN is a placental form which seems to be synthesized by a special type of placental cells which look morphologically different from the fibroblasts, endothelial, or epithelial cells.

The finding that collagenous and reticular fibres were stained for FN especially in the subepithelial layer in the chorionic plate is not a surprising phenomenon. Previous studies (Engvall, Ruoslahti and Miller, 1978; Rennard, et al, 1980; Engvall and Ruoslahti, 1977; and McDonald, et al, 1982), have shown that FN has a strong affinity for collagen fibres, thus it mediates the adhesion of cells as well as platelets to collagenous substrates.

So far, one could suggest that this FN is similar in its activities and functions to the other fibronectins present elsewhere in the body. For, from previous studies, it is well known that FN's function as an anchoring and adhesive glycoprotein material that mediates the adhesion and anchoring of cells to each other and to the extracellular matrix (Mosher, 1980; Ruoslahti, Engvall and Hayman, 1981; Rennard et al, 1980; Ruoslahti, Hayman and Engvall, 1981). On these bases, it seems reasonable to suggest that the presence of FN in those different regions of the placenta is in fact, to mediate the adhesion of cells to each other and to the extracellular matrix. In addition to this and according to previous data (Ruoslahti, Engvall and Hayman, 1981; Rennard, et al, 1980; and Mosher, 1980), FN has been shown to be incorporated into the clot in blood coagulation. This led to the proposal that the presence of FN in Nitabuch's layer, which is a heavy deposit of fibrinoid at the uteroplacental interface (Sutcliffe et al, 1982), might

be an important factor in providing a favourable attachment matrix for cells growing into the clot during the implantation process. Moreover, FN might be involved in promoting cell division and mobility for the invading trophoblastic cells towards the uterus endometrium. At the same time, FN might be involved in controlling the morphology of these cells, for it has been observed that FN is absent from the surface of many transformed cell lines and tumours. This activity of FN can be applied for placental septa, cell islands and the chorionic plate as well.

According to our results, staining for FN can be seen clearly in regions undergoing degeneration (Boyd and Hamilton, 1970). This would lead us to predict that this might be concerned with the opsonizing activity of FN (Ruoslahti, 1981; Yamada and Olden, 1978; and Mosher, 1980). Accordingly, FN might act as a nonspecific opsonin, designed to facilitate the uptake of tissue debris, and helps elimination of such harmful particles by exposing them to the reticuloendothelial system. Studies done by Rennard, et al (1980) have shown that cellular uptake of FN from the surroundings is influenced by cell shape and by the presence of collagen.

The localisation studies demonstrated a similar distribution for Pregnancy Associated Plasma Protein A (PAPP-A) and fibronectin. This might indicate that both proteins, PAPP-A and FN, might be synthesized by the same type of cell(s) for the contribution of fibrinoid formation. The significance of the presence of PAPP-A is not clear since previous studies claimed that PAPP-A can exert an antifibrinolytic activity in vitro by inhibiting both plasmin and its activator (urokinase) (Bischof, 1984). However, Gore and Sutcliffe (1984) using radiolabelled protein binding assays demonstrated that PAPP-A did not bind to plasmin

(or trypsin). They also pointed out that the protease-inhibitory activity in samples of PAPP-A could be due to the presence of small amounts of contaminating alpha-2-macroglobulin in Bischof's preparation. Alpha-2-macroglobulin is a major inhibitor of plasmin in blood. The inhibition of enzymes other than plasmin or trypsin by PAPP-A was not excluded (Gore and Sutcliffe, 1984). From this it could be argued that the presence of PAPP-A at the uteroplacental interface does not interfere with the turnover of fibrin, which is much more likely to be due to other proteins such as alpha-2-macroglobulin.

CHAPTER 3

CULTURE STUDIES ON ISOLATED PLACENTAL CELLS

3:1 INTRODUCTION

Previously, several attempts were carried out on early placentae for placental cell isolation. Low and Redman (1968), managed to isolate trophoblasts from 12-13 week old placentae obtained by hysterotomy from therapeutic abortions. Similar attempts were carried out on placentae at 11-12 days of pregnancy for the isolation of extra embryonic cells (Fager, Blank and Nebel, 1977; Yoder et al., 1978).

In this study, the previous results obtained from placental organ culture and immunohistochemical studies (Chapter 2), which were carried out on term placentae, encouraged me to isolate the stained cells in both the uteroplacental interface and the chorionic plate, by means of trypsinization, in order to study them and apply further studies including their morphology and ability to grow in vitro.

CHAPTER 3

CULTURE STUDIES ON ISOLATED PLACENTAL CELLS

3:1 INTRODUCTION

Previously, several attempts were carried out on early placentae for placental cell isolation. Loke and Borland (1970), managed to isolate trophoblasts from 12-13 week old placentae obtained by hysterotomy from therapeutic abortions. Similar attempts were carried out on placentae at 11-12 days of pregnancy for the isolation of mice trophoblastic cells (Toder, Blank and Nebel, 1977; Toder et al, 1983).

In this study, the previous results obtained from placental organ culture and immunohistochemical studies (Chapter 2), which were carried out on term placentae, encouraged me to isolate the stained cells in both the uteroplacental interface and the chorionic plate, by means of trypsinization, in order to characterize them and apply further studies including their morphology and ability to grow in vitro.

3:2 MATERIALS AND METHODS

3:2:1 Materials Culture media, fetal calf serum and culture supplements were obtained from Flow Laboratories (UK). Trypsin and versene were obtained from Virology Department in Glasgow. Serum fibronectin was obtained from BRL, Gelatin from Sigma Chemical Company. Human amniotic fluid from Yorkhill Hospital. Epidermal Growth Factor, 3-Aminobenzamide (3AB), and E-Aminocaproic Acid all were purchased from Sigma Chemical Company. ^{35}S -methionine was obtained from Amersham. Enhance, from New England Nuclear. Tris and SDS were obtained from Sigma Chemical Company. Percoll and Ficoll-Paque were obtained from Pharmacia Fine Chemicals. X-omat films were purchased from Eastman Kodak Company.

3:2:2 Methods

1. Trypsinization of Placental Tissue. Under sterile conditions throughout the trypsinization process, tissue pieces from both the chorionic plate and Nitabuch's layer were dissected carefully from freshly delivered placentae in order to avoid as much villous tissue and blood vessels as possible. The dissected tissue was transferred into a 250ml flask with 50-100ml complete media minus fetal calf serum (CM-FCS), and left at room temperature in the hood overnight. The next day, the tissue fragments were washed thoroughly 3 or 4 times with sterile PBS to remove blood excess. The tissue was then transferred into another flask with 50-60ml sterile trypsin to be shaken for one hour at room temperature. The supernatant from this first trypsinization (T1) was discarded, and the tissue was put in a sterile sieve over a 37°C waterbath for 5x15 min time periods, renewing the 80-100ml trypsin each time. These trypsinizations were termed T2/1-T2/5. The first

trypsinization supernatant (T2/1) was also discarded, whereas T2/2-T2/5 were filtered through sterile gauze and centrifuged at 1000 rpm for 5 mins using a bench centrifuge to remove the trypsin solution. The cellular pellets were resuspended in 4ml complete media plus fetal calf serum (CM+FCS) and stored at 4°C in order to inactivate any remaining trypsin.

The cellular pellets from T2/2-T2/5 were pooled and centrifuged out, washed once with CM+FCS and finally resuspended in 4ml CM+FCS. These suspended cells were carefully layered using a pasteur pipette onto a 30ml 15-52% percoll gradient then centrifuged at 1000 rpm for 20min. After centrifugation, 20mls of the suspended cells, which appeared through the percoll gradient as a broad band in the lower region of the gradient above the red blood cells pellet, were carefully removed using a pasteur pipette and dispersed into two tubes, each containing 25ml CM+FCS. After gentle mixing, the tubes were centrifuged at 1000 rpm for 20min to remove the remaining percoll. The cells were washed once more with CM+FCS and finally resuspended in 2ml CM+FCS.

2.Percoll Gradient Preparation. For making one percoll gradient , a stock solution of 7.5ml percoll was made by adding 6.75ml percoll to 0.75ml 10x(CM-FCS). Then 3.75ml of this solution were mixed with 21.25ml CM-FCS to make the 15% percoll solution, and 2.6ml were mixed with 2.4ml CM-FCS to make the 52% percoll solution. The 15% percoll was put first into a sterile 50ml blue-capped centrifuge tube, then very carefully the 52% percoll was pipetted beneath.

3.Ammonium Chloride Tris (ACT) Treatment. The ACT solution was prepared by the addition of 20.594g Tris base (Sigma) /100ml; pH 7.65 to 0.83g/100ml NH_4Cl (Sigma) at the ratio of 1 to 9.

To remove red blood cells in the cellular preparation, both the

isolated cells and 25ml ACT solution were warmed up to 37°C. This was followed by mixing the two and leaving them for 1min at the same temperature for red blood cell lysis. Then the cells were pelleted at 1000 rpm for 5min, washed in 10ml CM+FCS, spun down and finally resuspended in 4ml CM+FCS. The treatment with ACT was repeated in case of red blood cell lysis failure.

4. Cellular Count. 1% trypan blue solution was used for cellular count and viability assessment. 25ul trypan blue and 25ul of the suspended cells were added to 200ul of PBS. The solution was then left for 2-3min before applying onto a haemocytometer. Cellular viability was assessed by the dye exclusion; no blue staining should be observed inside the viable cells.

5. Placental Cell Culture. The freshly isolated placental cells were distributed at $2-3 \times 10^5$ cells per Linbro well in 0.5ml complete media (see Chapter 2 for media components). For ^{35}S -met experiments, met/5 culture media was used instead. The cells were left at 37°C in the incubator supplemented with 5% CO_2 . These cultured cells were left for about 2-3 weeks before becoming confluent, during which the media was changed every 4-5 days of culture. At this stage the cells were readily passaged by aspirating the culture media, washing the cells with versene, then adding 1ml trypsin : versene solution (4:1 ratio). The cells were put in the incubator for 2-3mins, then detached from the surface by means of gentle suction using a pasteur pipette. This is followed by the addition of CM+FCS for trypsin inactivation, centrifugation, and resuspension in 1ml CM+FCS before distributing them at $2-3 \times 10^5$ cells per well. After cell culture, the ^{35}S -met labelled supernatants were TCA precipitated (see Chapter 2) and then analysed using 5-15% SDS gels. These gels were incubated in Enhance for 1hr at

room temperature before being washed in water, dried and exposed to X-omat films.

6.Culture Substrates. Different culture substrates were used including human plasma clot and glass cover slips coated with 25ug/ml of either fibronectin or gelatin.

3:3 RESULTS

3:3:1 Cellular Viability. Cellular viability was assessed after trypsinization using the trypan blue exclusion method. As shown in Fig (1), the viability varied from one preparation to another; the maximum viability being 98%. This variation could be due to differences in the amount of tissue trypsinized and the trypsinization time.

3:3:2 Placental Cell Culture. The isolated placental cells were seeded on different substrates such as glass cover slips with or without fibronectin and/or collagen coat and human plasma clot to see the best substratum for them to grow on. The cells from either Nitabuch's layer or the chorionic plate seem to grow on any of these substrates. Nevertheless, Nitabuch's layer cells cultured on human plasma clot, started its digestion after 4-5 days of culture (Fig.2a), compared to the chorionic plate cells which digested the clot at a slower rate.

In addition, these isolated cells in culture showed a tendency to aggregate and thus to form a cellular clump, which once it attached itself to the substratum, cells from beneath started to extend and spread throughout the well until becoming confluent where they will be ready for passage (Fig.2b). The cultured cells were highly heterogeneous (Fig.2a); they showed variation in size, shape, and number of nuclei. Three main sizes were recognized; small, medium and

large. Some cells were highly vacuolated compared to others. These cultured cells were able to grow successfully for 2-4 months without stromal fibroblastic overgrowth.

3:3:3 Studies on the Incorporation of ^{35}S -Methionine into Cultured Placental Cells. Isolated placental cells were cultured on different substrates. The incorporation of ^{35}S -met into these cells was studied. Figure 3 shows the TCA precipitation counts during five days of culture. As can be seen, the highest incorporation for the placental cells grown on plastic alone was at day 3 which slightly decreased thereafter. The TCA counts for the cells grown on cover slips coated with fibronectin or gelatin as well as plasma clot showed continuous increase. However, the TCA counts for chorionic plate cells grown on plasma clot started to decrease after day 4. Moreover, chorionic plate cells cultured on cover slips coated with fibronectin or gelatin showed higher incorporation than those from Nitabuch's layer. On the other hand, Nitabuch's layer cells cultured on plasma clot showed higher incorporation than those from the chorionic plate. Analysis of the culture supernatants using SDS polyacrylamide gel electrophoresis, showed polypeptide patterns similar to those obtained from organ tissue culture (as shown in Chapter 2, Fig 1A). Figure 4 shows several ^{35}S -met labelled polypeptide bands from both chorionic plate and Nitabuch's layer cultured cells using the above mentioned substrates. One of the major polypeptides expected in these cultures has a molecular weight of 230K which corresponds to that of fibronectin.

3:3:4 Effect of Human Amniotic Fluid and Epidermal Growth Factor. To improve the culture conditions, human amniotic fluid and epidermal growth factor were used. The presence of human amniotic fluid in the culture media instead of fetal bovine serum did not cause a significant

change in cellular morphology and the cells failed to proliferate quickly. Nevertheless, the chorionic plate cells, in particular those cultured on plasma clot in the presence of human amniotic fluid, showed a relatively higher rate of clot digestion compared to those cultured in its absence. This could possibly be due to the cells naturally obtaining some benefit from the amniotic fluid, thus becoming stimulated to synthesize additional proteins or to increase the rate of protein synthesis. Such a result seems to be in accord with a previous study carried out by Toder et al (1983). In this study it was found that trophoblastic cells isolated from KR mice placentae at 11-12 days of pregnancy, when cultured in the presence of isolated alpha-fetoprotein or amniotic fluid in the media, showed an extensive increase in the number of tritiated thymidine labelled cells, indicating an increase of DNA synthesis. In addition, protein synthesis by these cells was also found to be about two to three times more. From this it was concluded that alpha-feto protein, being the main protein in the amniotic fluid, may be one of the fetal factors involved in promoting trophoblast differentiation. An SDS analysis for the ³⁵S-met labelled supernatants in the presence of human amniotic fluid showed the synthesis of mainly FN as well as other minor polypeptides (Figure 5, tracks 2 and 6).

The addition of 100ng/ml epidermal growth factor (EGF) to the cultured placental cells from both Nitabuch's layer and the chorionic plate did not reveal any significant change. This was consistent with previous study carried out on primary cultures of cells derived from human and bovine amniotic fluid (Gospodarowicz, Moran and Owashi, 1977).

3:3:5 Effect of 3-Aminobenzamide (3AB) and E Aminocaproic Acid (EACA).

The effect of 3AB and EACA on the cultured placental cells was also

studied. It was observed that the addition of 10mM 3AB to the cells from both Nitabuch's layer and the chorionic plate cultured on plasma clot showed an inhibition effect on clot digestion similar to that of E aminocaproic acid. This could be due to the possibility that 3AB has inhibited the cellular differentiation ,thus inhibiting them from synthesizing any proteolytic proteins. This observation is consistent with the finding of Farzaneh et al (1982) which was carried out on primary chick myoblasts in culture. In this study it was found that the addition of 3AB inhibits their fusion and differentiation but not their proliferation.

An SDS PAGE analysis for these culture supernatants in the presence of 3AB and EACA revealed several polypeptide bands indicating cellular synthetic activity (Figure 5, tracks 3,7 and 4,8 respectively).

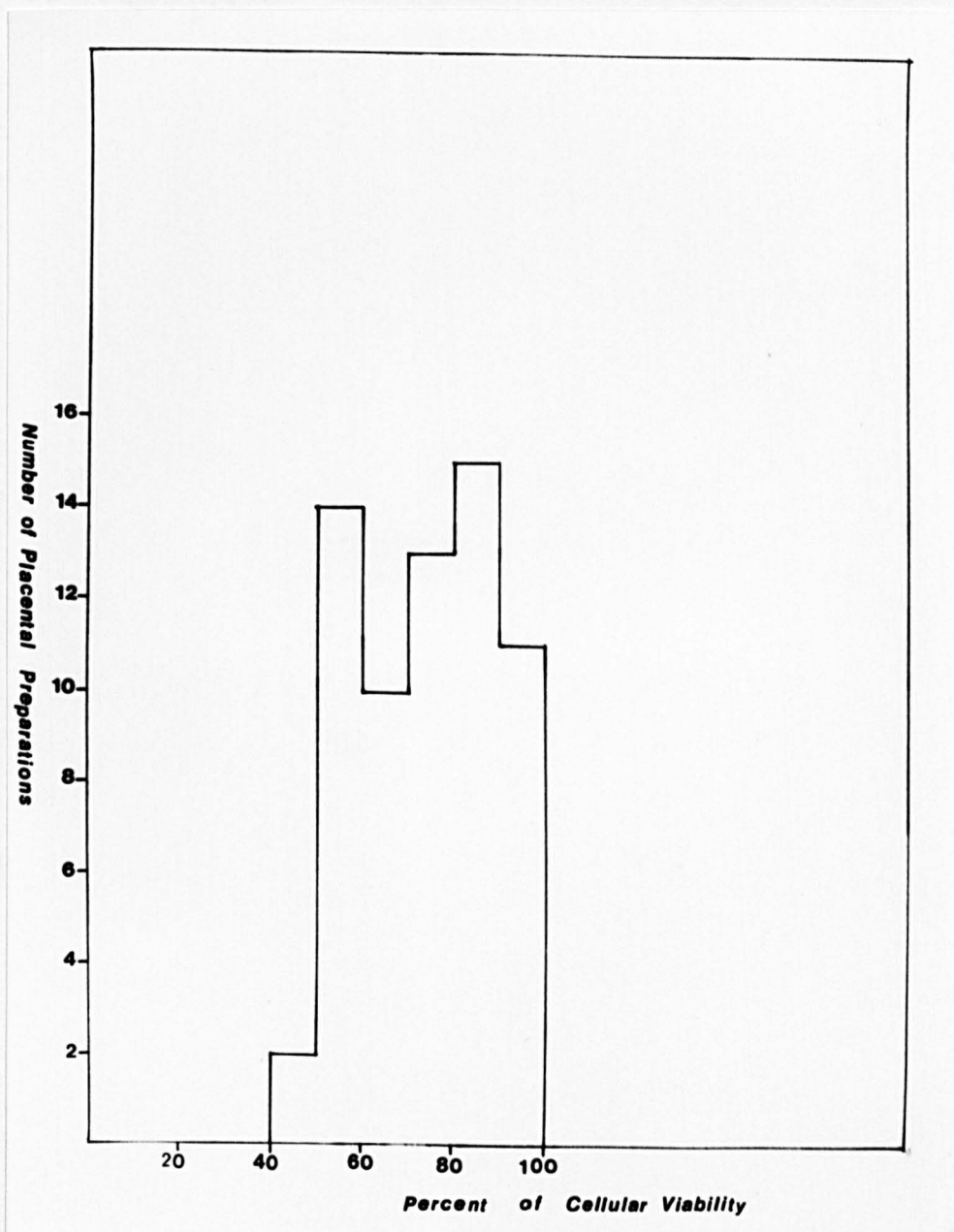


Figure 1. The percentage of viable placental cells after trypsinization as plotted against the number of placental preparations.

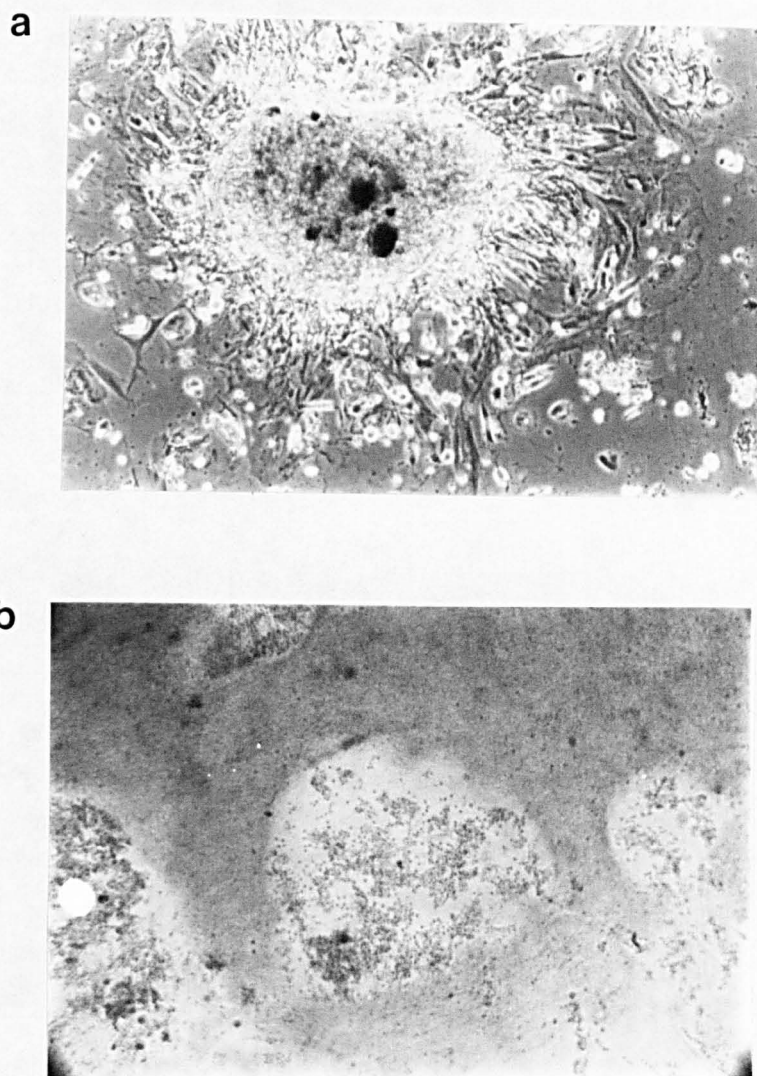


Figure 2. Isolated placental cells from Nitabuch's layer. (a) Cells grown on plastic showing cellular clump as well as other individual cells; (b) cells grown on human plasma clot; clot digestion by these cells is clear.

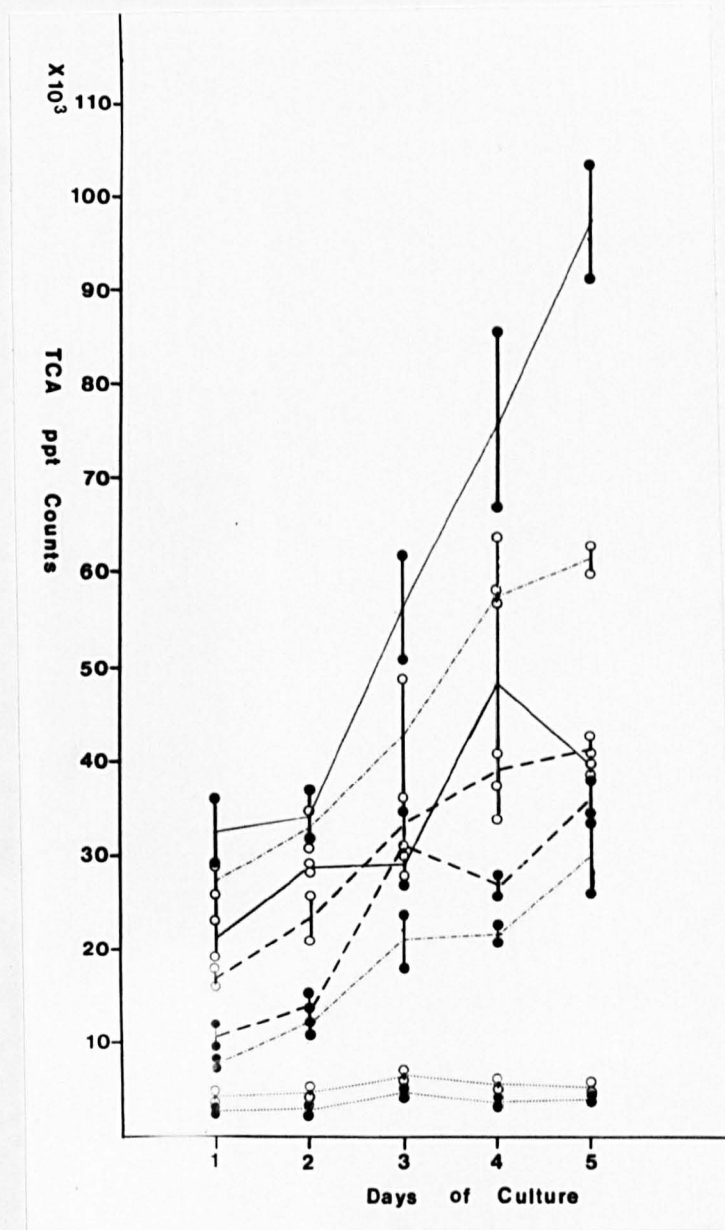


Figure 3. The effect of various culture substrates on the incorporation of ^{35}S -met into proteins. ML layer cells (●) and CP cells (○) were each set up in duplicate linbro wells. Culture substrates used are as follows: Plastic (.....), Gelatin (---), Fibronectin (----), and Plasma clot (—).

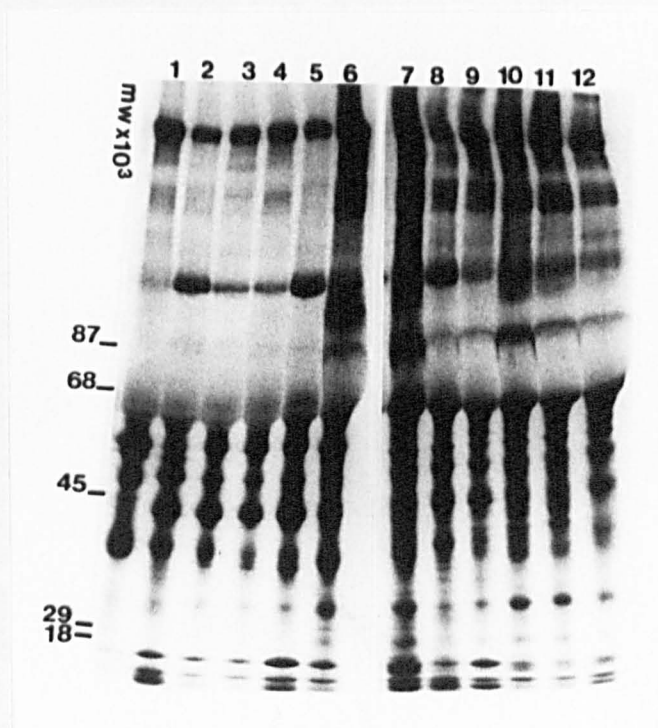


Figure 4. The autoradiograph of 5-15% SDS gel analysis for the culture supernatants of isolated placental cells from Nitabuch's layer and the chorionic plate after being cultured on different substrates. ^{35}S -methionine labelled proteins for cultured chorionic plate and Nitabuch's layer cells on, human plasma clot (tracks 1,2 and 7,8 respectively), fibronectin coated glass cover slips (tracks 3 and 9 respectively), gelatin coated glass cover slips (tracks 4 and 10 respectively), plastic alone (tracks 5 and 11 respectively), and glass cover slips alone (tracks 6 and 12 respectively).

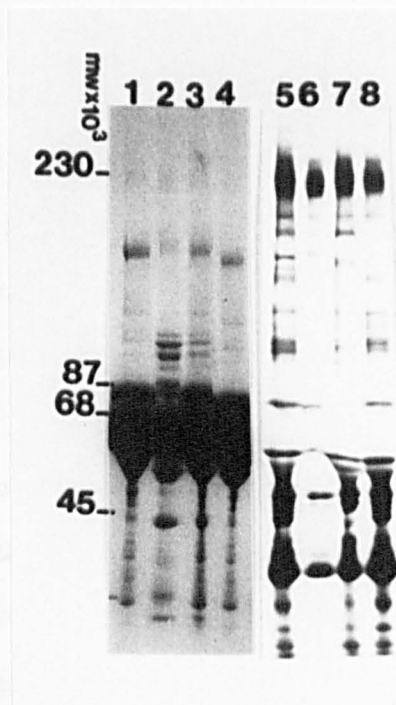


Figure 5. 5-15% SDS gel analysis for the culture supernatant of Nitabuch's layer cells after being cultured on human plasma clot in the presence of human amniotic fluid, 3AB and EACA. Coomassie blue stained proteins in the presence of ordinary complete media (track 1); human amniotic fluid instead of FCS (track 2); 3AB and EACA (tracks 3 and 4 respectively) . The corresponding autoradiograph for the 35 S-methionine labelled proteins are shown in tracks 5-8 respectively.

3:4 D I S C U S S I O N

It has been demonstrated in the results section that the isolated placental cells from both the basal and the chorionic plates showed cell populations heterogeneous for size and number of nuclei. The viability of these cells as shown varied from 45-98%. As mentioned earlier, these cells seem to be able to grow on different substrates. However, they showed higher TCA precipitation counts when grown on plastic cover slips coated with fibronectin or gelatin as well as on human plasma clot. It might be concluded that these cells prefer to grow on a substance which is relatively similar to their natural environment, nevertheless, there was no difference in the protein pattern synthesized by them when cultured in the presence or absence of different substrates.

In culture, most of these cells stay in suspension in the first 24-48 hrs; subsequently, they tend to attach to the substratum until becoming confluent ready for passage. As noted above, these cultured cells showed variation in morphology; mononucleate, binucleate and multinucleate cells were observed. These results are consistent with the findings of others. Aladjem and Lueck (1981), Toder *et al.*, (1983) described four main morphologically distinct cell types. These consist of polygonal cells (predominant type, 60-70%), mono- or multinucleated giant cells, small round cells and fibroblastic cells. Contractor, Routledge and Sooranna (1984), described the presence of three main cell types. These were found to be epithelial-, macrophage- and fibroblast-like cells. The epithelial-like cells were divided into the multinucleated and the small- and medium-sized round cells, and these were most likely to be

derived from the trophoblast, since they were stained with the monoclonal antibody anti-cytokeratin (LE61). Others described the presence of an intermediate cell type (Thiede, 1960). However, others described three distinct types of cells; spindle, epithelioid and multinucleated giant cells (Thiede, 1960; Chung, et al 1969 ; Lueck and Aladjem, 1980). These cells were able to grow successfully without stromal overgrowth for 2-4 months with passaging when necessary. Incubation with the amniotic fluid and the epidermal growth factor (EGF), did not stimulate cellular proliferation, although EGF is known to bind to choriocarcinoma cells and promote human chorionic gonadotrophin secretion (Benveniste et al ,1978). Moreover, it also binds the villous syncytiotrophoblast, possibly to provide a route for supplying underlying cytotrophoblast with differentiation signals ; a process that could be important during the early development of the placenta and generation of syncytiotrophoblast from cytotrophoblast (Richards et al, 1983). In addition, these cells showed a high tendency to form cellular clumps. This could be due to cellular secretion of a particular polypeptide, fibronectin, in order to protect themselves from any humoral or cellular attack and perhaps to act as an opsonin for the removal of any cellular or tissue debris.

Cell Culture on Fibronectin or Gelatin Coated Glass Cover Slips. It has long been appreciated that most adherent eukarotic cells *in vivo* are supported by extracellular matrices. Such adherence is required for cell replication, differentiation, migration, morphogenesis and disease. These matrices are composed of collagens, glycosaminoglycans, proteoglycans, glycoproteins such as fibronectin, and other minor components (Klienman, Klebe and Martin, 1981; Grinned, Head and Hoffpauir, 1982; Hsieh and Chen , 1983). Accordingly, gelatin and

fibronectin coated glass cover slips were used as culture substrates for the placental cells. As mentioned in the results section, no difference was observed in the morphology and alignment of the placental cells compared to those cultured on glass or plastic alone. Although these cells were isolated from tissue that showed extensive synthesis of fibronectin as noted in chapter 2, the presence of these proteins in the surrounding of these cells in vivo might not be only for attachment and adhesion but also for protection from maternal cells by being involved in the reticuloendothelial system for clearing away any tissue debris by means of opsonization (Yamada and Olden, 1978; Ruoslahti et al, 1982a).

Human plasma clot as a substrate for placental cell culture. The ability of trophoblasts to produce proteolytic enzymes has been studied previously (Strickland et al, 1976; Kubo et al, 1982; Glass et al, 1983). It was found that the production of such enzymes by placental cells would be a logical mechanism for implantation. Among these proteolytic enzymes was plasminogen activator, which has been identified in the postimplantation mouse embryo (Strickland et al, 1976; Kubo et al, 1982), and in cultured human trophoblastic cells prepared from 8-12 week old placentae (Martin and Arias, 1982). Our results are consistent with the previous studies; trophoblastic cells from the uteroplacental interface showed clear plasma clot digestion after 4-5 days of culture. This might be due to the fact that the components of such substratum are similar to those of the fibrinoid at the uteroplacental interface (Sutcliffe et al, 1982). Thus, the former cells which could be of the endovascular type might be provoked to secrete proteolytic enzymes mimicking the preparation for the implantation process. On the contrary, the cells from the chorionic plate showed less proteolytic activity,

probably due to differences in protein synthesis according to cellular position in the tissue (Boyd and Hamilton,1970).

The addition of E-aminocaproic acid (EACA) and 3-aminobenzamide (3AB) which has been shown to inhibit differentiation and cellular fusion (Farzaneh et al,1982) revealed failure of placental cells to digest the plasma clot. These results are comparable with previous studies (Dubin et al,1980; Kubo ,Spindle and Pedersen ,1981) where it was found that EACA exerts an effect on attachment and outgrowth in culture. These findings might suggest that the addition of 3AB seems to prevent the cells from further differentiation, thus inhibiting them from synthesizing different proteins including plasminogen activator (PA) which subsequently leads to clot digestion. The addition of 0.2M EACA to these cells seems to inhibit the digestion due to its binding to plasminogen activator (Deutsch and Mertz,1970; Quigley, Ossowski and Reich,1974), thus preventing its activation to plasmin. The production of PA by trophoblastic cells as mentioned in previous studies (Sheppard and Bonnar, 1978; Martin and Arias, 1982; Glass et al ,1983) seems to play an important role in placental implantation which is known to be an invasive process limited by time and space. Glass and colleagues (1983), suggested that the entry of trophoplast into the endometrium occurs by displacing the cells rather than destroying them which could in part explain the manner in which trophoblast passes through the epithelial layer in the uterus. It is also possible that these cells would use or synthesize fibronectin (Chapter 2) which would help the cells to stay together and to anchor them to the extracellular matrix. Once reaching the utero-placental spiral arteries it would gradually diminish their occlusion and replacement of the endothelial cells by an initial lining of endovascular cytotrophoblastic cells (Sheppard and Bonnar,1978). The

action of PA might be local in these arteries so that no clot formation would take place in order to provide enough blood supply to the fetus. The deposition of fibrin in the media of decidual spiral arteries (Sheppard and Bonnar, 1978) might take place in order to provide scaffolding to which the penetrating trophoblasts attach. In addition, it could be possible that at the end of pregnancy, this deposition of fibrin and fibronectin being one of the major substrates for factor XIII might help in blood coagulation at the parturition time. Moreover, the binding of fibronectin to fibrin might enhance phagocytosis of cellular debris.

CHAPTER 4

CHARACTERIZATION OF THE PLACENTAL CELLS IN THE INTERPLACENTAL INTERFACE AND THE CHORIONIC PLATE

CHAPTER 4

CHARACTERIZATION OF THE PLACENTAL CELLS IN THE UTEROPLACENTAL INTERFACE AND THE CHORIONIC PLATE

4:1 INTRODUCTION

From the previous chapter, it was shown that the isolated placental cells were highly heterogeneous, with different sizes and morphologies. It became necessary to characterize and identify these cells to check the degree of contamination with other cell types such as endothelial cells, leucocytes, and fibroblasts.

Accordingly, immunohistochemical staining studies were carried out on cytospin preparations and cryostat sections from both the uteroplacental interface and the chorionic plate to identify and quantify the cell types in these preparations. Different antibodies against the intermediate filaments (cytokeratin), the placental proteins (HCG alpha and beta subunits, hPL, SP1, PAPP-A and FN), and other trophoblast antigenic determinants (H315 and H316) were used. In addition, the distribution of the MHC antigens (class I and class II) was studied using the monomorphic monoclonal antibody W6/32 and the monoclonal antibody DA6.231.

4:2 MATERIALS AND METHODS

4:2:1 Materials Frozen placental sections were prepared in the Royal Infirmary Hospital in Glasgow. Paraffin-wax embedded early placental sections were obtained from the Pathology Department, Glasgow University. The cytospin preparations were prepared using a Shandon Cytospin 2 centrifuge. Xylene was obtained from Seelze-Hannover Ltd. Haematoxylin from BDH Chemicals Ltd. H_2O_2 was obtained from FISONS. Normal swine serum, swine anti-rabbit immunoglobulins, peroxidase-antiperoxidase (soluble complex of horseradish peroxidase and rabbit anti-horseradish peroxidase), goat anti-mouse IgG (H and L), peroxidase conjugate was obtained from P-L Biochemicals, Inc. Rabbit anti-mouse immunoglobulins, rabbit anti-PAPP-A, rabbit anti-hPL, rabbit anti-SP1, rabbit anti-B₂M and mouse anti-LCA were all purchased from DAKO (Denmark). Rabbit anti-FN was obtained from BRL Laboratories Inc. INN-hCG-17 and INN-hCG-2 were obtained from Serotec Ltd (England). W6/32 and 19/9 were obtained from Sera-lab (Sussex-England). Lectin and alpha-L-fucose were purchased from Sigma Chemical Company. Rabbit anti-UEA I lectin was obtained from E.Y. Laboratories Inc. (USA). PKK1 and PK-V were obtained from Labsystems (Finland). TR1 was generously obtained from Dr. Christopher Sunderland, Bristol. H315 and H316 were generously obtained from Dr. Peter Johnson, Liverpool. 3-Diaminobenzidine was obtained from Sigma Chemical Company, haematoxylin was obtained from BDH Chemicals Ltd. DA6.231 was obtained from Dr. Guy, MRC Clinical Population and Cytogenetics Unit, Western General Hospital, Edinburgh.

4:2:2 Methods

1.Cytospin preparations. Freshly prepared placental cells from both Nitabuch's layer and the chorionic plate were suspended in complete media minus fetal calf serum (CM-FCS). After counting the total number of cells, an appropriate number of cells was diluted with CM-FCS to a final concentration of $2-4 \times 10^4$ cell per 0.3ml which was applied per slide. Before use the slides were wiped with absolute alcohol then labelled at one end using a diamond pencil. White filter paper was applied on top and both were inserted into a slide clip, ready to be placed in the centrifuge head.

Using a Cytospin 2 centrifuge (Shandon), the suspended cells were put into the centrifuge chambers using a 1ml syringe. At the end of this stage, the centrifuge was set up at 1200rpm for 5min. The slides thereafter were removed carefully, left to dry, then fixed in acetone for 10 sec before being kept in the slide box until use.

2.Immunoperoxidase staining. This was carried out on cytospin preparations and cryostat sections from both Nitabuch's layer and the chorionic plate. The cryostat sections were left at room temperature for 10 min, followed by acetone fixation for 2-4 min. The cytospin endogenous peroxidase was abolished using 3% H_2O_2 in H_2O for 10 min at room temperature. The sections and cytospins were washed briefly in TBS and incubated with 1/5 NSS for 30 min at room temperature, followed by incubation with monoclonal antibodies (100ul) at the following dilutions in TBS; PAPP-A, FN, hPL, SP1 1/100; hCG alpha and beta subunits 1/50; PKK1, TR1 1/40; 7A11 1/40; H315, H316 1/50; W6/32, B₂M, 19/9, DA6.231 1/40; PK-V 1/50; anti-LCA 1/100. This was detected after washing by incubation with rabbit anti-mouse antibodies 1/100 followed by the antibody series described in Chapter 2. The detection of the first antibody was also carried out by a second incubation with peroxidase-

conjugated goat anti-mouse immunoglobulin. In both methods the stain developed using diaminobenzidine/H₂O₂ as mentioned in Chapter 2. Tissues and cytopins were counterstained with haematoxylin and mounted in DPX.

The staining procedure for anti-UEA I lectin was as follows. Tissue sections were incubated with 1/5 NSS for 30min, followed by incubation with UEA lectin 30mg/ml for another 30 min. The lectin excess was removed and rabbit anti-lectin was applied for 40 min at room temperature. After washing with TBS, the sections were incubated with swine anti-rabbit 1/100 and the rest of the procedure was followed as described in Chapter 2. The staining was abolished completely when UEA I lectin was incubated with 0.2M alpha-L-fucose for 30 min before application.

Negative controls were carried out in every experiment. These include normal mouse serum (NMS), normal rabbit serum (NRS), adsorption with pure antigen (for FN and lectin) and exclusion of the first antibody.

4:3 RESULTS

4:3:1 Localization studies on sections from the basal and the chorionic plates. Using the immunoperoxidase staining technique, different antigenic determinants were studied and localized on cryostat placental sections from both the uteroplacental interface and the chorionic plate. These determinants include intermediate filaments, placental proteins, trophoblastic markers and markers for other cell types such as endothelial cells, fibroblasts and leucocytes. The expression of MHC antigens on the surface of these trophoblasts was also considered.

Negative controls for the frozen sections and the wax embedded sections are shown in Figure 1 .

1.Intermediate filaments localization. In this study trophoblastic cells were identified by their content of intermediate filaments. Two mouse monoclonal antibodies (PKK1 and TR1) against cytokeratin were used. Both monoclonal antibodies showed similar results. Positive staining was detected in the chorionic plate cryostat sections; strongly stained cells were observed, some were detected around blood vessels. The same was observed in placental wax embedded sections in which staining for the trophoblastic cells in the chorionic plate was discernable (Figure 2A a,b). Both frozen and wax-embedded placental sections showed similar results in the uteroplacental interface layer. In addition, marked staining for the villous syncytiotrophoblastic layer was observed (Figure 2A c). However, no detectable staining was displayed by the villous stroma.

Clear villous cytotrophoblast staining was detected in wax-embedded sections of early placentae. Staining was also observed in the endometrial glandular epithelium (Figure 2B).

2.Placental proteins

Human Chorionic Gonadotrophin Alpha and Beta subunits. Cells in both the uteroplacental interface and the chorionic plate revealed positive staining for the alpha subunit of placental hCG. The villous stroma and syncytiotrophoblasts were also positive (Figure 3). The staining for beta subunit was less intense and the number of stained cells was also less in both regions (Figure 4).

3.Trophoblast markers.

The murine monoclonal antibodies H315 and H316 were applied on frozen sections from both the uteroplacental interface and the chorionic

plate. The two mAbs stained villous syncytiotrophoblasts; however, no staining was observed in the villous stroma (Figure 5). A larger subpopulation of the chorionic plate cells were stained with H316 than with H315. Only H316 reacted with the uteroplacental interface cytotrophoblasts and endovascular cells (Figure 5a).

4.The hybridoma antibody 7A11

This hybridoma antibody was prepared in our laboratory after the injection of isolated chorionic plate trophoblasts into mice. Positive staining for the trophoblastic cells was observed in the chorionic plate; the staining was prominent at the edges of the cells with faint cytoplasmic staining. This was also observed on paraffin wax-embedded chorionic plate sections (Figure 6a). One of the characteristic features of this staining is the staining for the amnion epithelial cells. Furthermore, staining was detected with precision in the syncytiotrophoblastic layer, the intensity of which seems more at the basal membrane as shown in Figure 6b. The staining pattern is very similar to that for anti-cytokeratin (PKK1).

Using early placentae, this Ab reacted with the villous cytotrophoblasts, cytotrophoblast cell islands and columns (Figure 6c). In addition, clear staining was also observed in the columnar epithelium of the endometrial glands as well as scattered cells in that uterine tissue (Figure 6d).

5.Other cellular markers

Vimentin , Leucocyte Common Antigen and UEA I Lectin. No staining was observed for leucocytes, fibroblasts and endothelial cells when frozen sections from the chorionic plate and the uteroplacental interface were used. Positive staining was displayed only in the endothelial cell lining of the blood vessels when anti-UEA I lectin was applied on wax

embedded sections (Figure 7a). The staining was completely abolished when rabbit anti-UEA I lectin was complexed with 0.2M alpha-L-fucose (Figure 7b). No staining for vimentin was detected in both the chorionic plate and the uteroplacental interface (Figure 7c), however, weak staining was observed in the villous stroma (Figure 7d).

6. Localization of MHC antigens

a. HLA-Class I and B₂M. The non-villous trophoblastic cells in both the uteroplacental interface and the chorionic plate showed positive staining for these antigenic determinants. The monoclonal antibody W6/32 was used for the detection of the HLA class I monomorphic determinants. HLA class I staining was comparable to that for B₂M. No discernable staining was detected in the villous syncytiotrophoblasts. However, positive reaction was observed in the villous stroma for both antigens (Figure 8).

b. HLA-Class II. Trophoblastic cells in Nitabuch's layer and the chorionic plate did not display any positive staining. The villous syncytiotrophoblast were also negative. Occasional cells within the villous stroma and the subepithelial layer in the chorionic plate. In addition, a few spindle-shaped cells at the uteroplacental interface were positive. The morphology and the size of these cells were different from those for the cytotrophoblastic cells (Figure 9).

4:3:3 Localization studies on the isolated placental cells. Cytospin preparations proved to be essential for the identification of the highly heterogeneous isolated placental cells from either Nitabuch's layer or the chorionic plate. Using peripheral blood lymphocytes as a control, the HLA detection by the immunoperoxidase staining method failed when the endogenous peroxidase was blocked with 3% H₂O₂ and methanol. However, positive staining for these antigens was achieved when PBS or

H₂O were used instead of methanol which seems to either mask the antigenic determinants or alter their structure (Figure 10).

Accordingly, different antibodies were applied on the placental cells from both Nitabuch's layer and the chorionic plate. Table (1) shows the percentage of stained cells. A substantial number of these cells display positive staining for the intermediate filament markers TR1 and PKK1 (markers for the epithelial cells and cells of epithelial origin) (Figure 11a). In addition, a high percentage of these cells stained for the placental proteins SP1, PAPP-A, FN and hPL (Figure 14 a,b,c and Figure 13c respectively). The percentage of stained cells for the alpha and beta subunits of hCG was less. Moreover, the cells stained for beta subunit were also less than those stained for alpha subunit as shown (Figure 13). Besides this, the cells stained clearly with H316 and H315 (Figure 12 c,d). The percentage of the stained chorionic plate cells with H316 (36-58%) was higher than that with H315 (17-25%). Furthermore, positive staining was also detected for HLA class I antigens and B₂M (fig. 11 c,d)). The stained cells for HLA class II using 19/9 and DA6.231 mAbs were few. These cells look different from the trophoblasts; they were smaller in size. Further, they look larger than lymphocytes (Figure 12 a). In Nitabuch's layer 11-28% of the cells were positive for this antigen, whereas 6-21% were stained in the chorionic plate using DA6.231. Similar results were obtained with 19/9 mAb.

These isolated placental cells were studied for other cellular contamination, such as fibroblasts, leucocytes and endothelial cells. Only a small proportion of the cells reacted with PK-V, anti-LCA and anti-UEA I lectin. The stained cells look different in size and morphology from the trophoblastic cells (Figure 15).

Negative controls were set up with every experiment. They included NRS, NMS as the first antibodies, exclusion of the first antibody or antibody adsorption when possible (Figure 10).

Placentae	A		B		C		D	E	F	G
Antibodies	NL	CP	NL	CP	NL	CP	CP	CP	CP	CP
TRI	55	57	60	59	79	80	-	-	-	-
PKKI	62	68	55	69	75	86	61	65	83	70
anti-vimentin (PK-V)	-	4	-	6	-	11	4	9	10	8
anti-LCA	-	1	-	3	-	4	4	7	5	7
anti-Lectin	7	10	8	8	14	12	2	10	11	6
W6/32 (class I)	57	50	58	62	85	79	58	84	89	76
B ₂ M	55	59	69	70	79	75	53	60	75	70
DA6.231 (class II)	17	15	28	21	11	5	-	-	-	-
INN-hCG-17 (anti-alpha-hCG)	25	19	23	25	30	23	13	10	22	23
INN-hCG-2 (anti-beta-hCG)	18	12	10	13	6	9	2	9	10	11
anti-FN	50	50	64	69	73	78	-	-	-	-
anti-PAPP-A	54	60	62	70	69	73	-	-	-	-
anti-SP1	30	42	47	51	50	58	-	-	-	-
anti-HPL	43	39	50	52	53	56	-	-	-	-
H316	-	36	-	49	-	58	-	-	-	-
H315	-	17	-	19	-	25	-	-	-	-
7A11	-	-	-	-	-	-	55	60	62	73

Table1. % of Stained Trophoblastic Cells for different specificities after isolation from Nitabuch's layer and the chorionic plate. For more details see appendix table 1.

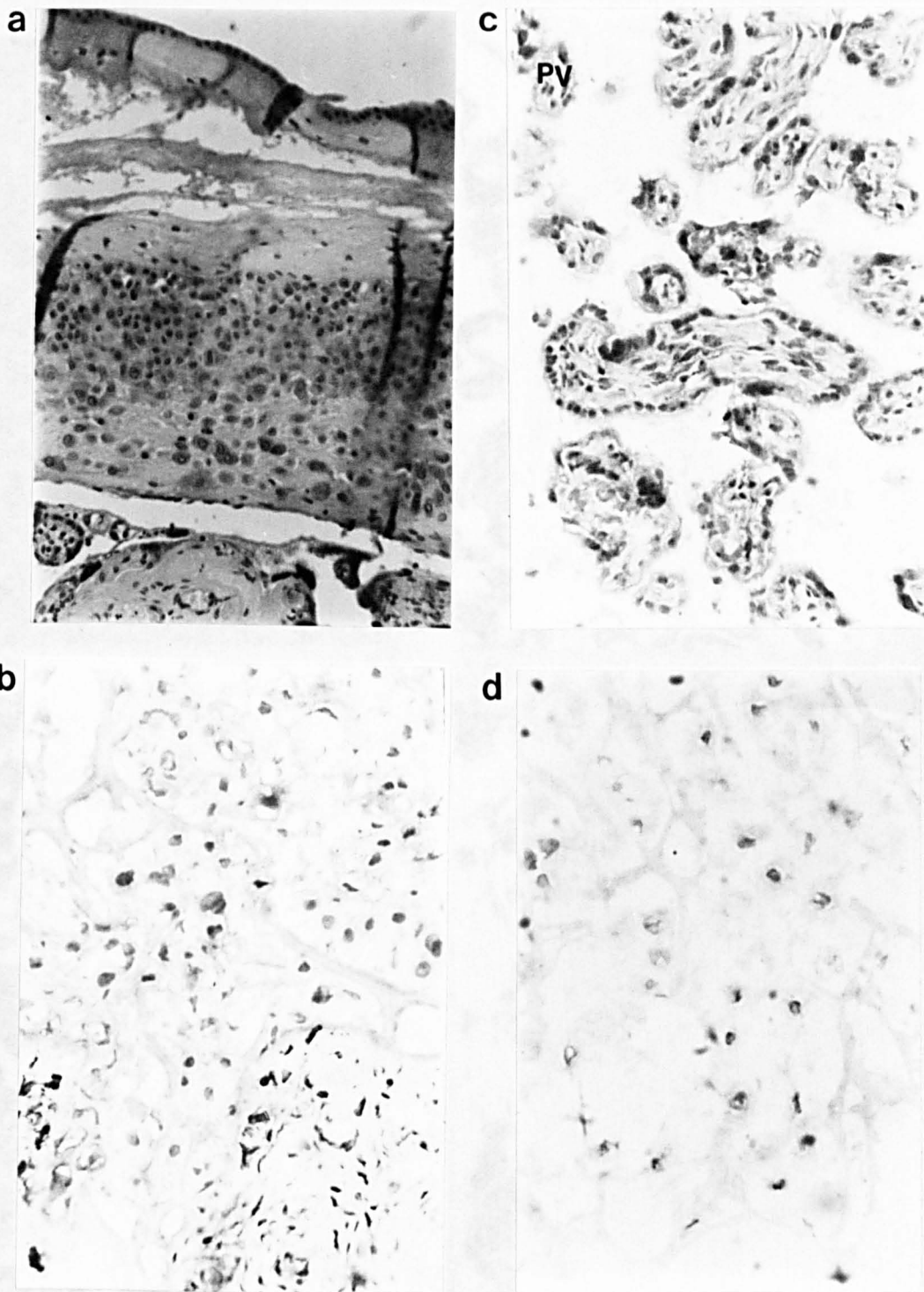


Figure 1. Human term placenta. (a) Wax embedded section of the chorionic plate stained with Haematoxylin and Eosin (magnification X50); (b), (c) and (d) frozen sections of the chorionic plate, placental villi and the uteroplacental interface respectively, stained using the indirect immunoperoxidase method excluding the first antibody. (Magnification X80).

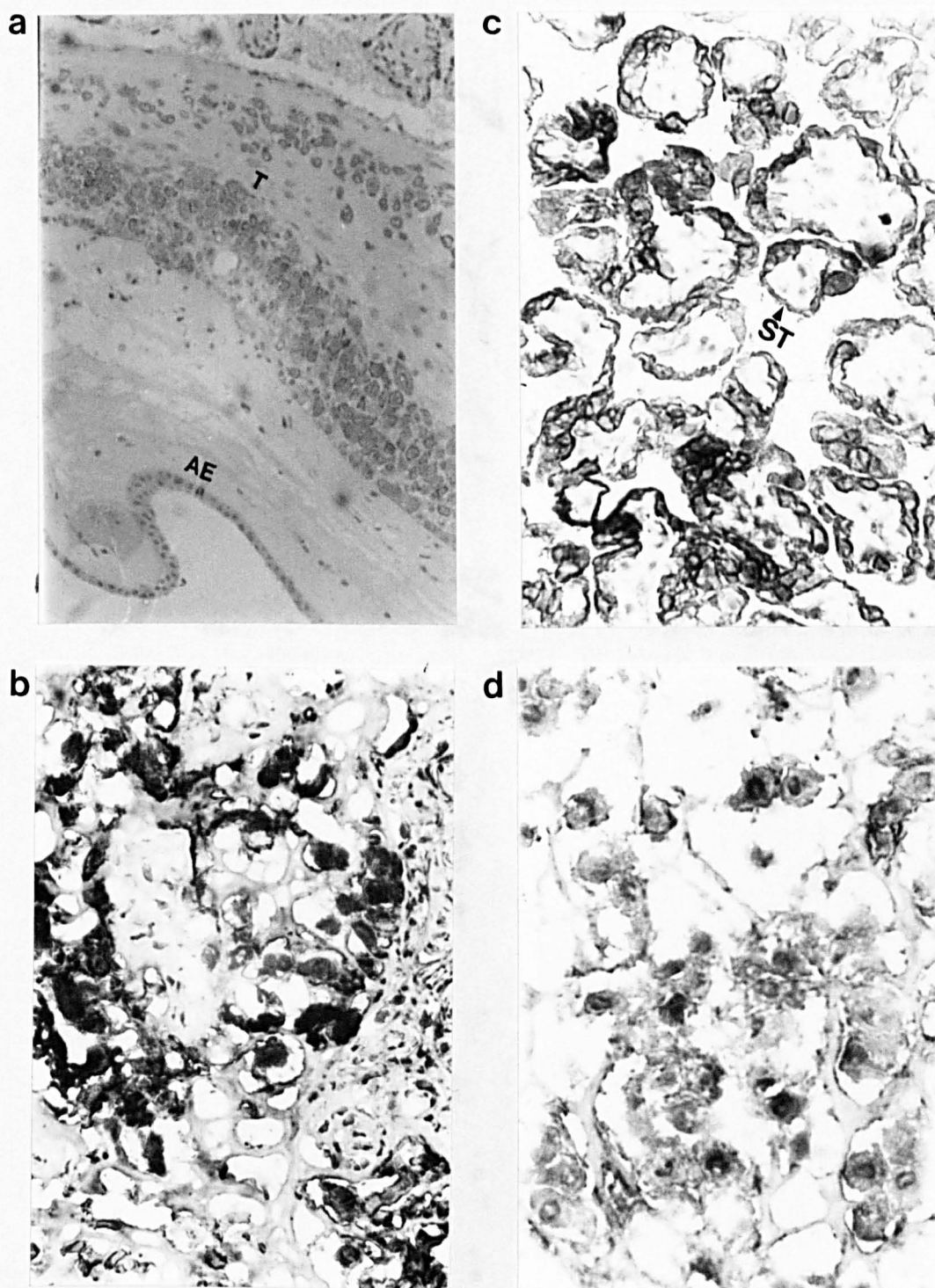


Figure 2A. Term placental tissue. Indirect immunoperoxidase staining using the monoclonal antibody mouse anti-cytokeratin PKK1. (a) Wax embedded section for the chorionic plate showing positively stained cells (T) with the amniotic epithelium (AE) displaying no reactivity (magnification X50) ; (b) frozen section for the chorionic plate (magnification X80) ; (c) the placental villi showing positively stained syncytiotrophoblasts (ST) (magnification X100) ; (d) the uteroplacental interface (magnification X100).

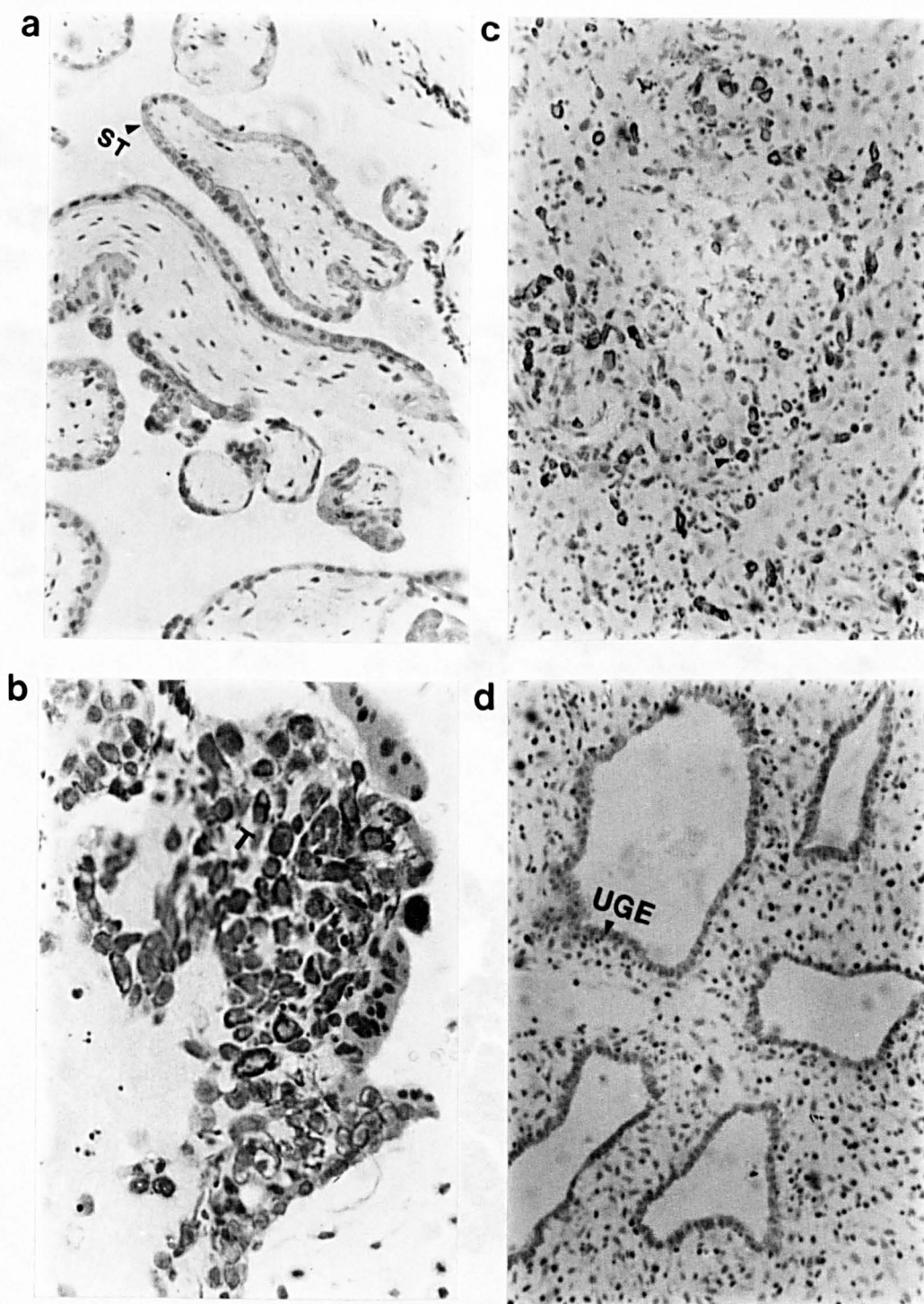


Figure 2B. Wax-embedded section for an early human placenta (8-11 weeks old). Indirect immunoperoxidase staining using the mAb anti-cytokeratin PKK1 showing reactivity with (a), (b) the syncytiotrophoblasts (ST) and cells (T) in the the cytotrophoblast cell islands (magnification X80 and X120 respectively); (c) with placental cells in the endometrium (magnification X63);(d) uterine glandular epithelium (UGE) with positively stained cells (magnification X50).

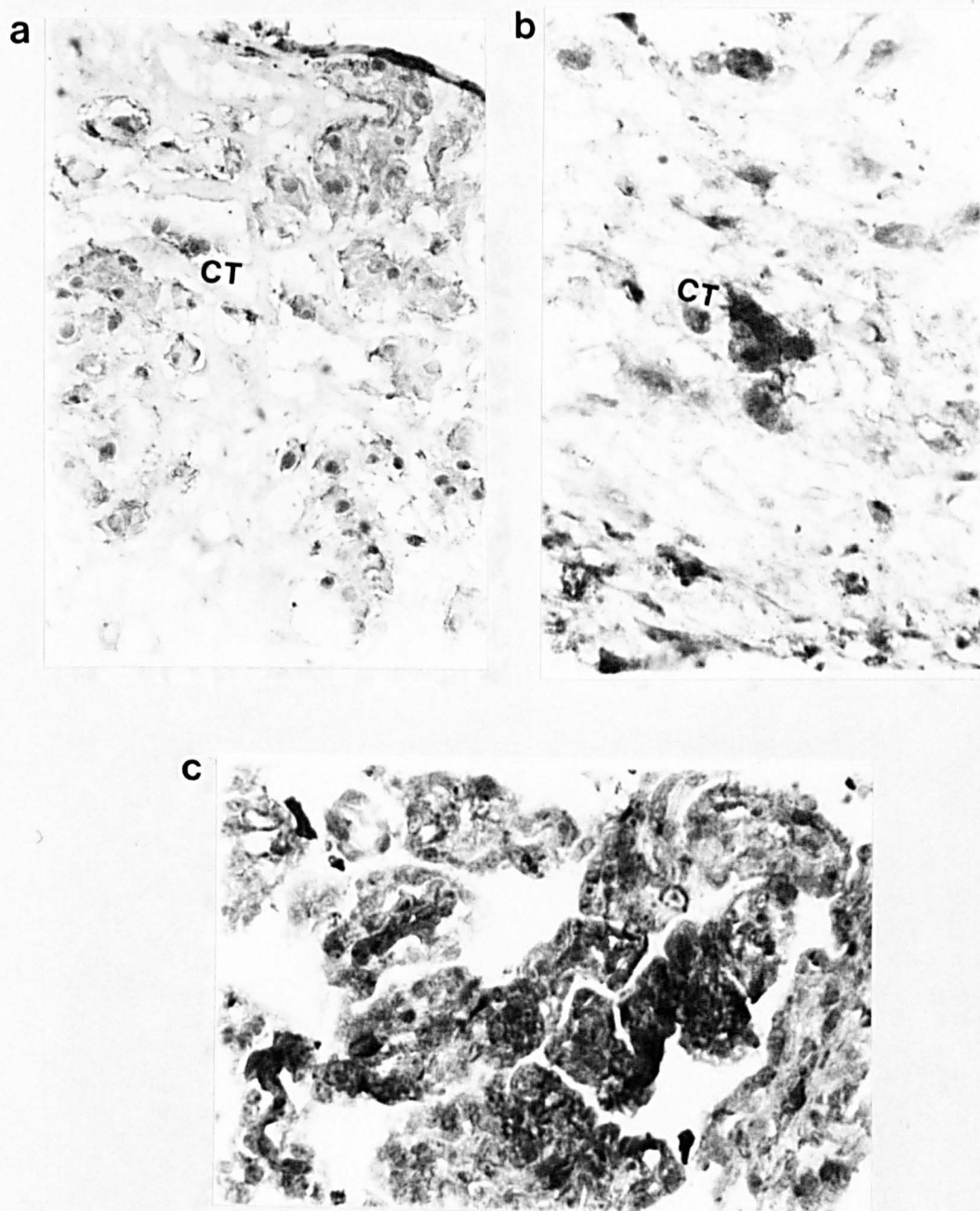


Figure 3. Indirect immunoperoxidase labelling of frozen sections from different term placental regions using the mAb INN-hCG-17 (anti-hCG-alpha). (a) The chorionic plate (magnification X80); (b) the uteroplacental interface (magnification X128); (c) the placental villi (magnification X100). (CT) cytotrophoblasts.

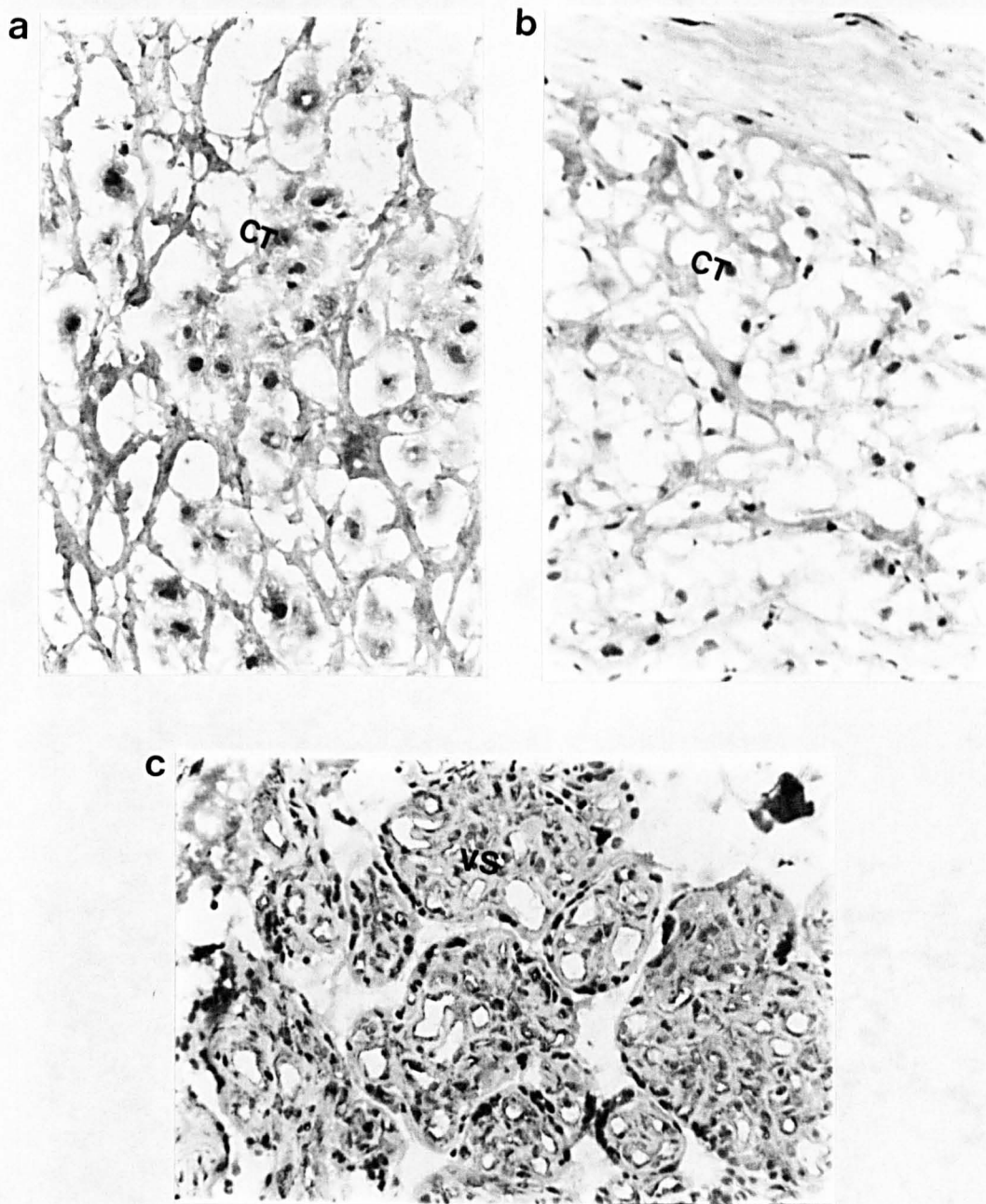


Figure 4. Frozen sections for human term placenta. Indirect immunoperoxidase labelling using the mAb INN-hCG-2 (anti-hCG-beta), showing weakly stained cells (CT) in the (a) uteroplacental interface (magnification X80); (b) chorionic plate (CT) (magnification X100); (c) villous stroma (VS) (magnification X80).

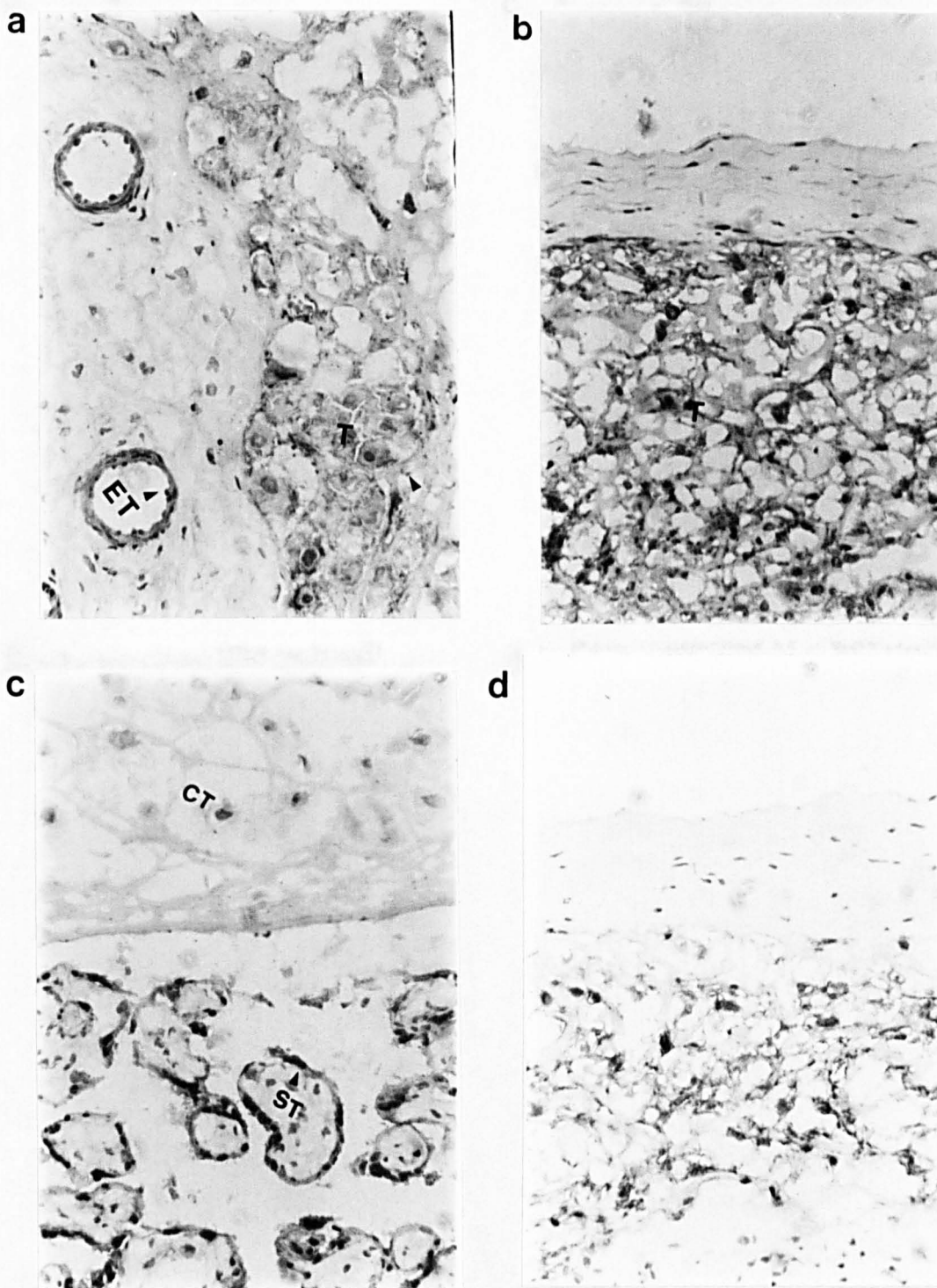


Figure 5. Frozen section of human term placenta. Indirect immunoperoxidase staining using (a) H316 showing positive cells (T) in the uteroplacental interface, note the staining of the endovascular trophoblasts (ET) ; (b) H316 showing positive cells in the chorionic plate below the fetal mesenchyme (T); (c) H315 showing positive syncytiotrophoblasts (ST) and negative cells (CT) in the uteroplacental interface, the syncytiotrophoblast staining is similar to that for H316; (d) H315 showing positive cells in the chorionic plate (magnification X80).

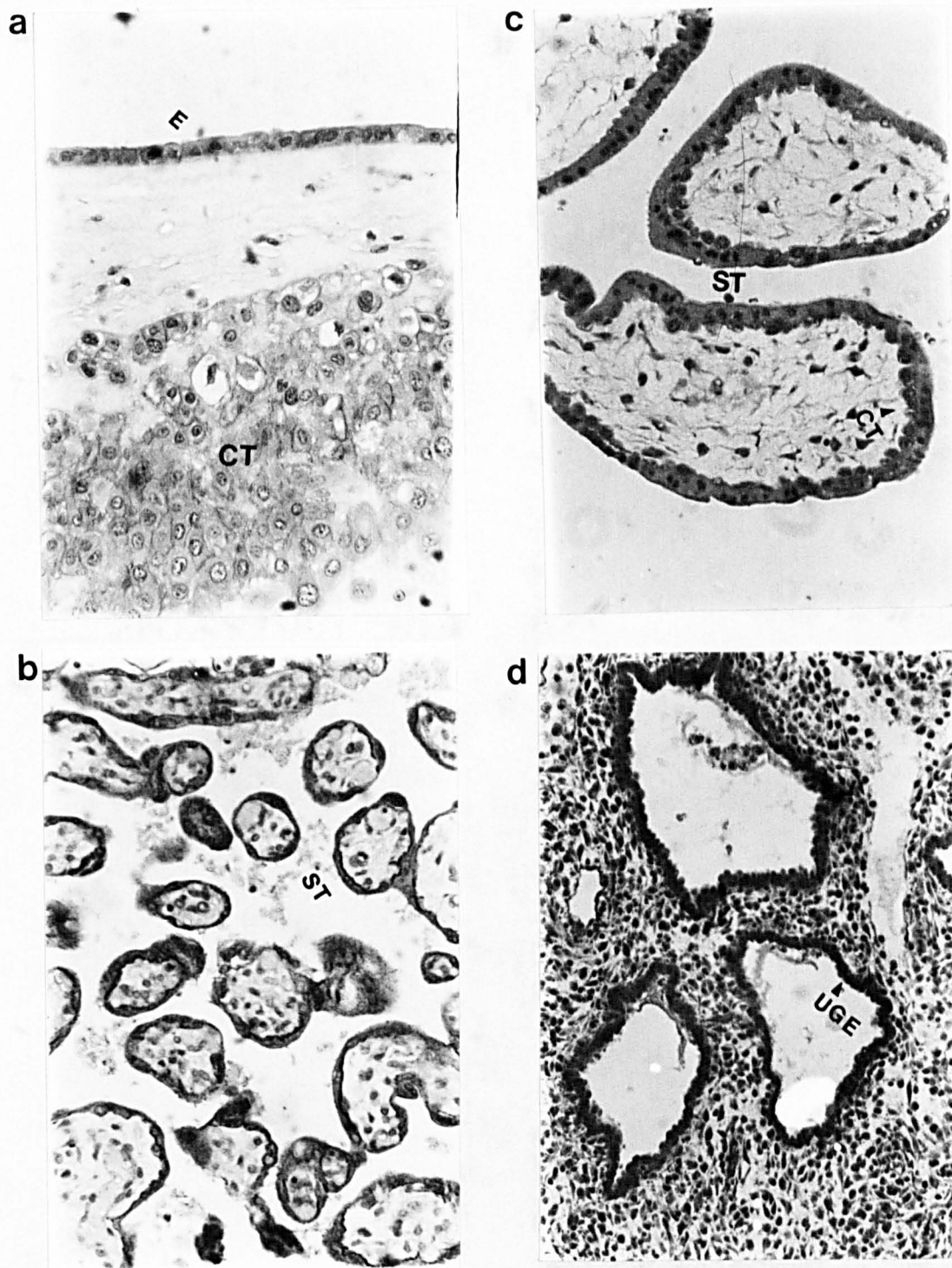


Figure 6. Indirect immunoperoxidase staining using the hybridoma antibody 7A11. (a) The chorionic plate of human placenta at term (magnification X100); (b) the placental villi at term displaying positively stained syncytiotrophoblasts (ST) (magnification X80); (c) placental villi of human early placenta showing positively stained cytotrophoblasts (CT) (magnification X80); (d) the uterine glandular epithelium (UGE) with scattered positive cells (magnification X50).

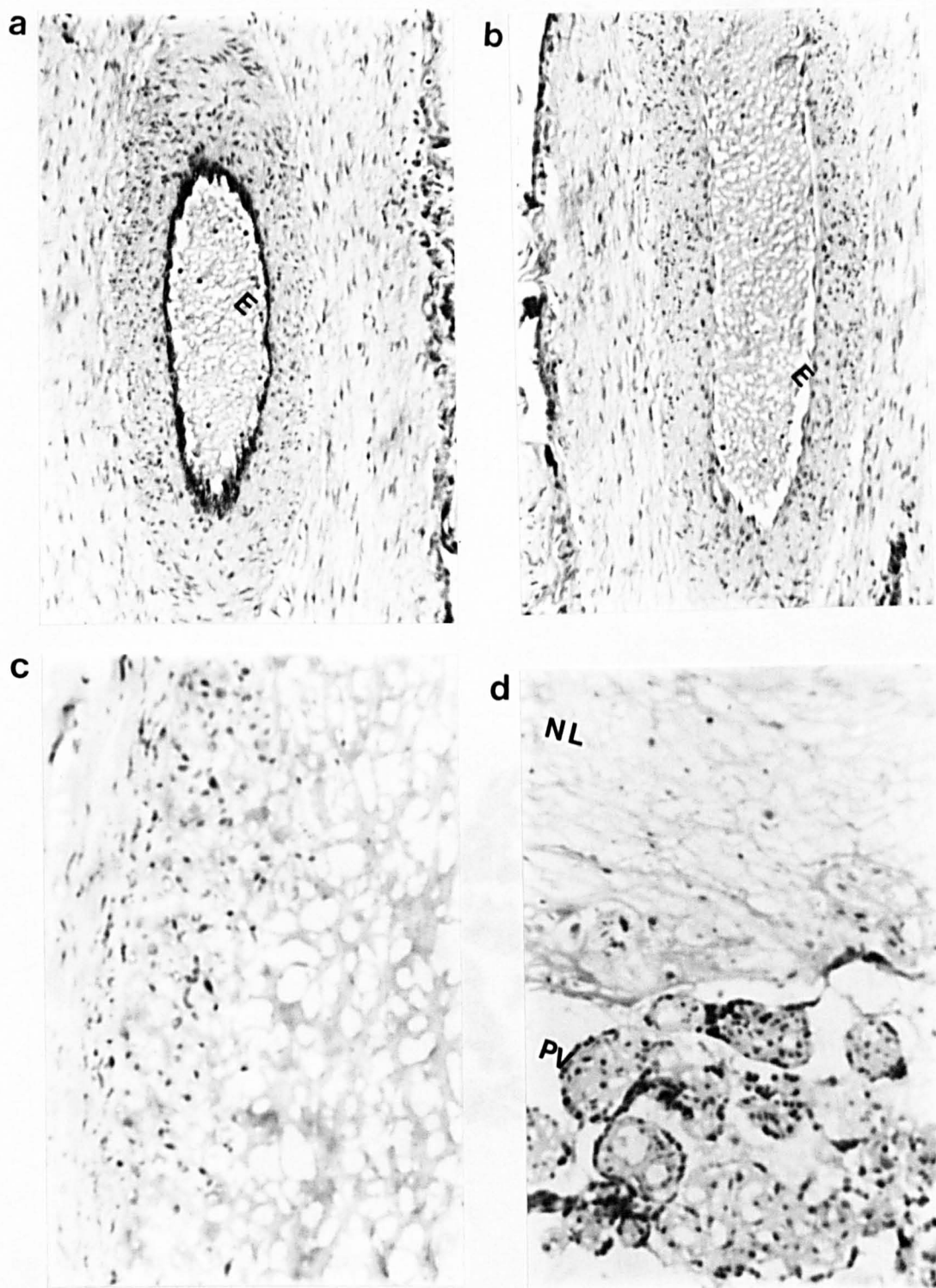


Figure 7. Wax-embedded sections of term placenta. (a) Indirect immunoperoxidase staining using *Ulex europaeus* I agglutinin lectin (UEA I) for the endothelial cells identification (E) (magnification X50); (b) adsorbed lectin using 0.2M alpha-L-fucose (magnification X100). (c) Frozen section of the chorionic plate using PK-V (magnification X80); (d) the uteroplacental interface (NL) and the placental villi (PV) (magnification X80).

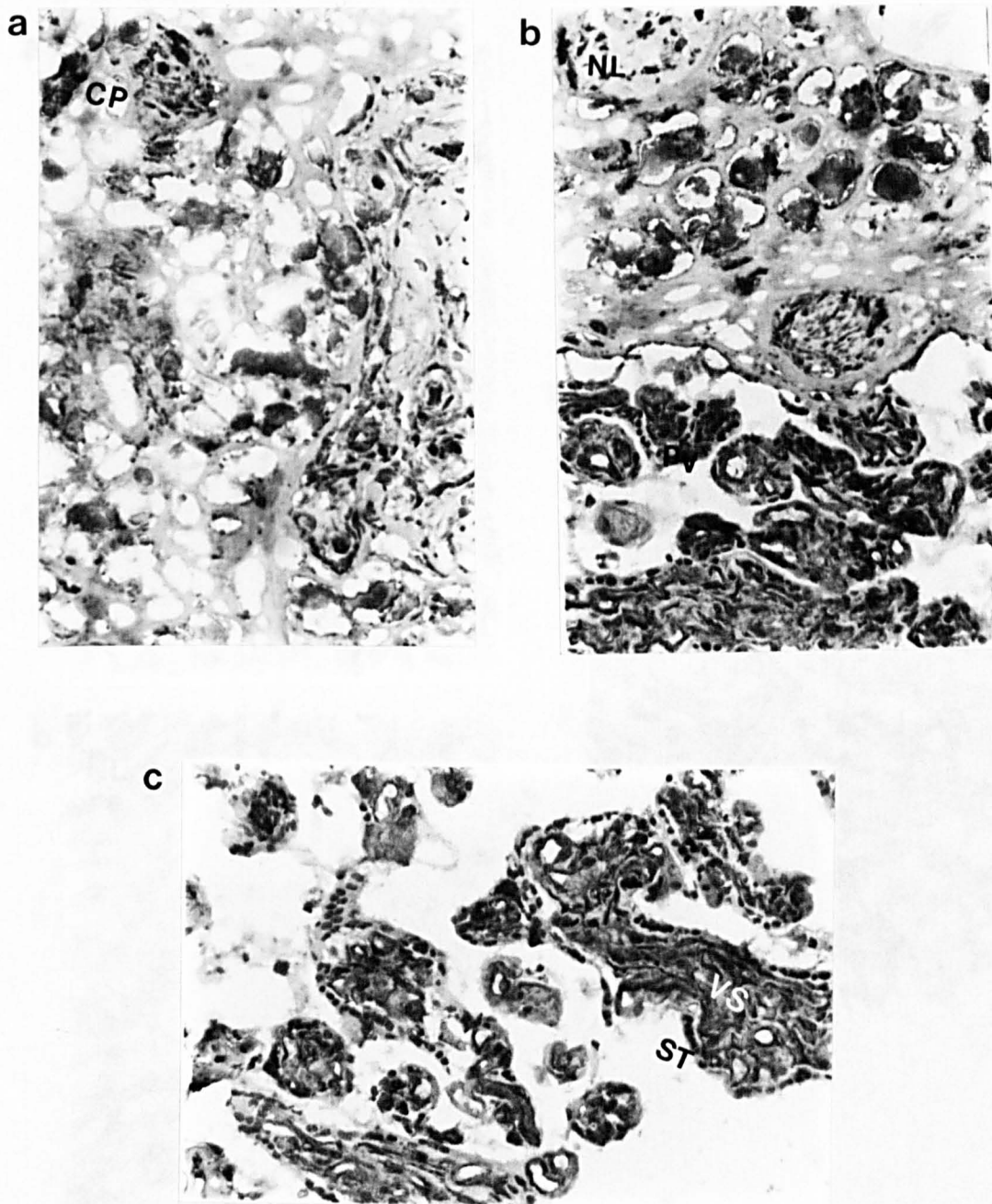


Figure 8. Indirect immunoperoxidase staining using the monomorphic monoclonal antibody W6/32. (a) Frozen section from the chorionic plate of the human placenta at term (CP). (b) The uteroplacental interface (NL) and the placental villi (PV). (c) The placental villi with the syncytiotrophoblasts (ST) are negatively stained, compared to the villous stroma (VS). This distribution is similar to that for beta-2-microglobulin. (Magnification X80).

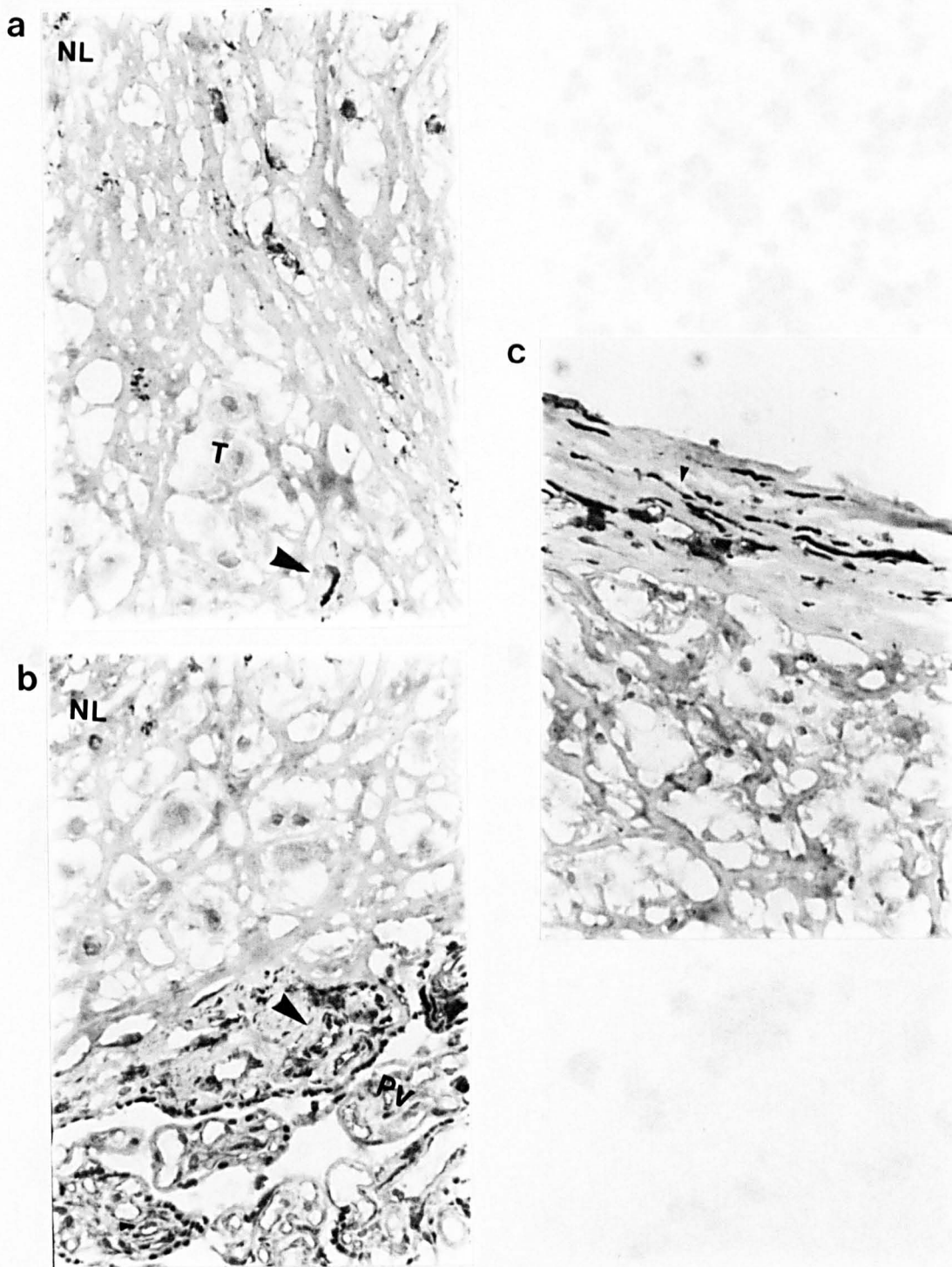


Figure 9. Frozen sections of human term placentae. Indirect immunoperoxidase staining using the mAb DA6.231 (anti-HLA-DR). (a) The uteroplacental interface (NL) showing negatively stained trophoblastic cells (T) with the exception of some spindle shaped cells showing positive reaction as indicated (see arrow); (b) the uteroplacental interface (NL) and the placental villi (PV); the syncytiotrophoblasts (ST) are showing no reactivity, however, some positively stained cells can be seen in the villous stroma (as indicated); (d) The chorionic plate with the subepithelium showing positive reaction (as indicated). (Magnification X80).

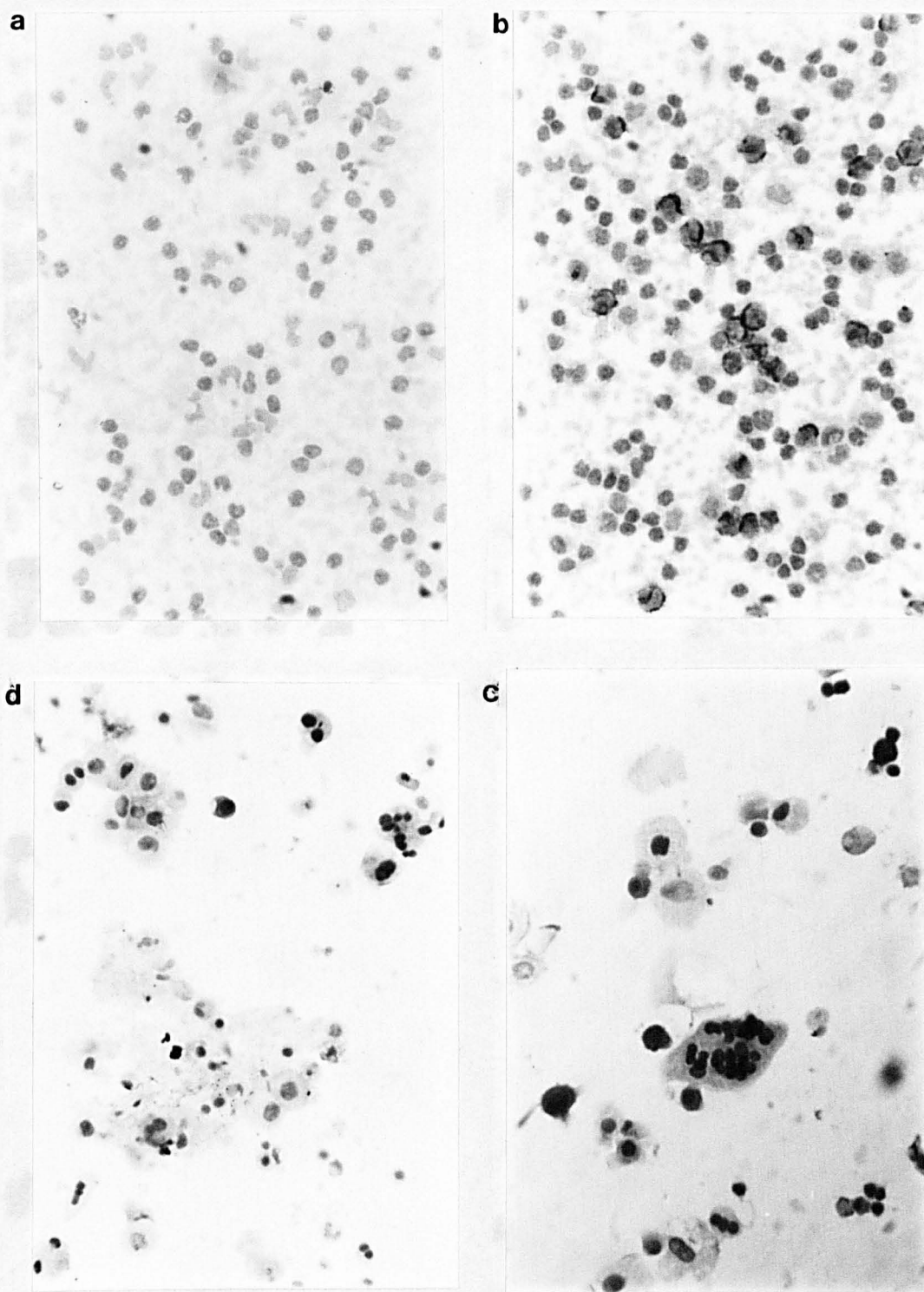


Figure 10. Cytospin preparations for the peripheral blood lymphocytes and the placental cells. Indirect immunoperoxidase staining applied on peripheral blood lymphocytes cytospin preparations using the monoclonal antibody W6/32. (a) No reaction with W6/32 as methanol was used for the endogenous peroxidase blocking ; (b) positive reaction as methanol was replaced with either PBS or H_2O (magnification X100). Negative controls for the chorionic plate cytospin preparations (c) using normal mouse serum (NMS); (d) excluding the first antibody. (Magnification X80).

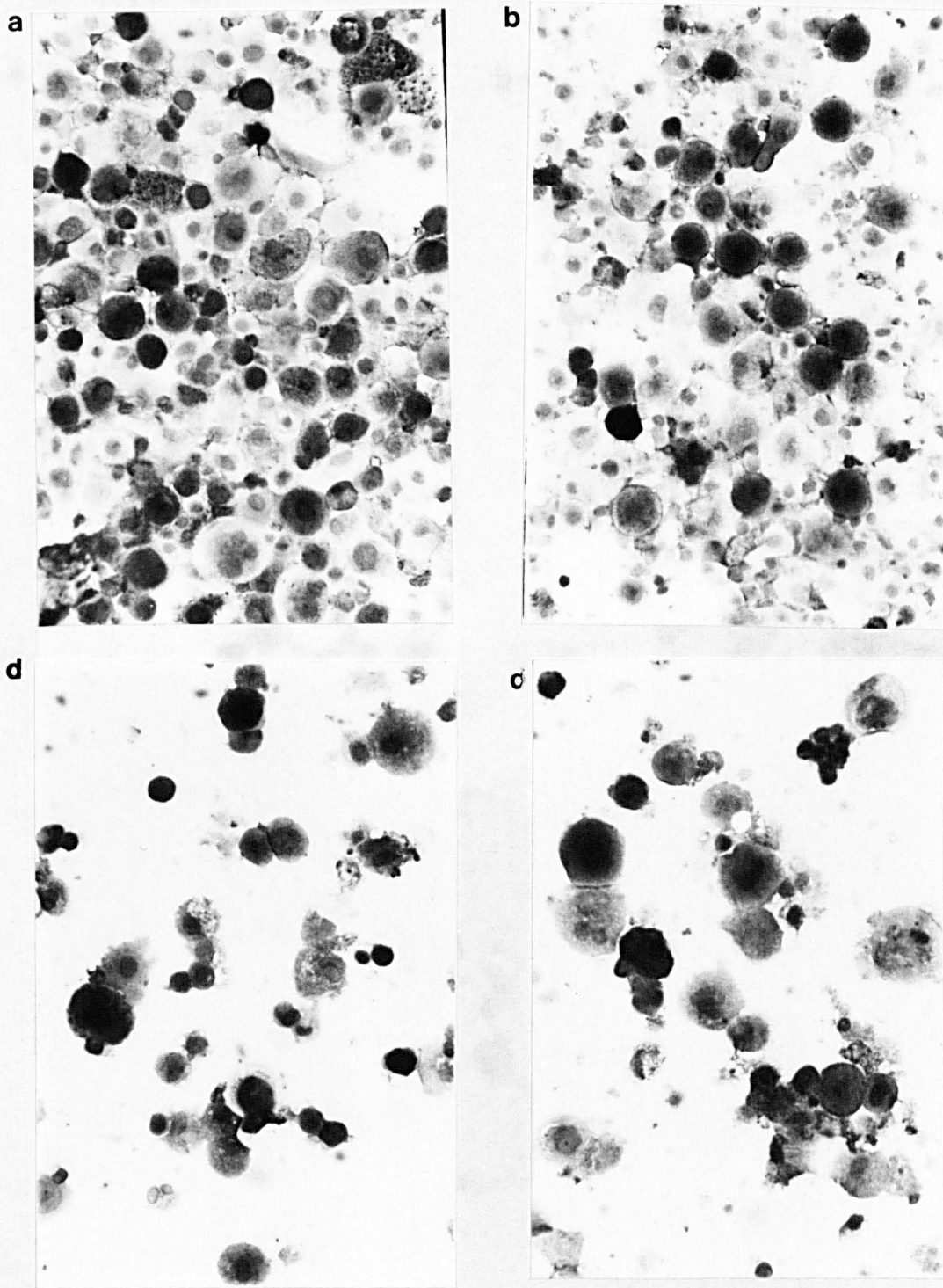


Figure 11. Chorionic cytopsin preparations. Indirect immunoperoxidase staining using (a) PKK1 (anti-cytokeratin) ; (b) 7A11 (hybridoma antibody) ; (c) W6/32 ; (d) anti-beta-2-microglobulin . (Magnification X80).

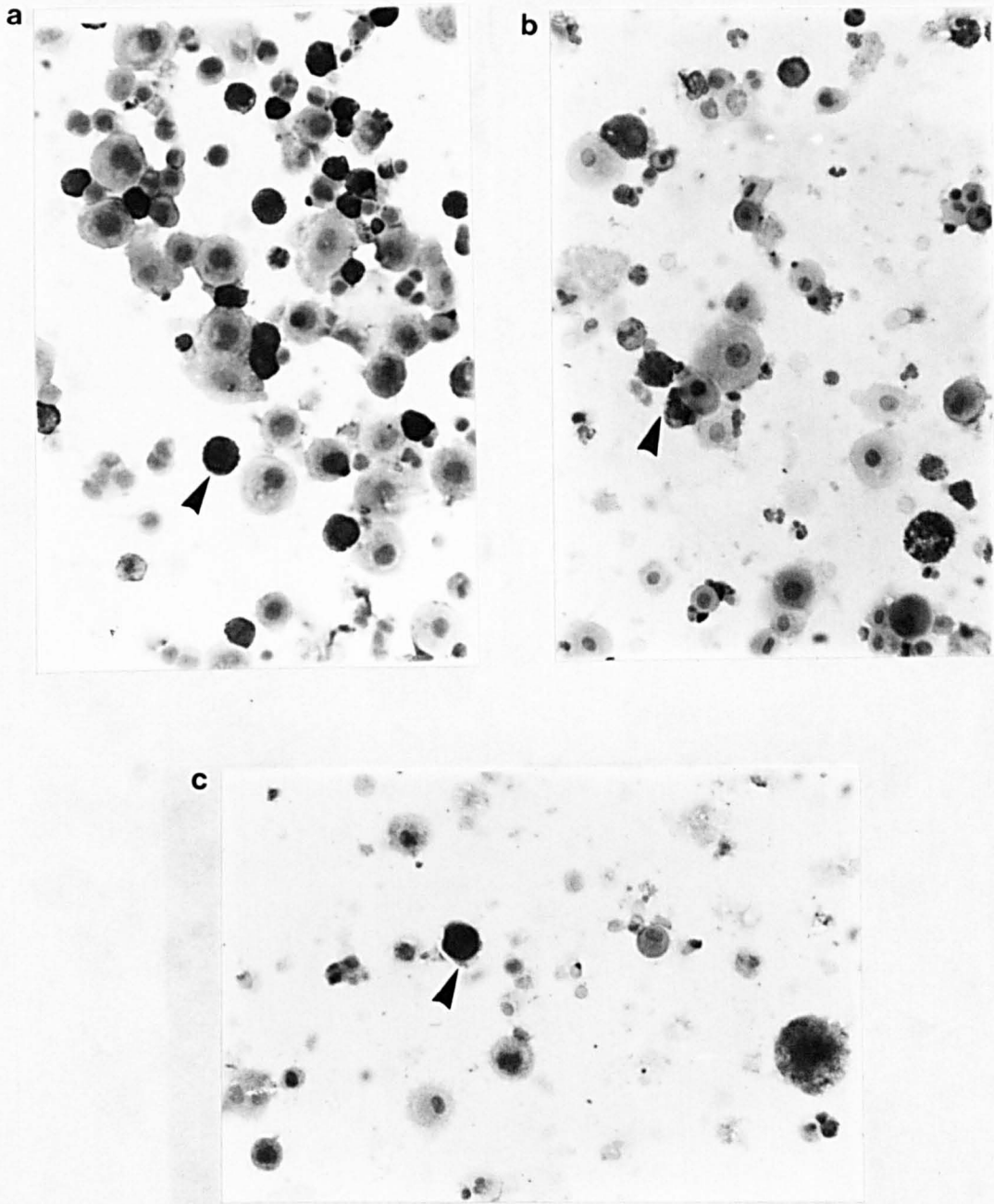


Figure 12. Chorionic plate cytospin preparations. Indirect immunoperoxidase labelling using (a) DA6.231 (anti-HLA-DR) (magnification X80); (b) H316 (magnification X100); (c) H315 (magnification X80).

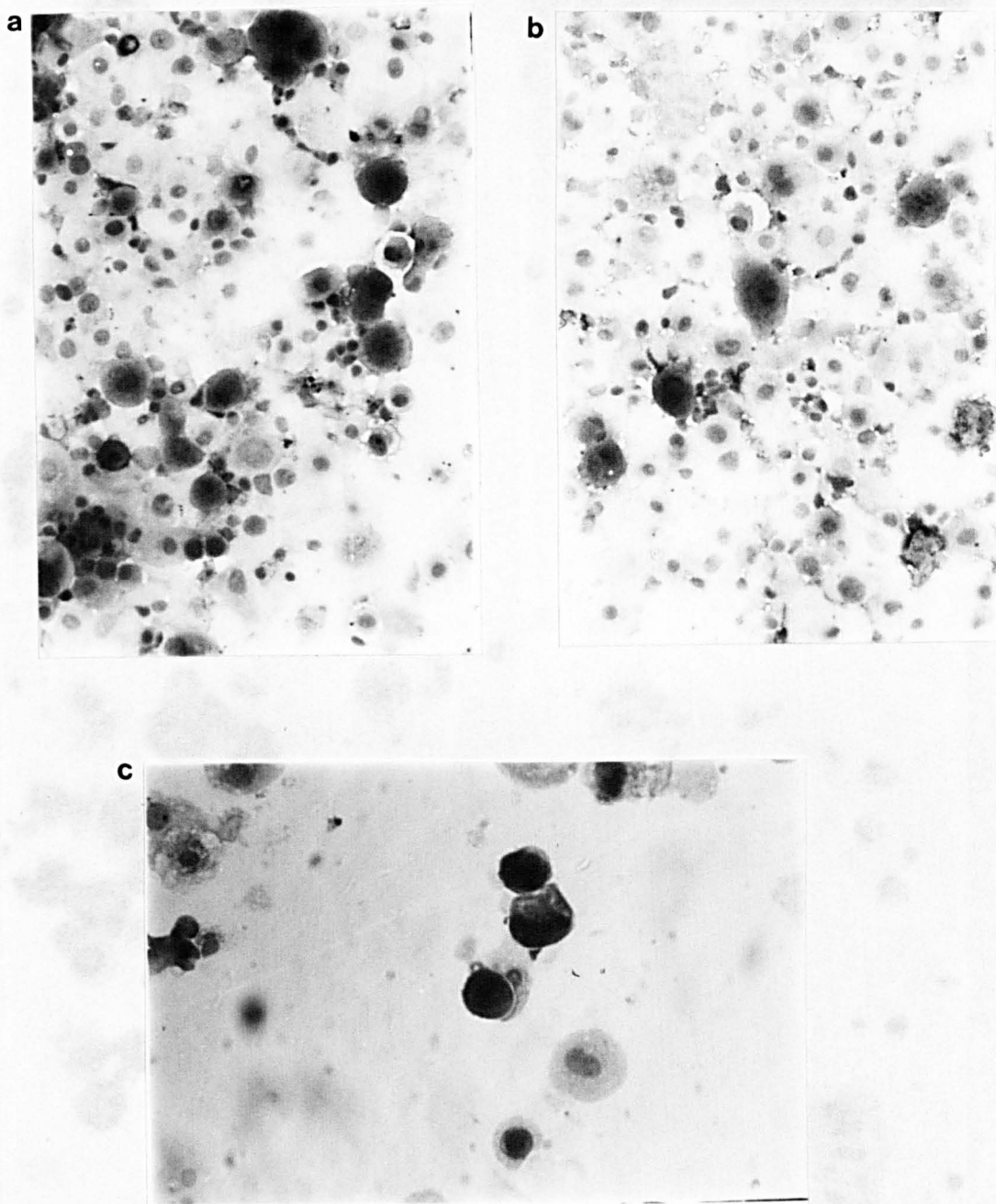


Figure 13. Chorionic plate cytospin preparations. Indirect immunoperoxidase labelling using (a) IMM-hCG-17 (anti-alpha-hCG) (magnification X80); (b) IMM-hCG-2 (anti-beta-hCG) (magnification X80); (c) anti-hPL (magnification X80).

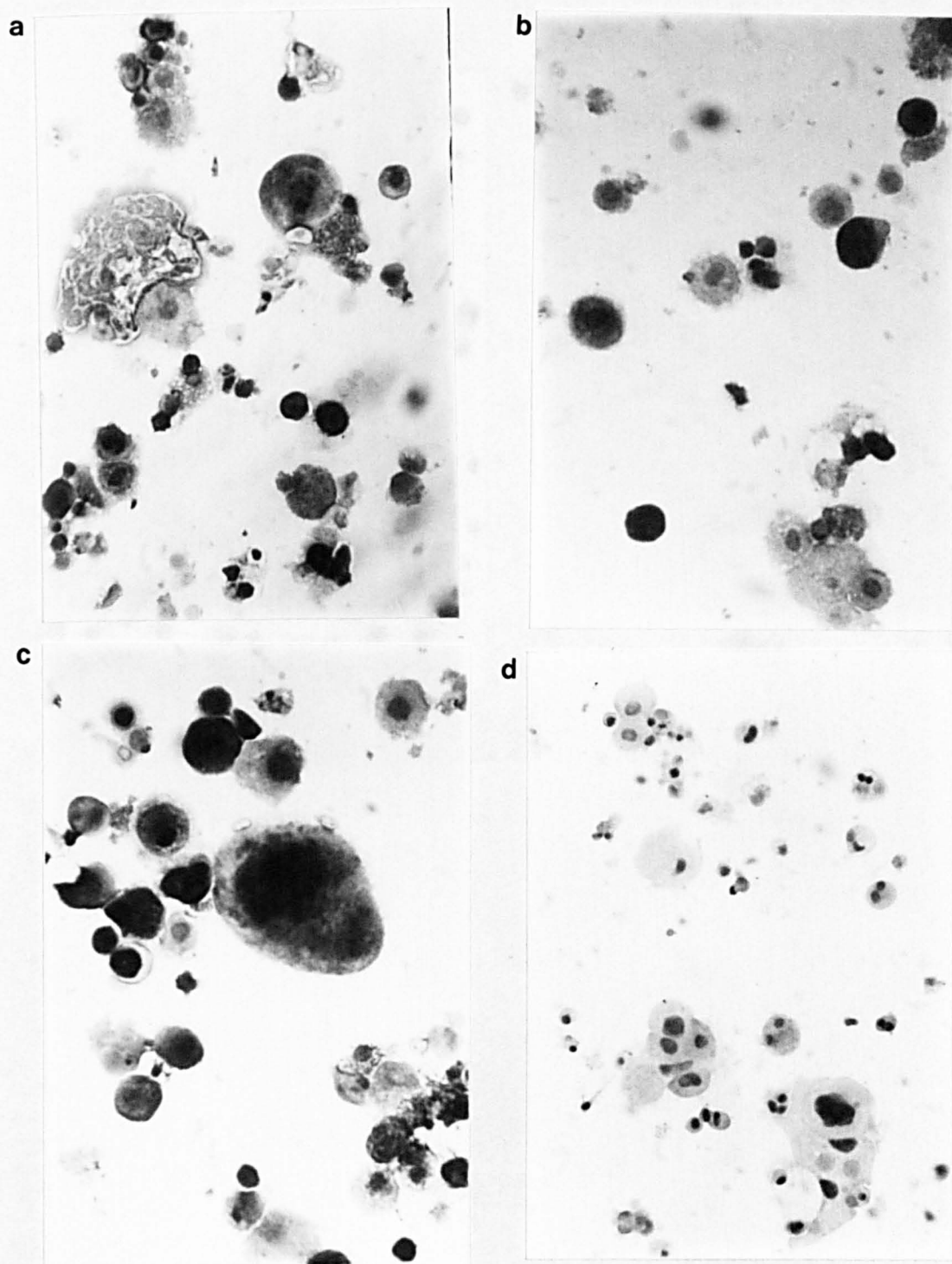


Figure 14. Chorionic plate cytopspin preparations. Indirect immunoperoxidase labelling using (a) anti-SP1 ; (b) anti-PAPP-A ; (c) anti-fibronectin ; (d) adsorbed fibronectin; no staining is observed. (Magnification X80).

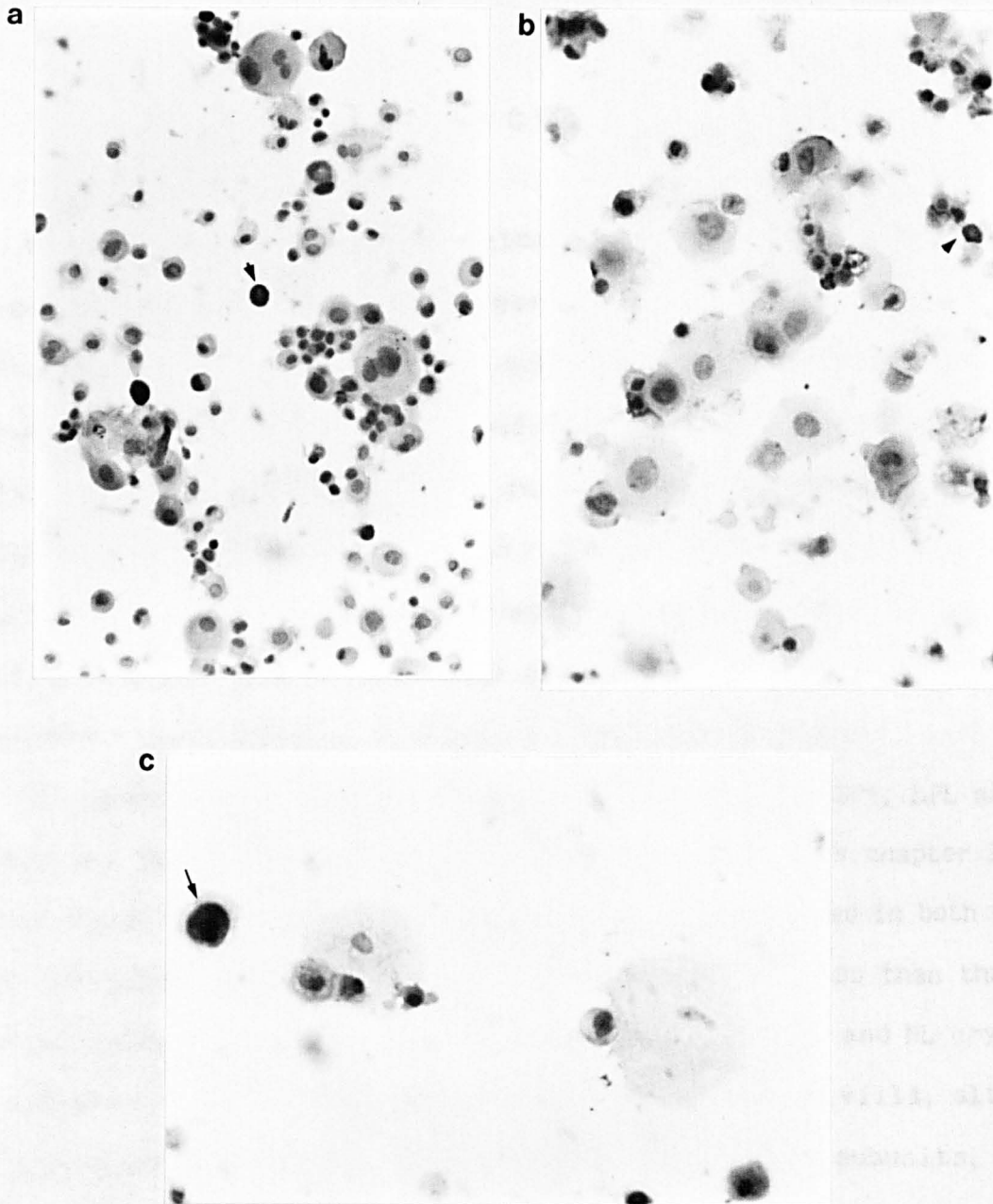


Figure 15. Chorionic plate cytospin preparations. Indirect immunoperoxidase staining using (a) UEA I lectin for the detection of endothelial cells. Cells were incubated with UEA I lectin followed by rabbit anti-lectin and immunoperoxidase staining (magnification X80); (b) anti-LCA (magnification X80); (c) PK-V (anti-vimentin) (magnification X100).

4:4 DISCUSSION

The previous immunohistochemical studies on placental cytospin preparations from Nitabuch's layer and the chorionic plate showed clearly positive staining for human placental proteins such as fibronectin (FN) and pregnancy associated plasma protein-A (PAPP-A). The stained cells look quite similar in morphology. These results are in accord with the previous results obtained in fixed placental sections discussed in Chapter 2. The unstained cells could be maternal (decidual) cells or other type of placental cells which do not synthesise such proteins.

In addition to these two proteins, the staining for SP1, hPL and the alpha and beta subunits of hCG was also studied. In this chapter it has been shown that alpha and beta hCG subunits were located in both NL and CP. The number of stained cells for beta subunit was less than that for alpha-subunit. Staining for both subunits in the CP and NL cryostat sections was displayed by few cells. In the placental villi, although the villous stroma showed positive staining for both subunits, only a few cells were stained for beta subunit. However, the syncytiotrophoblastic layer was positive only for alpha subunit. The latter observations are consistent with previous studies (Gaspard *et al*, 1980). The negative staining for beta-hCG in the syncytiotrophoblast could be due to the marked diminution of hCG from the end of the first trimester to term (Kurman *et al*, 1984). Moreover, the trophoblast staining observed in the cytospin preparations for alpha and beta subunits, SP1, and hPL might permit the tentative conclusion that these cells could be of the intermediate trophoblastic cell type described by

Kurman et al (1984).

Beside these placental proteins, it was found that the placental cells in cryostat sections ,wax-embedded sections and cytopsin preparations reacted very strongly with mAbs against intermediate filaments (anti-cytokeratin TR1 and PKK1). Both mAbs reacted with villous and non-villous trophoblasts, which is consistent with the finding of Redman et al, (1984) and Contractor, Routledge and Sooranna (1984). They showed that JMB2 and LE61 respectively reacted with all trophoblast populations throughout pregnancy. In addition, the reactivity of this antibody was also confined to epithelial cells of the endometrial glands in the uterine tissue. The latter finding was also observed when PKK1 and the hybridoma antibody 7A11 were applied to uterine endometrial tissue. In addition, other mAbs H315 and H316 were demonstrated by Bulmer, Billington and Johnson,(1984) to give similar results . Furthermore, in this study it was shown that these latter mAbs reacted with trophoblastic cells in both the CP and NL as well as villous syn-, and cytotrophoblasts which is consistent with their distribution in 8-10 weeks placentae (Bulmer, Billington and Johnson, 1984). This pattern of staining seems quite similar to that obtained by our hybridoma 7A11, which has been shown to react with villous and non-villous trophoblasts at term and early placentae. In addition, syncytial sprouts and intervillous syncytial elements, as well as cytotrophoblastic cell columns and cell islands associated with the chorionic villi were also stained. No observed staining was confined to the syncytiotrophoblastic layer of early placentae.

From these results it might be concluded that these different mAbs, although they show almost the same distribution in early and term placentae, do not recognise the same antigenic determinant on the

surface of these cells. For, as far as the villous trophoblasts are concerned, both H315 and H316 reacted with syn- and cytotrophoblastic cells of early placentae, whereas PKK1 and 7A11 reacted only with the cytotrophoblasts. Additionally, the staining of the syncytiotrophoblastic layer at term using PKK1 and 7A11 can be explained by the hypothesis that this layer has been formed by the fusion of the early villous cytotrophoblastic cells. These studies show that placental cells express a variety of antigenic determinants which therefore can be recognized and detected by different monoclonal antibodies.

Furthermore, 7A11 and PKK1 were shown to stain a distinctive layer of placental cells below the fetal mesenchyme in the chorionic plate. This layer reacted positively with PAPP-A and FN as demonstrated in Chapter 2, therefore supporting the suggestion that these two proteins are synthesized by placental cells. However, another set of cells that reacted with anti-cytokeratin (PKK1) and 7A11, did not reveal positive staining for either FN or PAPP-A. From this it could be argued that these cells might be another type of trophoblastic cells that do not synthesize and secrete placental proteins, or they might be more differentiated trophoblasts where they no longer synthesize such proteins. Several studies carried out on the amniochorionic membrane, revealed a similar structure to the chorionic plate. In these studies, it was demonstrated that the layer beneath the trophoblastic cells is composed of decidual cells (Kuman *et al*, 1984; Hsi, Yeh and Faulk, 1982; Bulmer, Billington and Johnson, 1984). Although such similarity does exist between the two regions, it can be argued that this decidual layer does not exist in the chorionic plate since there is no direct contact with maternal tissue. In addition, PKK1 staining as mentioned above

might support this , since the decidual cells do not react with anti-cytokeratin antibodies.

Additional cellular marker antigens were studied to check the degree of other cellular contamination in these isolated chorionic plate cells. In the cryostat sections no staining was observed in any of the villous or non-villous trophoblastic cells, when antibodies against leucocytes (anti-LCA), endothelial cells (anti-UEA I lectin) and fibroblasts (PK-V) were applied.

As far as the staining for endothelial cells is concerned, apart from the cytopsin preparations, wax-embedded placental sections were also used. UEA I lectin which has a nominal specificity for the alpha-L-fucose residues of sugar moities that preferentially react with the ABH blood group substance of the vascular endothelium,irrespective of the blood group type, has been used as a marker for these cells (Holthofer et al, 1982). The only stained cells were those lining the blood vessels; no staining elsewhere was observed. The protocol mentioned in this chapter for the localization of endothelial cells is the first to be reported. Other workers used immunofluorescent and immunoperoxidase staining techniques using anti-Factor VIII and FITC conjugated UEA I lectin (Holthofer, et al 1981; Holthofer, et al 1982).

Cytopsin preparations revealed small number of cells stained for LCA (1-7%), UEA I lectin (2-12%) and Vimentin (4-11%). The stained cells look different from the trophoblastic cells apart from being smaller. Apparently, these results indicate low contamination with leucocytes, endothelial cells and fibroblasts. These results confirm our previous cell culture experiments in which no fibroblast overgrowth was observed.

MHC antigens on trophoblastic cells. Different biochemical and

immunohistochemical studies have clearly demonstrated that human villous trophoblast does not express class I MHC antigens (Goodfellow et al, 1976; Sunderland et al, 1981; Faulk and Temple, 1976), whereas others showed their expression on non-villous trophoblasts (Sunderland, Redman and Stirrat, 1981a; Redman et al, 1984). In this study, using anti-B₂M and W6/32 which binds monomorphic determinants, it was found that our results are consistent with the previously mentioned results. No staining was observed in the villous syncytium, however, membranous elements of the villous stroma, and cells in both chorionic plate and Nitabuch's layer were stained. Moreover, a substantial number of the placental cells in the cytopsin preparations were stained for these antigens. Because of the difficulty in identifying the MHC-bearing trophoblastic cells among the highly heterogeneous isolated cells in the cytopsin preparations and even in the placental sections, mAbs against the intermediate filaments (anti-keratin) were used as mentioned earlier in this section. The pattern of staining for these HLA class I antigens was quite similar to that for cytokeratin, thus confirming the placental origin of the stained cells. This observation is in accord with previous study carried out by Redman et al, (1984). In addition, the staining pattern was also similar to that for 7A11, H315 and H316 mAbs. These results were ample evidence to show that the isolated cells, especially those from the chorionic plate, are mostly trophoblasts. This finding might help in doing further experiments on these cells. Although the majority of these heterogeneous cells stained for cytokeratin, it is still difficult to distinguish between the different cellular types due to the minor cellular contamination with endothelial cells, leucocytes and fibroblasts.

An important point worth noting at this stage is the reaction of the

extra-villous trophoblast with W6/32 monomorphic monoclonal antibody. Redman et al (1984) revealed in his study that the cells identified as extra-villous trophoblast on the basis of position, morphology and JMB2 antigen expression, and which expressed the monomorphic determinants of HLA-A,B,C, were not maternal in origin, for they failed to react with maternal-specific antibodies , MA2.1 and ME.1, which recognize HLA-A2 and HLA-B7 respectively. Moreover, it was revealed that these trophoblasts which reacted with antibodies to monomorphic class I MHC (W6/32, PA2.6 and BB7.7) failed to bind HLA-A or -B antibodies specific for the fetal phenotype. This suggested that class I MHC antigens are qualitatively different, either belonging to the HLA-C series or to a novel MHC class. Johnson (1983) reported the possibility that W6/32 ,when compared to other mAb reactions, might be recognizing antigenic determinants other than class I MHC antigens that could possibly be encoded by the T1a region. This might be likely since murine class I MHC genes, from which 31 map to the Qa and T1a regions, are homologous to those of the human (Steinmetz, 1984; Goodenow et al,1982). The staining for HLA-DR in the frozen sections showed similar results to Sunderland, Mason and Redman (1981) and Sutton et al (1983). In these studies little staining was observed in the placental villous stroma or in the fetal blood vessels. Moreover, HLA-DR positive cells were found in the connective tissue underlying the amniotic epithelium. It was pointed out in these studies that these cells which are HLA-DR positive do resemble the dendritic cells which have been described in other tissues (Steinman et al, 1979; Hart et al, 1981). Although it is still uncertain whether these cells are trophoblasts, a study has been carried out by Bami and co-workers on human trophoblast from nine week to twelve-week aborters both in culture and cryostat sections using immunofluorescence

techniques. They found HLA-DR on cells which have been identified as trophoblasts due to hCG secretion. In my study, the cytospin preparations revealed heavily stained cells for the HLA-DR antigens. These cells did not look like dendritic cells nor did they look like lymphocytes. Further studies are required to check whether these cells are trophoblasts or another cell type such as endothelial cells.

So far, none of the trophoblastic cells studied displayed HLA-DR (Redman, et al 1984; Bulmer and Johnson, 1985a). Although class II MHC antigens stimulate the strongest allograft rejection reactions, class I differences are sufficient for tissue rejection (Klein, 1975). The trophoblast bearing fetal class I MHC antigens in early and mature pregnancy lies in direct contact with maternal tissue. Although a rejection reaction might be expected, it has been suggested by Gill (1983), that the maternal immune response to trophoblast MHC products may be essential for the normal development of the implanting conceptus, and that a failure of this response occurring with histocompatible matings would cause abortion.

CHAPTER 5

IMMUNOLOGICAL STUDIES ON CHORIONIC PLATE ISOLATED CELLS

5:1 INTRODUCTION

In Chapter 4 it was shown that the isolated placental cells from either Nitabuch's layer or the chorionic plate express B₂M, and the monomorphic determinants for class I HLA antigens, as well as other antigenic determinants. These results encouraged me to carry out tissue typing experiments in order to study the HLA phenotypes expressed by these placental cells.

Cells from the chorionic plate were used for this purpose so as to avoid any maternal cell contaminants, since these cells lack the contact with the uterine tissue. Microcytotoxicity assay was applied using antibody and rabbit complement. Both eosin dye exclusion and ³⁵S-methionine incorporation were used for the assessment of cell viability.

5:2 MATERIALS AND METHODS

5:2:1 Materials F(ab')₂ fragment of sheep anti-mouse IgG (whole molecule) FITC conjugate and sheep anti-rabbit IgG (whole molecule) FITC conjugate were obtained from Sigma Chemical Company. FITC conjugate sheep anti-human IgG, FITC conjugate sheep anti-human IgM, rabbit anti-human IgG and mouse anti-human C3 were purchased from the Scottish Antibody Production Unit. Rabbit complement was obtained from the Buxted Rabbit Co. Ltd. W6/32, anti-B₂M, 19/9 and anti-LCA were obtained from Sera-lab. H316 (anti-TLX) was generously given by Dr. Peter Johnson, Liverpool. The mouse xenogeneic antibodies were prepared in our laboratory. Anti-HLA-A7 and anti-HLA-B8 were generously given by Professor Heather Dick, Royal Infirmary, Glasgow. Culture media, fetal bovine serum and culture supplements were obtained from Flow Laboratories. ³⁵S-methionine was purchased from Amersham. Enhance was obtained from New England Nuclear. Scintillation counts were measured in a SL30 Liquid Scintillation Spectrometer (Intertechnique). All samples were prepared in a volume of high efficiency emulsifier cocktail (Biofluor, New England Nuclear). Tris and SDS were obtained from Sigma Chemical Company. High salt buffer; 10mM Tris-HCL, pH 7.4, 1.5M KCl, 0.14M NaCl, 0.5% Triton X-100, and 0.5mM PMSF (all were purchased from Sigma Chemical Company). EDTA and EGTA were obtained from BDH.

5:2:2 Methods

1. Antibody Dependent Complement Mediated Cytotoxicity Assay. Freshly prepared lymphocytes and trophoblasts were washed and resuspended at 1-2X10⁶ cell / ml in complete media without fetal calf serum. Cell viability was assessed by incubating 2ul of suspended cells with 2ul of

heat inactivated normal human serum (NHS), and 2ul of eosin. The cell preparations were left at room temperature for 5 min and then counted in Kora slides or haemocytometer chambers. Viable cells appeared refractile, non-viable cells stained red with eosin. The viability for lymphocytes usually exceeds 95% and this provided a suitably low background value against which to assay the effect of cytotoxic antibodies.

The assay was performed by adding to each well of a Terasaki plate one drop of liquid paraffin to prevent any evaporation of the media during and after the assay. This was followed by the addition of 2ul of cell suspension and 2ul of neat antiserum, and the cells were incubated for 30 min at room temperature. At the end of this period, 5ul of neat rabbit complement was added to each well and the cells were incubated for a further 60 min. 3ul of eosin were then added and left for 3-5 min followed by the addition of 5ul of formalin. A coverslip (50x75mm) was placed on top and cells were left to settle at 4°C for at least 10 min. The viable cells were observed using Leitz Wetzlar (Diavert) microscope.

2.Immunofluorescent Staining. Indirect immunofluorescent experiments were carried out to detect TLX, LCA, B₂M, and class I and class II monomorphic determinants on the surface of viable cells. Since this method has fewer steps than the immunoperoxidase staining method, it was more convenient to use on un-fixed cells in suspension. For this purpose freshly prepared cells at $1-2 \times 10^5$ cells /tube suspended in 30-40 ul CM-FCS were used. After 30-40 min incubation with the appropriate antibody, the cells were rinsed in PBS, spun down and resuspended in 25ul (1/10) rabbit anti-mouse IgG (Fab) conjugated with fluorescein isothiocyanate (FITC). After 30 min incubation the cells were washed in

PBS and resuspended in a 1:1 ratio of glycerol-PBS in a total volume of 30ul / tube, and examined using Leitz Wetzlar microscope.

3. Localization of Human Complement and Immunoglobulins. Using indirect immunofluorescent staining, cells at $1-2 \times 10^5$ suspended in 30-40 ul complete media without fetal calf serum were used per tube. 30 ul of the monomorphic mAb W6/32 used at the dilution of 1/4 was added to the cells and left for 30-40 min. This was followed by a further 90 min incubation with 70 ul of human complement (NHS). At the end of this period, cells were rinsed in PBS then incubated for 30 min with mouse anti-human C3 followed by another rinse in PBS. Rabbit anti-mouse IgG (Fab) conjugated with FITC was added to the cells at the dilution of 1/10. After 30 min incubation the cells were washed in PBS and resuspended in 50% glycerol ready to be observed using the Leitz Wetzlar microscope (filter number 3) .

The detection of human immunoglobulins IgG and IgM on the surface of placental cells was carried out as mentioned above. For the immunoglobulin IgG both direct and indirect immunofluorescent staining were carried out. In the indirect method rabbit anti-human IgG was used at the dilution of 1/10 followed by incubation with 1/10 sheep anti-rabbit IgG(Fab) conjugated with FITC. The direct immunofluorescent method was applied for both IgG and IgM immunoglobulins; sheep anti-human IgG and IgM conjugated with FITC were used at the dilution of 1/10.

Specificity controls were performed in each case; they consisted of :
1-Omitting the specific antiserum (exclusion of non-specific staining).

2-Using NMS as an alternative for the specific antiserum.

For the localization of complement:

1-Omitting all the stages except for the FITC conjugate.

2-Incubation only with NMS and the FITC conjugate.

4. Antibody Mediated Complement Cytotoxicity Assay Using ^{35}S Methionine for Cell Viability Assessment. During this assay all steps were carried out under sterile conditions. 30 μl of freshly prepared placental cells and peripheral blood lymphocytes were used at a concentration of $1-2 \times 10^5$ cells/tube. The appropriate antibody was added at the dilution of 1/4 (30 μl /tube). After 30-40 min incubation, 70 μl of neat rabbit complement were added per tube and the cells were incubated for 60-90 min at room temperature. This was followed by rinsing the cells of the excess of antibody and complement using CM-FCS, then resuspending them in 500 μl of met/5 media. 15-20 μCi of ^{35}S -met were added per tube, and the cells were incubated for 48 hrs at 37°C . Freshly prepared human lymphocytes were used as a control. At the end of the incubation time, the cells were pelleted and resuspended in 250 μl of 2% SDS followed by freezing all the samples until use.

Controls for this assay were performed as follows:

1-CM alone.

2-CM + either antibody or complement.

3-Cells alone.

4-Cells + either antibody or complement.

5. Complement Mediated Assay; Application on Mixed Lymphocytes and Placental Trophoblastic Cells. Placental cells and lymphocytes were mixed together at the ratios of 1:4, 1:10, 1:20, respectively. Trophoblastic cells were used at the concentration of 1×10^5 cell per tube per 30 μl of CM-FCS. The mixed cells were incubated for 14-16 hrs

at 37°C before applying the complement mediated cytotoxicity assay as described above.

6.Keratin Extraction of Labelled Cells. According to the method described by Oshima et al, (1983), placental trophoblastic cells were cultured in plastic tubes ($2-5 \times 10^5$ cells per tube) in the presence of 15 uCi ^{35}S -met. This was followed by incubation for 3-4 days at 37°C. At the end of incubation, the cells were pelleted and resuspended in 1 ml of high salt buffer, followed by sonication for 30 seconds and then homogenization. The homogenized cells were spun down at 10,000 xg for 15-20 min. Another 1 ml of the same buffer was added followed by a second homogenization and centrifugation steps. The pellet was subsequently washed in 200 ul of the same buffer followed by centrifugation. These extraction steps were repeated five times. At the end of the final extraction step, the extracted pellet was resuspended in 50 ul of boiling mix followed by heating for 30 min at 80 °C (water bath). The heated extract was spun down and only the supernatant was used for analysis on 10% SDS polyacrylamide gels.

5:3 RESULTS

5:3:1 Complement mediated killing assay using eosin dye exclusion.

To study the effect of complement in the presence of antibody on isolated placental trophoblastic cells, neat antibody was added to the cells followed by the addition of neat rabbit complement. Eosin dye exclusion was used to assess the viability of cells after treatment. For this purpose, 23 experiments were carried out on trophoblastic cells isolated from different placentae. These experiments showed that no observed killing was taking place; the percentage of viable cells after

antibody and complement treatment was quite similar to that for the controls. However, peripheral blood lymphocytes under the same conditions showed a very high percentage of cellular death compared to the controls which as shown in Table 1 include cells alone, cells + either antibody or complement and cells + heat inactivated normal human serum (NHS). Results shown in Table 1 are for the placentae with the highest viability percentages.

5:3:2 The accessibility of antigenic determinants on the trophoblastic cell surface. The expression of placental and histocompatibility antigens on the surface of trophoblastic cells has already been studied using immunoperoxidase staining method as mentioned in Chapter 4 . However, the present study was set up on unfixed cells to confirm that these antigens could be bound by antibodies in the complement mediated cytotoxicity assay and that the resultant mixture complexes were available to bind complement. For that purpose different antibodies were used including W6/32, DA6.231, B₂M, and others as shown in Table 2. Both trophoblastic cells and lymphocytes showed very strong staining for class I HLA when the monomorphic monoclonal antibody W6/32 was used. 68-85% of the trophoblasts were stained. Similar results were obtained for B₂M. The pattern of staining for W6/32 and B₂M was mainly across the cellular surface (cytoplasmic staining). Figure 1 (a,b), shows size variation of the stained cells. No obvious staining for class II HLA was observed.

Anti-TLX, anti-LCA, and mouse xenogeneic antibodies were also studied. Both trophoblastic cells and peripheral blood lymphocytes showed positive staining for the xeno. antibodies. The percent of stained cells and the pattern of staining were quite similar to that for W6/32; 69-81% of the trophoblasts showed positive reaction with W6/32.

Only 0-3% of the heterogeneous trophoblastic cell population were positive for LCA (Fig.2c). The staining for LCA was evident for lymphocytes compared to that for trophoblastic cells. 62-71% of the lymphocytes showed positive reaction. 10-19% of the trophoblasts were stained for TLX (Table 2). The staining pattern was mainly across the cell membrane (Fig.2a).

No background staining was observed when the cells were incubated with FITC conjugate alone or normal mouse serum plus FITC conjugate.

5:3:3 Complement binding to the trophoblastic cells in the presence and absence of antibody. To further interpret the complement mediated killing assay results, it was necessary to study the complement binding to the cells after antibody treatment. Both trophoblastic cells and lymphocytes were studied, and normal human serum (NHS) was used as the complement source. Complement binding was assayed by using the Leitz Wetzler microscope. Table 3, summarises the percentages of stained cells under different treatments. As can be seen in Fig.3a, negative controls in which cells were incubated with FITC conjugate alone did not show any staining due to non-specific binding. However, trophoblastic cells showed positive staining for human complement binding after incubation with W6/32. The staining pattern varies from staining at the edges to staining across the cellular surface (Fig.4d). Heat inactivation of human complement in the presence of antibody did not cause a marked change in the percentage of stained trophoblastic cells. These results were similar to those obtained after incubation only with anti-human C_3 and FITC conjugate (Fig. 4a). In addition to this, the presence of normal human serum with both anti-human C_3 and FITC conjugate did not cause any significant increase in the percent of stained cells.

5:3:4 Effect of EGTA and EDTA on placental trophoblastic cells. The effect of both EGTA and EDTA on trophoblastic cells was studied. Both reagents were added to the cells at the complement stage. As shown in Fig.4 (b,c), the intensity of the stain did not change due to the presence of such reagents. Beside this, the percentage of stained cells looks relatively similar to that of the controls (Table 3). The percentage of trophoblasts reacting with both reagents look similar, although normally they react in different ways; EDTA removes both Ca and Mg ions from the media, thus inactivates the entire complement pathway system, whereas EGTA specifically removes Ca ions leaving the alternative complement pathway intact.

5:3:5 Detection of human IgG and IgM immunoglobulins on the surface of trophoblastic cells. In order to study the presence or absence of alloantibodies on the surface of the trophoblastic cells to which complement might bind in the absence of added antibody, both immunoglobulins were studied using immunofluorescent staining. As shown in Fig.3, few cells showed positive staining for IgG immunoglobulin. The stained cells were medium to large in size and the pattern of staining was across the cell surface. No staining was detected for IgM immunoglobulin. Table 3, shows that 32-42% of the trophoblastic cells reacted with IgG using the indirect immunofluorescent staining, however, 17-19% of the cells were stained when the direct immunofluorescent method was applied. No detectable staining was observed for IgM.

5:3:6 The use of more than one antibody specificity in the cytotoxicity assay. From the previous results, it appeared that the placental cells are insensitive to the complement attack in the presence of only one antibody. One reason for this might be that the antigenic determinants on the large trophoblastic cell surface are insufficient density to

allow the bound antibodies to fix the complement. Accordingly, to check this probability both monoclonal and polyclonal antibodies were used. These included the monoclonal antibodies W6/32, 19/9, and polyclonal antibodies for B₂M as well as alloantibodies for HLA-A7 and B8. The results were similar to those when only one antibody was applied. As shown in Table 4, the percent of viable cells after subjection to antibodies and complement was not different from those for the controls (incubation with either antibody or complement).

5:3:7 Effect of NaN₃ on trophoblastic cells. Since the trophoblastic cells did not show a marked change in the percentage of cell viability after subjection to either one or more antibodies, it was proposed that these cells might possess a mechanism(s) by which they manage to escape such attack. Such mechanism(s) might involve the masking of the antigen-antibody complex by means of secreted glycoprotein, or the patching formation on the surface of the cell for this complex, followed by the capping process which usually end up by either endocytosis or shedding of that particular region. Such phenomenon is known as antigenic modulation.

In order to study whether these cells demonstrate such phenomena, it was necessary to add to these cells a substance which inhibits the occurrence of that process. Accordingly NaN₃ was used as an inhibitor at a final concentration of 10 mM (Gordon and Stevenson, 1981). One set of the isolated chorionic plate cells was pre-incubated with W6/32 and 10mM NaN₃ before the addition of rabbit complement. Another set of cells was incubated with both W6/32 and rabbit complement followed by the addition of NaN₃ at the times indicated. As can be seen in Table 6, there is no detectable change on the percent of viable cells compared to the controls.

Peripheral blood lymphocytes were treated with NaN_3 in order to check its effect on cellular viability in the absence of antibody and complement after incubation for different periods of time. As shown in Table 5, no detectable effect on cellular viability was seen after 60 min incubation with NaN_3 compared to the controls.

5:3:8 Complement killing assay using ^{35}S -methionine for the assessment of cell viability. To ensure the dye exclusion assessment of trophoblast viability, the incorporation of ^{35}S -met into the cells and supernatants was used. Previously, the effect of complement on trophoblast cells had been assessed by eosin dye exclusion. The results suggested that the cells are substantially complement resistant. Since this was a potentially important deduction, I thought it necessary to develop an independent assay of cell viability. I selected the measurement of protein synthesis (Chapter 2), as an appropriate assay. The initial experiments revealed several difficulties and complications due to the incorporation of ^{35}S -methionine into fetal calf serum proteins. To find out whether this was a serious problem, the assay was first set up using peripheral blood lymphocytes, since these were more convenient to prepare. From the TCA precipitation counts (Table 7), it can be seen that the TCA counts were incorporated into complete media (without cells) in the presence of FCS and FCS+C. The addition of 50ug/ml chloramphenicol and 100ug/ml cycloheximide did not alter the percentage of incorporation (Table 7). However, the addition of dithiothreitol (DTT) to the media in the presence of FCS and FCS+C reduced the counts by about 50% as shown in Table 8. The removal of FCS showed similar reduction in the percentage of ^{35}S -met incorporation when 10mM DTT were added (Table 8). Similar results were also obtained when peripheral blood lymphocytes were cultured in CM-FCS. Analysis

for the CM either alone or in the presence of chloramphenicol, cycloheximide and DTT using 5-15% SDS gels were carried out. Figure 5, showed the presence of a major labelled polypeptide with a molecular weight corresponding to that of albumin (68K). No change in the intensity of this radiolabelled band was observed when both chloramphenicol and cycloheximide were added to the media. However, the intensity was reduced when 10mM DTT was added. From this it could be argued that the presence of FCS in the CM seems to cause a high level of background due to non-biosynthetic reactions. Since my concern was to study the labelled proteins as an evident for cellular viability, I directed my study towards the structural proteins obtained by cell lysis with 2% SDS , rather than the secreted proteins to avoid any misinterpretations, due to the presence of non-specific labelling as mentioned above.

In addition to this, it was observed that 20-24 hrs of incubation with or without treatment were sufficient for ^{35}S -met incorporation into the peripheral blood lymphocytes.

5:3:9 Application of complement mediated cytotoxic assay on trophoblastic cells. Trophoblastic cells from different placentae showed variations in the percent of ^{35}S -met incorporation as shown in Figure 6. The addition of the monomorphic mAb W6/32 to the cells caused an increase in the incorporation compared to that for complement alone. However, a relative increase in the percent of incorporation was observed when both W6/32 and complement were incubated with the cells. Peripheral blood lymphocytes were used as a positive control; they were incubated with either antibody, complement, or both. As expected, the percent of incorporation was very low when these cells were incubated with both W6/32 and complement (Figure 6).

5:3:10 Effect of heat inactivated complement on trophoblastic cells.

Because it is difficult to ascertain the effect of complement under the previous conditions, it was necessary to study its effect after heat inactivation. For that purpose rabbit complement was heated at 56°C for 30 min, then applied to the trophoblastic cells after being subjected to the appropriate antibody. Figure 7A, shows the primary results for three placentae as the total counts for the cellular pellets were plotted against their corresponding TCA counts. As can be seen there are an unexpected variations in the cellular total counts that could be due to either cellular clump formation, cellular viability, or other unknown reasons. Figure 7B, represents the percentages of the TCA precipitable counts for the same placentae. As shown, the incubation with heat inactivated serum caused a relative increase in the percent of incorporation compared to that without heat inactivation. Discernable effect on lymphocytes treated with heat inactivated complement in the presence of W6/32 was also observed; the percent of incorporation into lymphocytes was much higher than those treated with W6/32 and complement without heat inactivation.

5:3:11 Effect of more than one antibody specificity on trophoblastic cells using the cytotoxic assay. The percentage of ^{35}S -met incorporation into trophoblastic cells after incubation with 2 antibodies in the presence of complement are shown in Figure 8. The lowest percent of incorporation, as can be seen, was when cells were incubated with W6/32, B₂M, and complement. On the other hand, the highest percentage of incorporation was observed when trophoblastic cells were incubated with both heat inactivated normal human serum (NHHS) + complement and complement alone. The incubation with these antibodies in the presence of complement did not cause a significant change in the

percentage of TCA precipitation as compared to the trophoblasts alone (Figure 8). Incubation of trophoblasts with either antibody or complement alone were used as a control.

5:3:12 Studies on mixed trophoblasts and peripheral blood lymphocytes.

In these experiments, both trophoblastic cells and peripheral blood lymphocytes were mixed at different ratios. The mAb W6/32 and rabbit complement were added to the cells as mentioned in the methods section. Figure 9, shows variation in the percentage of ^{35}S -met incorporation into three different placentae. As shown, 1 placenta out of 3 demonstrated high percentages of incorporation at the three different ratios (1:4, 1:10, and 1:20) as compared to that for cells alone. In addition one placenta showed a decrease in the percentage of TCA precipitation at the ratio 1:20 as compared to the ratios 1:4 and 1:10. The percentage of ^{35}S -met incorporation when trophoblasts from two placentae were incubated with the monomorphic monoclonal antibody W6/32 and complement was less than that for mixed trophoblasts and lymphocytes as well as trophoblasts alone. As far as the peripheral blood lymphocytes are concerned, a significant drop in the TCA percentage can be seen after incubation with both antibody + complement.

5:3:13 SDS / polyacrylamide gel electrophoresis. 5-15% SDS polyacrylamide gels were used for qualitative studies on the collected supernatants and the 2% SDS treated cell pellets. As can be seen in Fig.10, the autoradiographic film revealed several polypeptide bands shared between trophoblastic cells and lymphocytes without W6/32 and complement treatment (tracks 3,4 and 5,6 respectively). Tracks 1 and 2, showed lymphocytes treated with W6/32 and complement. As can be seen it is evident that no biosynthetic process occurred due to the absence of polypeptide bands. On the contrary, trophoblastic cells showed several

protein bands even after subjection to both W6/32 and complement (tracks 13 and 14). Mixed trophoblastic cells and lymphocytes using the 1:4, 1:10 and 1:20 ratios (tracks 7-12), showed several polypeptide protein bands. However, a discrete 51k radiolabelled polypeptide band in the total trophoblast cellular extraction which is absent from the total extract of lymphocytes can be observed (tracks 3,4 and 5,6).

5:3:14 Keratin extraction. From the previous experiments, it has been noticed that the autoradiographic films revealed the presence of a polypeptide band with a molecular weight of 51K. Because this molecular weight lies within the molecular weight range of cytokeratins, several extraction experiments were carried out on ^{35}S -met labelled trophoblastic cells. 10% SDS polyacrylamide gels were used for running the extract. Figure 11, track 1, showed the total placental cellular extract, tracks 2 and 3, showed the first and the last washes respectively. Track 4, showed the final extract in which two major polypeptide bands with molecular weight of 45K and 51K can be observed, in addition to this other minor bands were detected. Cytoskeletal lymphocyte extraction is shown in track 5, as compared to the total lymphocyte cellular extraction (track 6). As can be seen, no bands with the molecular weights of 45K and 51K are observed.

Treatment	Trophoblasts			Peripheral Blood Lymphocytes non-pregnant				
	% of viab. before assay	Average total cell no/well	Average viable cell no/well	Average % of viable cells	% of viab. before assay	Average total cell no/well	Average viable cell no/well	% of viable cells
Complement alone (C')	65	1692	1032	61	85	28108	20800	74
	63	1948	1128	58	80	21744	15220	70
	76	1516	1060	70	97	38168	35876	94
Normal Human Serum (NHS) heat inactive		1596	1016	60		27800	21404	77
		1828	1080	59		28312	21984	75
		1824	1296	71		38244	35568	93
NHS + C'		1652	992	60		27800	21404	77
		1772	1028	58		28168	21408	76
		1824	1296	71		37792	35904	95
B ₂ M		1692	1032	61		27948	20124	72
		1912	1148	60		28692	20048	70
		1824	1296	71		39012	36672	94
B ₂ M+C'		1852	1112	60		28608	572	2
		1952	1132	58		29320	28	0.1
		1744	1220	70		37912	37	0.1
W6/32		1696	1000	59		27948	21240	76
		1844	1012	55		29240	21344	73
		1836	1228	67		38712	37164	96
W6/32 + C'		1748	960	55		28900	28	0.2
		1812	1068	59		27948	52	0.2
		1919	1320	69		39512	64	0.2
19/9		1584	964	61		28192	21144	75
		1632	964	59		29240	21344	73
		1788	1216	68		38312	36396	95
19/9 + C'		1452	784	54		28900	2890	10
		1816	1036	57		28320	6514	23
		1768	1236	70		37312	18656	50
DA6.231		1756	1088	62		27900	20088	72
		1866	970	52		28140	19698	70
		1812	1288	71		38712	32905	85
DA6.231 + C'		1539	8116	55		28188	197	0.7
		1768	919	52		27958	58	0.2
		1892	1268	67		37912	38	0.1
Xenogeneic Ab. (Mouse)		1662	831	50		27492	19794	72
		1785	1053	59		26651	18389	69
		1804	1173	65		32397	25918	80
Xenogeneic Ab. + C'		1593	924	58		28716	29	0.1
		1771	903	51		26391	264	1
		1680	1008	60		36015	540	1.5
H316		1675	888	53		27118	21423	79
		1690	862	51		26375	18726	71
		1908	1317	69		33977	28201	83
H316 + C'		1735	954	55		22931	23	0.1
		1821	911	50		26572	531	2.0
		1657	1094	66		30988	620	2.0

Table1. Isolated human chorionic trophoblasts ($1-2 \times 10^3$ cell per well) and peripheral blood lymphocytes ($2-4 \times 10^4$ cell per well) were incubated with antibody at room temperature for 30 min. Rabbit complement was added and the cells were incubated for further 90 min. The percentage of eosin exclusion was used as an estimate of cell viability. Controls with antibody or complement alone are shown. These results represent three experiments.

Antibodies	A		B		C	
	Lymphocytes	Trophoblasts	Lymphocytes	Trophoblasts	Lymphocytes	Trophoblasts
NHS	-	-	-	-	-	-
B ₂ M	82	80	85	67	87	70
W6/32 (class I HLA)	80	85	88	68	89	73
19/9 (class II HLA)	61	9	57	4	79	14
DA6.231 (class II HLA)	71	7	68	11	82	8
Xenogeneic Antibodies	82	81	75	69	83	70
Anti-LCA	62	0	71	3	67	1
Anti-TLX	65	10	79	19	80	11

Table 2. Percentage of stained chorionic plate cells using indirect immunofluorescent staining method for the above specificities.

Treatment	Complement		Antibody				Antibody and Complement			
	Average total cell no/well	Average total cell no/well	A	Average total cell no/well	B	Average total cell no/well	C	Average total cell no/well	Average total cell no/well	Average total cell no/well
	Total no of cells	No of stained cells	% of stained cells	Total no of cells	No of stained cells	% of stained cells	Total no of cells	No of stained cells	% of stained cells	
Lymphocytes + FITC	-	-	-	-	-	-	-	-	-	-
Lymph+W6/32+NHS+R.anti-HC3+FITC	59	30	51	79	37	47	83	37	45	
Trophoblastic Cells (Troph)+FITC	-	-	-	-	-	-	-	-	-	-
Troph+R.anti-HC3+FITC	40	20	51	39	16	40	36	16	45	
Troph+NHS+R.anti-HC3+FITC	43	28	65	39	23	58	70	36	51	
Troph+NHS(HI)+R.anti-HC3+FITC	58	33	57	37	23	62	74	48	65	
Troph+W6/32+NHS+10mM EGTA+1mM Mg +R.anti-HC3+FITC	55	30	55	67	37	55	49	22	45	
Troph+W6/32+NHS+10mMEDTA+R.anti-HC3+FITC	36	24	67	28	16	57	14	7	50	
Troph+W6/32+NHS+R.anti-HC3+FITC	30	22	73	57	35	61	31	19	60	
Troph+R.anti-H-IgG+FITC	26	11	42	53	17	32	64	22	36	
Troph+Sh.anti-H-IgG"FITC"	28	5	18	16	3	19	35	6	17	
Troph+Sh.anti-H-IgM"FITC"	-	-	-	-	-	-	-	-	-	-

Table 3. The percentage of stained un-fixed chorionic plate trophoblasts using indirect immunofluorescent staining. The cells were stained for human complement C3 and IgG. Direct immunofluorescent staining was carried out to localize IgG and IgM.

Treatment	Complement			Antibody			Antibody and Complement		
	Average total cell no/well	Average viable cell no/well	Average % of viable cells	Average total cell no/well	Average viable cell no/well	Average % of viable cells	Average total cell no/well	Average viable cell no/well	Average % of viable cells
NHS	2092±297	1756±271	84	2044±220	1656±232	81	2492±221	2116±257	85
	2788±213	1532±211	55	2812±252	1604±217	57	2808±240	1684±158	60
	1160±240	636±227	55	1108±149	632±134	57	1060±211	616±142	58
W6/32+19/9				2488±283	2088±115	84	2488±216	2016±242	81
				2796±251	1480±290	53	2744±194	1564±229	57
				1076±194	568±89	53	1092±231	600±98	55
W6/32+B ₂ M				2436±231	2020±210	83	2144±199	1736±198	81
				2844±193	1564±230	55	2732±215	1476±159	54
				1044±250	552±101	53	1076±247	548±83	51
W6/32+19/9+B ₂ M				2292±199	1972±273	86	2257±183	1835±247	81
				2824±173	1580±211	56	2796±197	1480±225	53
				1124±223	616±214	55	1116±264	592±211	53
anti-HLA-A7				2144±250	1864±217	87	2392±159	1985±264	83
				2732±197	1584±260	58	2728±217	1072±201	54
				1124±231	608±92	54	1184±291	664±122	56
anti-HLA-B8				2496±220	2148±194	86	2568±214	2184±235	85
				2576±213	1572±225	57	2840±142	1476±121	52
				1180±183	636±119	54	1144±234	604±91	53
anti-HLA-A7+ anti-HLA-B8				2480±198	2060±266	83	2088±219	1672±293	81
				2680±175	1473±172	55	2796±199	1480±211	53
				1184±112	616±135	52	1068±246	564±108	53

Table 4. Effect of complement on the trophoblasts in the presence of more than one antibody. Isolated trophoblasts were incubated for 30-40 min at room temperature with antibodies for different specificities. This was followed by the addition of rabbit complement and incubation at room temperature for 90 min. Cellular viability percentage and the controls with either antibody or complement are shown. Each value is the mean of five determinations \pm the standard deviation.

Treatment	Peripheral Blood Lymph (90%)			Peripheral Blood Lymph (93%)		
	Average total cell no/well	Average viable cell no/well	Average % of viable cells	Average total cell no/well	Average viable cell no/well	Average % of viable cells
Normal Human Serum + C'	20193 \pm 3184	17972 \pm 2983	89	22240 \pm 3248	20016 \pm 2976	90
W6/32 + C'	21731 \pm 2987	4 \pm 1.6	0.02	21084 \pm 3111	4 \pm 0.9	0.02
Pre-incubation with 10mM NaN ₃ for 30 min before C' addition	20399 \pm 3021	6 \pm 1.9	0.03	22704 \pm 2840	2 \pm 1.2	0.01
10mM NaN ₃ (incubation for 15 min)	20971 \pm 2314	18245 \pm 2934	87	20990 \pm 3192	18891 \pm 3043	90
Incubation for 30 min	21852 \pm 3394	18574 \pm 3087	85	21394 \pm 3221	19255 \pm 3087	90
Incubation for 45 min	21608 \pm 2187	18799 \pm 3015	87	22725 \pm 3215	19998 \pm 3166	88
Incubation for 60 min	20912 \pm 2253	18403 \pm 3321	88	21798 \pm 2915	18964 \pm 2827	87

Table 5. Effect of Sodium Azide (NaN₃) on the peripheral blood lymphocytes. Peripheral blood lymphocytes at $2-4 \times 10^3$ cell per well were pre-incubated with W6/32 and NaN₃ for 30 min before the addition of rabbit complement. Another set of cells were incubated only with sodium azide at a final concentration of 10mM at the times indicated during the 60 min incubation. Each value is the mean of five determinations (wells) \pm the standard deviation.

room temperature, followed by incubation with rabbit complement. Sodium azide was then added to a final concentration of 10mM at the times indicated during the 60 min incubation. Cellular viability was assessed by eosin dye exclusion. Each value is the mean of five determinations (wells) \pm the standard deviation.

Treatment	Placenta A (71%)			Placenta B (74%)		
	Average total cell no/well	Average viable cell no/well	Average % of viable cells	Average total cell no/well	Average viable cell no/well	Average % of viable cells
MHS + C`	1908±254	1144±244	60	2114±210	1374±233	65
W6/32 + C`	1542±230	1018±149	66	1752±266	1191±120	68
W6/32 + 10mM NaN ₃ + C` (pre-incubation 30 min)	1812±210	1160±192	64	2137±231	1389±224	65
W6/32 + 10mM NaN ₃ + C` (incubation for 15 min)	1897±199	1271±245	67	1983±185	1249±252	63
W6/32 + 10mM NaN ₃ + C` (30 min)	1693±215	1100±227	65	2019±267	1353±197	67
W6/32 + 10mM NaN ₃ + C` (45 min)	1691±183	1032±219	61	1838±221	1213±139	66
W6/32 + 10mM NaN ₃ + C` (60 min)	1732±192	1126±235	65	1568±223	1019±261	65

Table 6 . Effect of Sodium Azide (NaN₃) on the isolated chorionic plate trophoblasts in the presence of antibody and complement. Isolated trophoblasts from the chorionic plate were pre-incubated with 10mM NaN₃ and W6/32 for 30 min before the addition of rabbit complement. Other set of cells were incubated with W6/32 for 30-40 min at room temperature, followed by incubation with rabbit complement. Sodium azide was then added to a final concentration of 10mM at the times indicated during the 60 min incubation. Cellular viability was assessed by eosin dye exclusion. Each value is the mean of five determinations (wells) +/- the standard deviation.

Supplements to Complete Media	No Additives			Cycloheximide 100 ug/ml			Chloramphenicol 50ug/ml		
	Total Counts cpm	ppt. Counts cpm	% of inco.	Total Counts cpm	ppt. Counts cpm	% of inco.	Total Counts cpm	ppt. Counts cpm	% of inco.
FCS	274122 (257121-283200)	2570 (2350-2529)	0.9	280171 (260988-283982)	2623 (2429-2701)	0.9	309487 (289127-321190)	2693 (2576-2723)	0.87
FCS + C [*]	263147 (245631-273622)	2369 (2129-2590)	0.9	309750 (281193-314973)	3081 (2831-3120)	0.9	280969 (260120-298537)	2529 (2290-2579)	0.9

Table 7. Effect of cycloheximide and chloramphenicol on the incorporation of ^{35}S -methionine into the media. A final concentration of 100ug/ml cycloheximide and 50ug/ml chloramphenicol were added to the media in the presence and absence of complement. The culture supernatant total and precipitable counts were studied. The percentage of ^{35}S -methionine incorporation into the media are shown. The mean of three different determinations and the range are shown above.

Supplements to Complete Media	No DTT			10 mM DTT		
	Total Counts cpm	pp. Counts cpm	% of Inco.	Total Counts cpm	ppt. Counts cpm	% of Inco.
FCS	466882 (452983-483217)	5364 (5211-5821)	1.0	469097 (457785-482100)	2572 (2318-2620)	0.5
FCS +C	462110 (431187-479385)	5587 (5278-5821)	1.0	459426 (439487-469283)	3501 (3372-3562)	0.8
no FCS	306682 (271293-317294)	1706 (1532-1754)	0.5	325970 (301119-329920)	1386 (1223-1576)	0.4
C without FCS	453419 (435271-462781)	4828 (4522-4900)	1.0	356674 (321795-367293)	3778 (3312-3820)	0.9
C without FCS	187751 (165273-197255)	1877 (1742-1953)	1.0	ND	ND	ND
PBL (no FCS)	192137 (182911-203379)	1329 (1200-1423)	0.7	ND	ND	ND
PBL+C (no FCS)	186442 (162276-198399)	1703 (1621-1780)	0.9	ND	ND	ND

Table 8. Effect of dithiothriitol (DTT) on ^{35}S -methionine incorporation into the media. DTT was added in a total concentration of 10mM to the media under the mentioned conditions. Total and precipitable counts for the supernatant were studied. The percentage of ^{35}S -methionine incorporation into the TCA precipitable counts are shown. Both the mean of three determinations and the range are shown above.

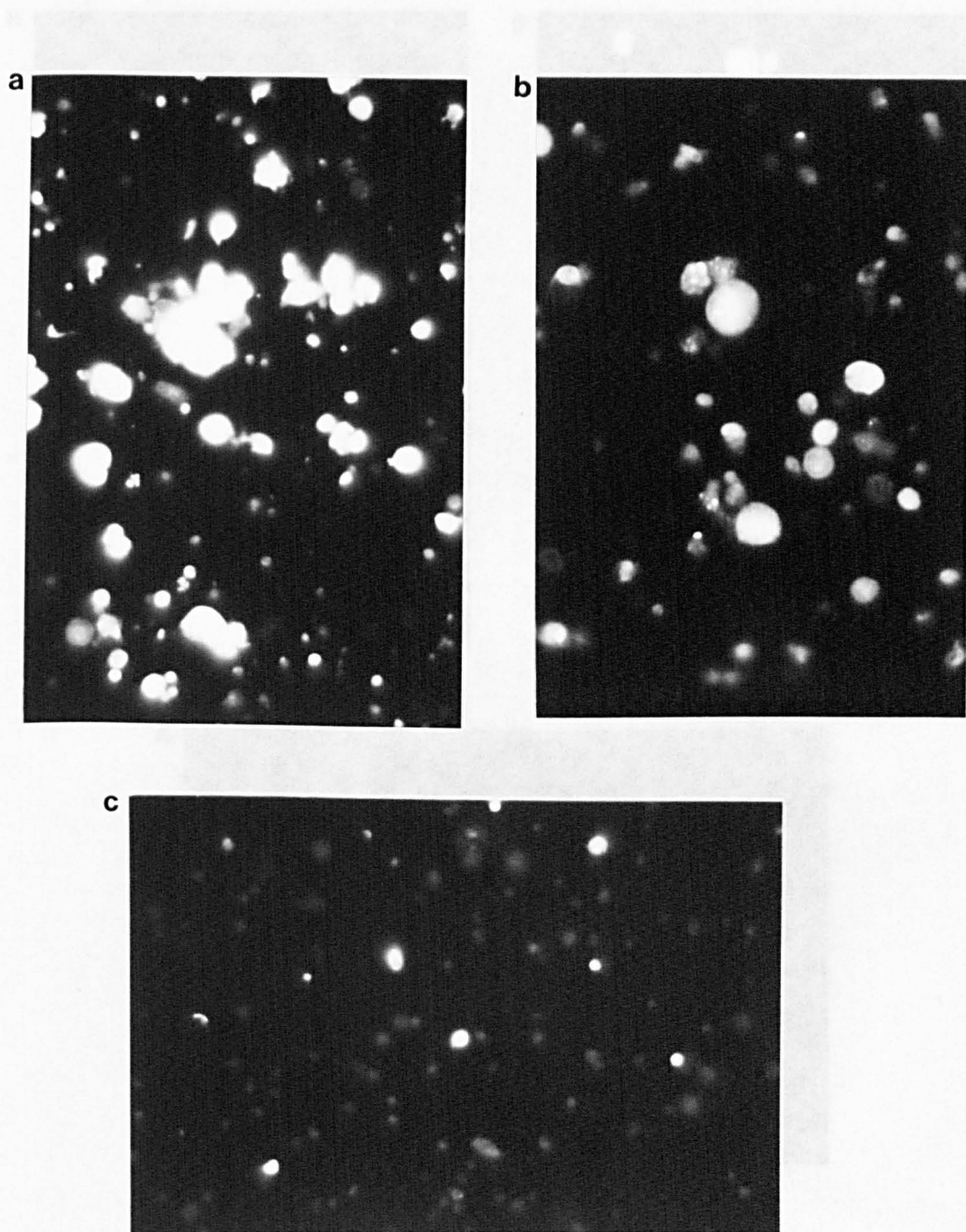


Figure 1. Indirect immunofluorescent staining on unfixed chorionic plate cells using (a) W6/32 (magnification X50); (b) anti-B₂M (magnification X100); (c) DA6.231 (anti-HLA-DR) (magnification X50).

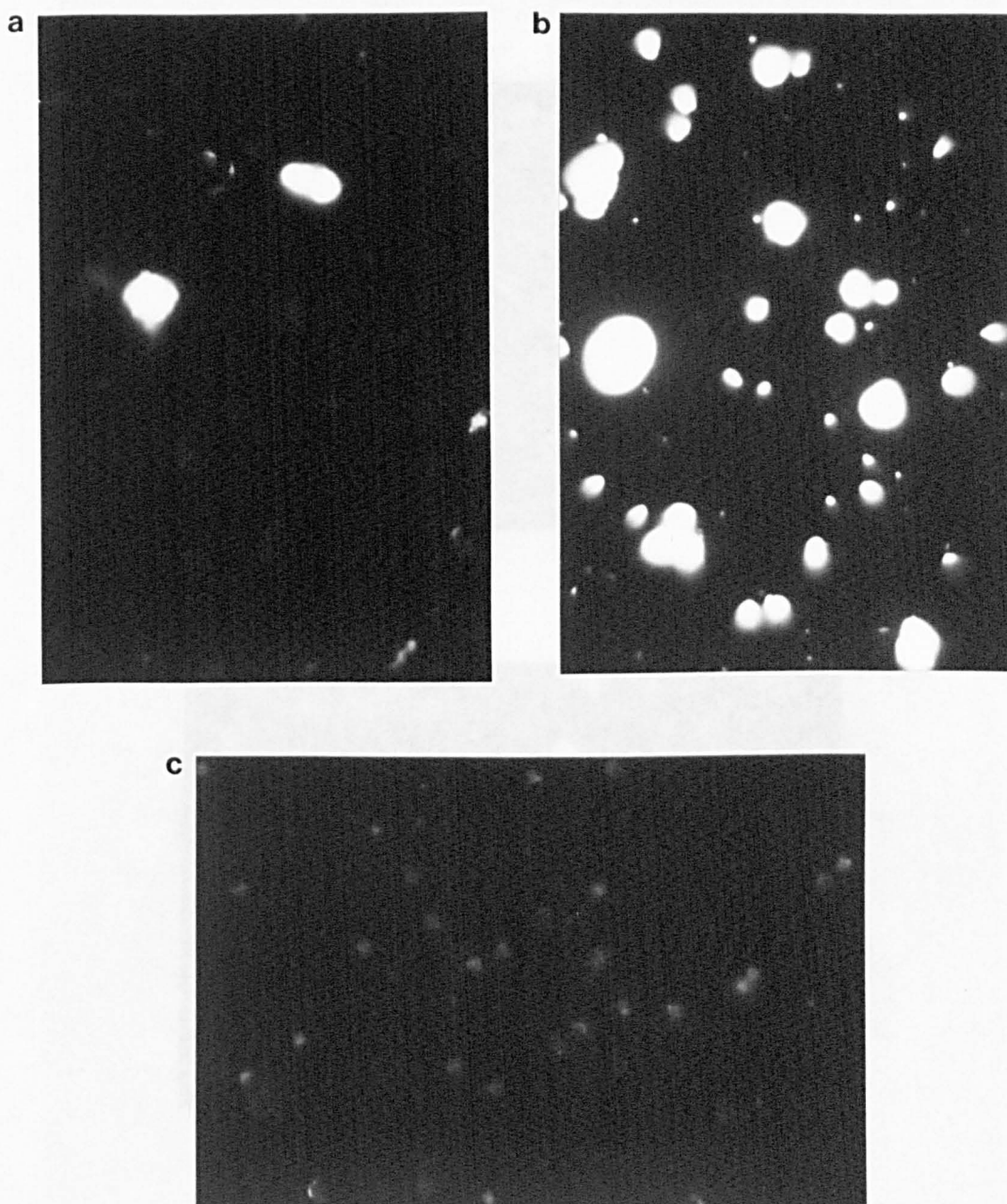


Figure 2. Indirect immunofluorescent staining on unfixed chorionic plate cells using (a) H316 (anti-TLX) (magnification X80); (b) mouse xenogeneic antibody (magnification X80); (c) anti-LCA (magnification X50).

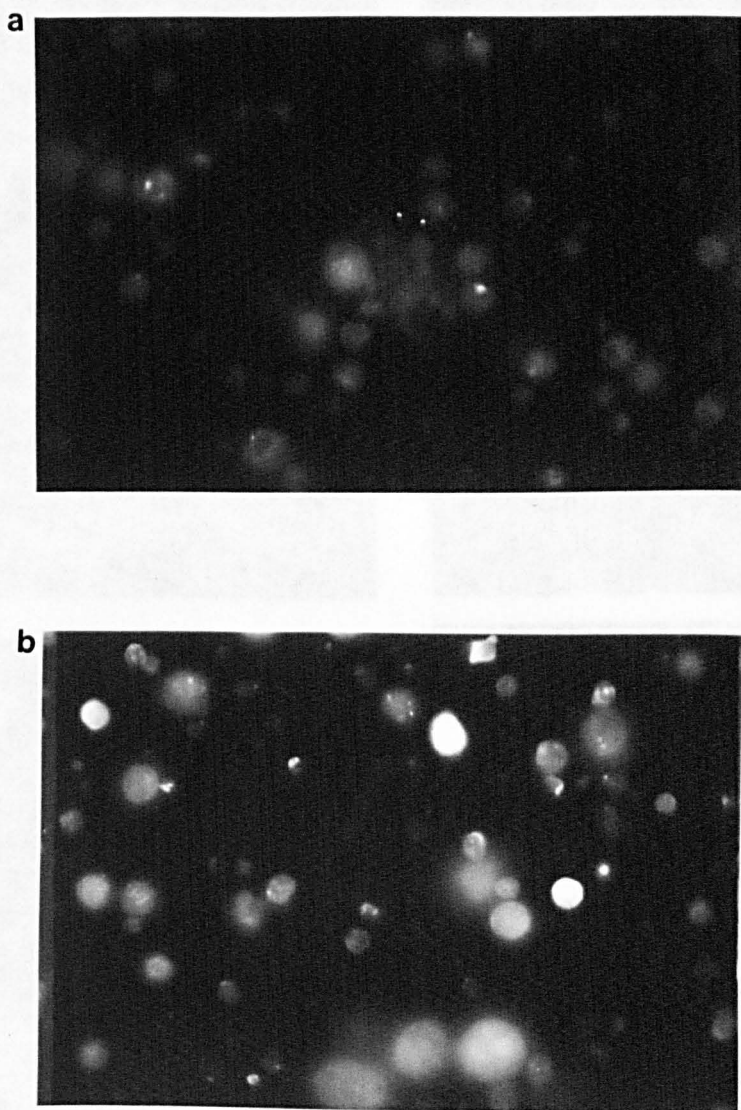


Figure 3. Indirect immunofluorescent staining on unfixed chorionic plate cells using (a) FITC conjugate alone (negative control) (magnification X100); (b) rabbit anti-human IgG (magnification X100).

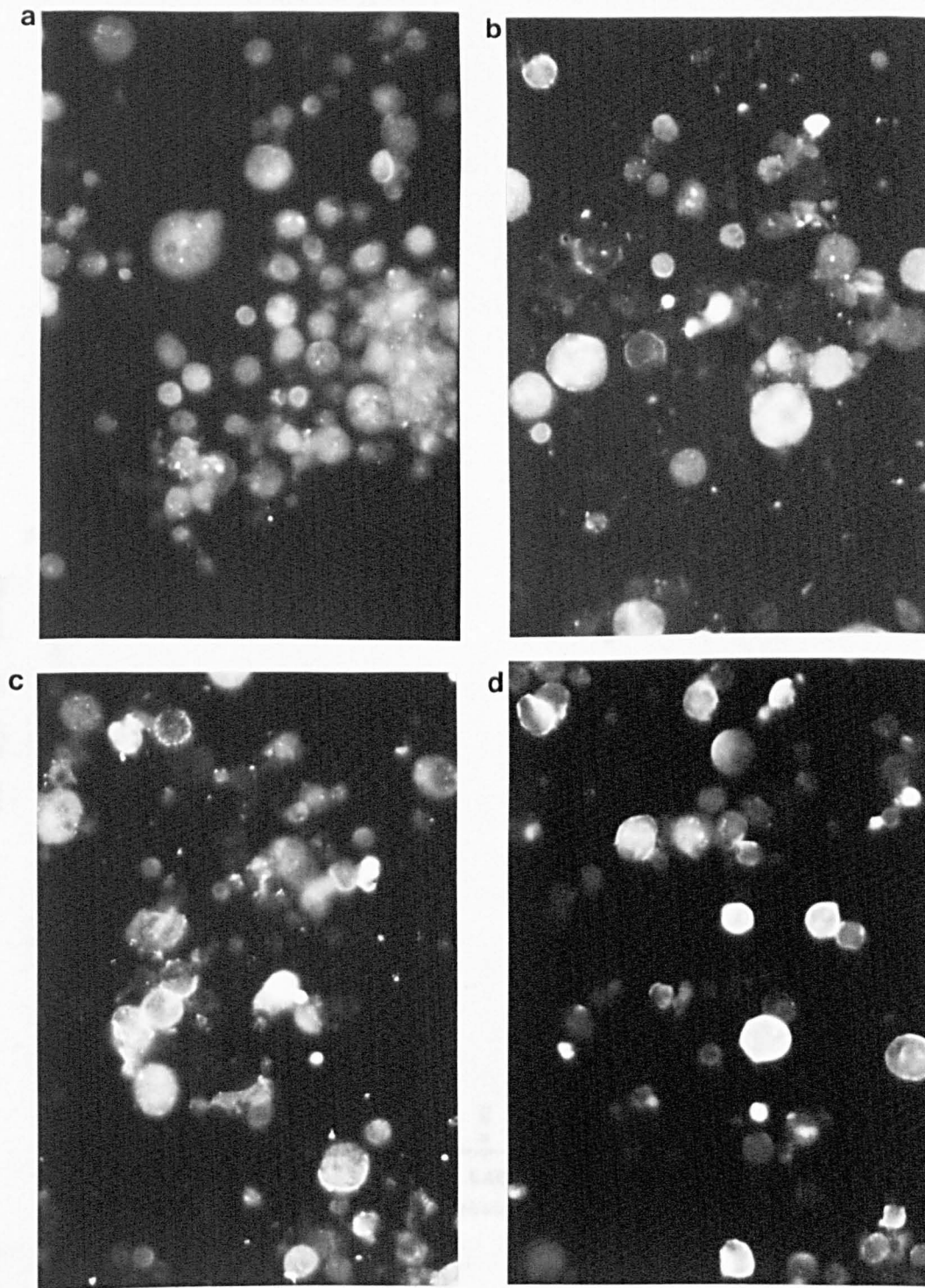


Figure 4. Indirect immunofluorescent staining on unfixed chorionic plate cells using (a) rabbit anti-human C3 (R anti-HC3) (magnification X100); (b) W6/32 + NHS + 10mM EGTA + 1mM Mg^{++} + rabbit anti-human C3 (R anti-HC3) (magnification X100); (c) W6/32 + NHS + 10mM EDTA + rabbit anti-human C3 (R anti-HC3) (magnification X100); (d) W6/32 + NHS + rabbit anti-human C3 (R anti-HC3) (magnification X100).

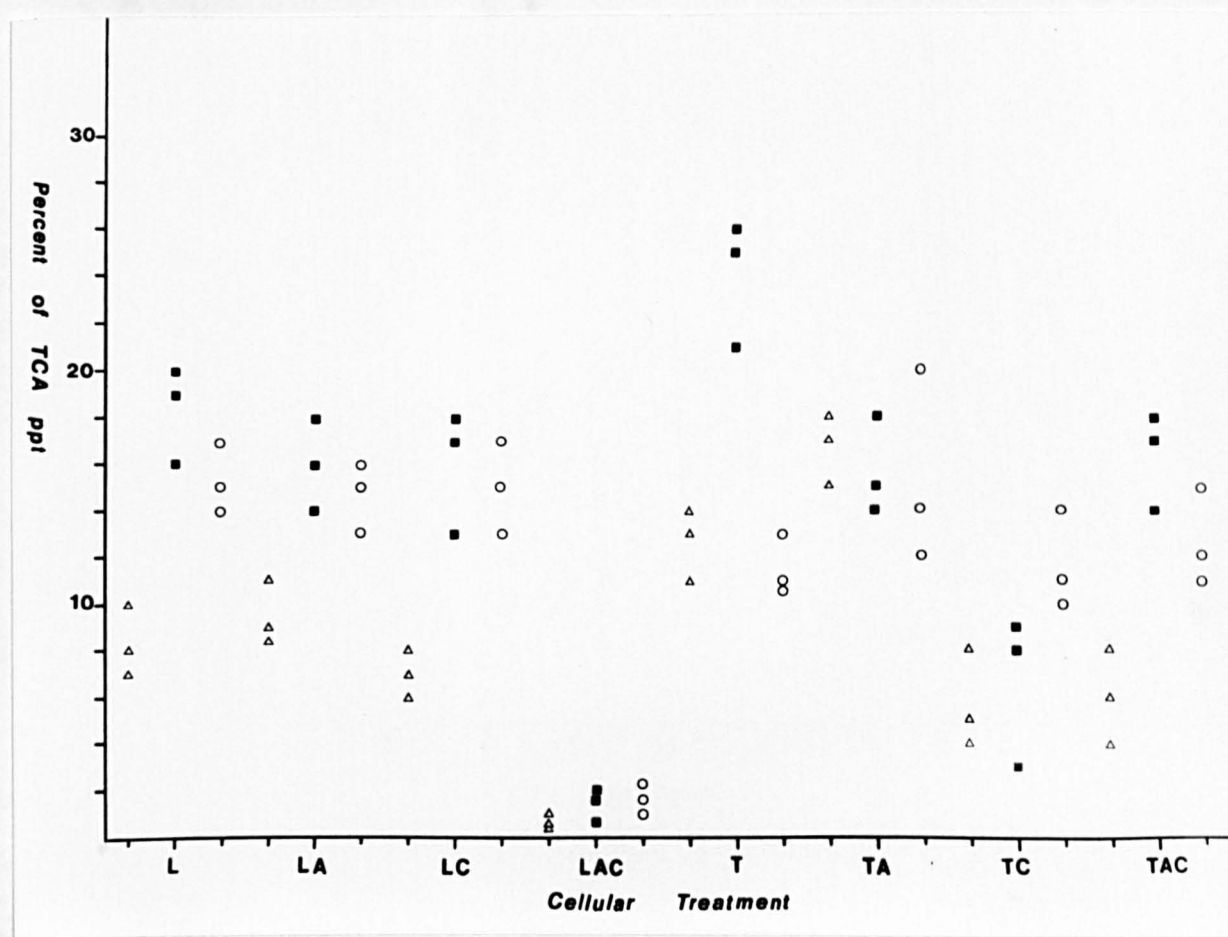


Figure 6. The percentage of ³⁵S-methionine incorporation into the TCA precipitated proteins. Lymphocytes (L) were used as a control; they were incubated with mAb W6/32 (LA), rabbit complement (LC), and W6/32 + rabbit complement (LAC). Isolated chorionic plate trophoblasts (T) were incubated with W6/32 (TA), rabbit complement (TC), and W6/32 + rabbit complement (TAC). The above results represent 3 experiments (■, ○, △). The cells were set up in triplicates.

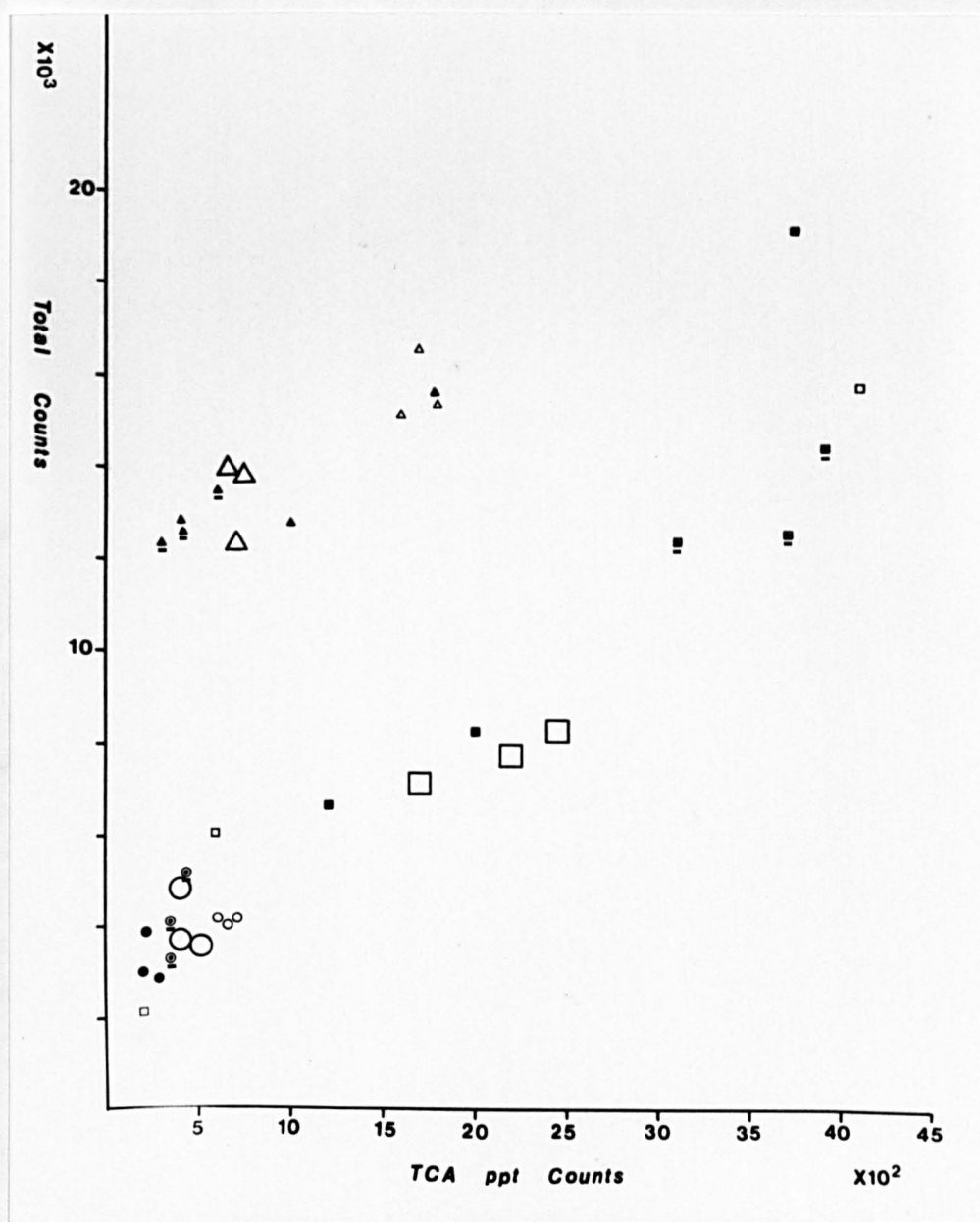


Figure 7. The effect of complement (C), heat inactivated complement (C_h), and W6/32 + complement (AC) on isolated chorionic plate cells (T) and peripheral blood lymphocytes (L). **A.** The data is drawn from three placentae (○, ■, ▲) and the total counts in each cell button are plotted against the corresponding TCA precipitated counts. The cells were set up in triplicates. The treatments of each cell pellet are as follows: T alone (○, □, △), TC (○, □, △), TC_h (○, □, △), and TAC (●, ■, ▲).

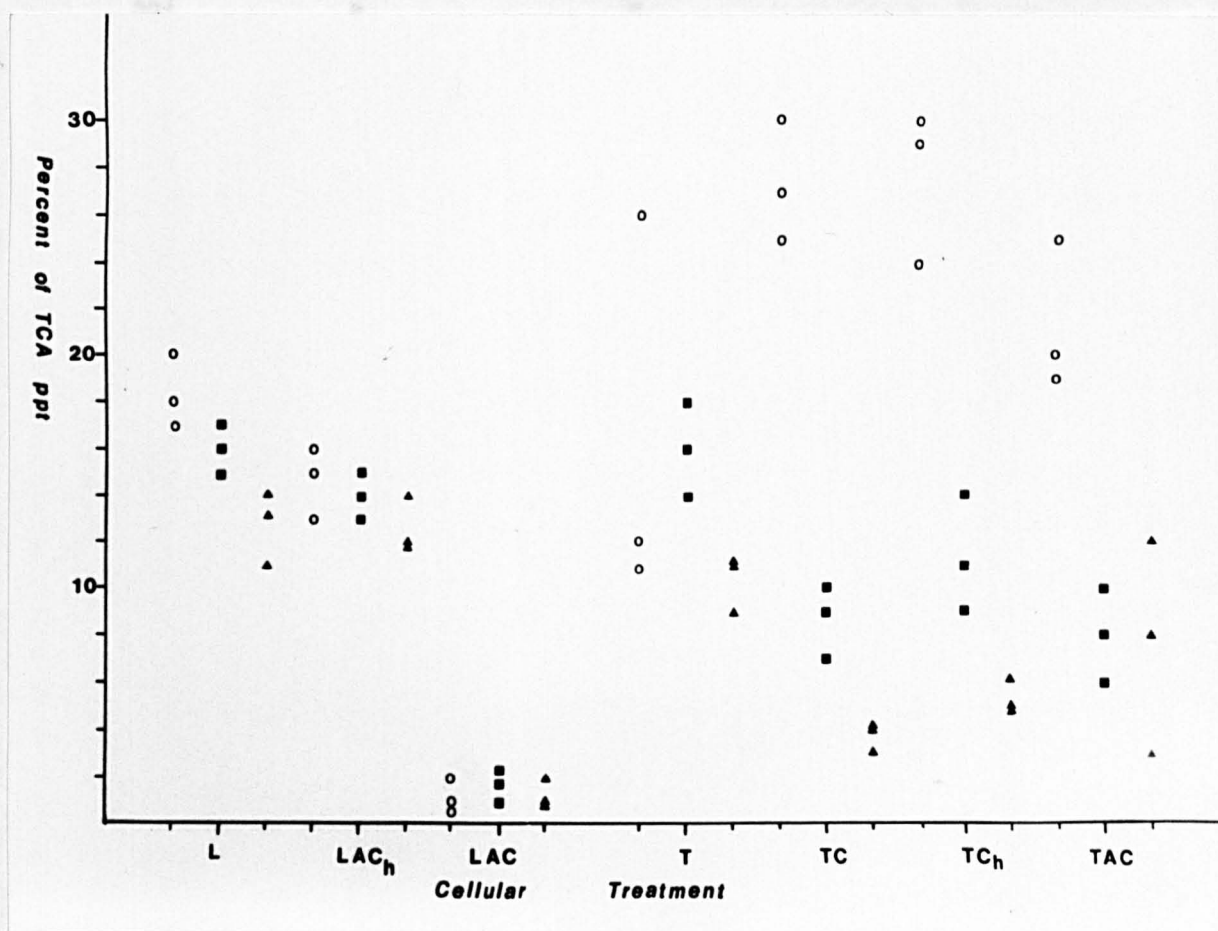


Figure 7B. The data is represented as the percentae of the TCA precipitable counts. Treatments are as shown in the graph.

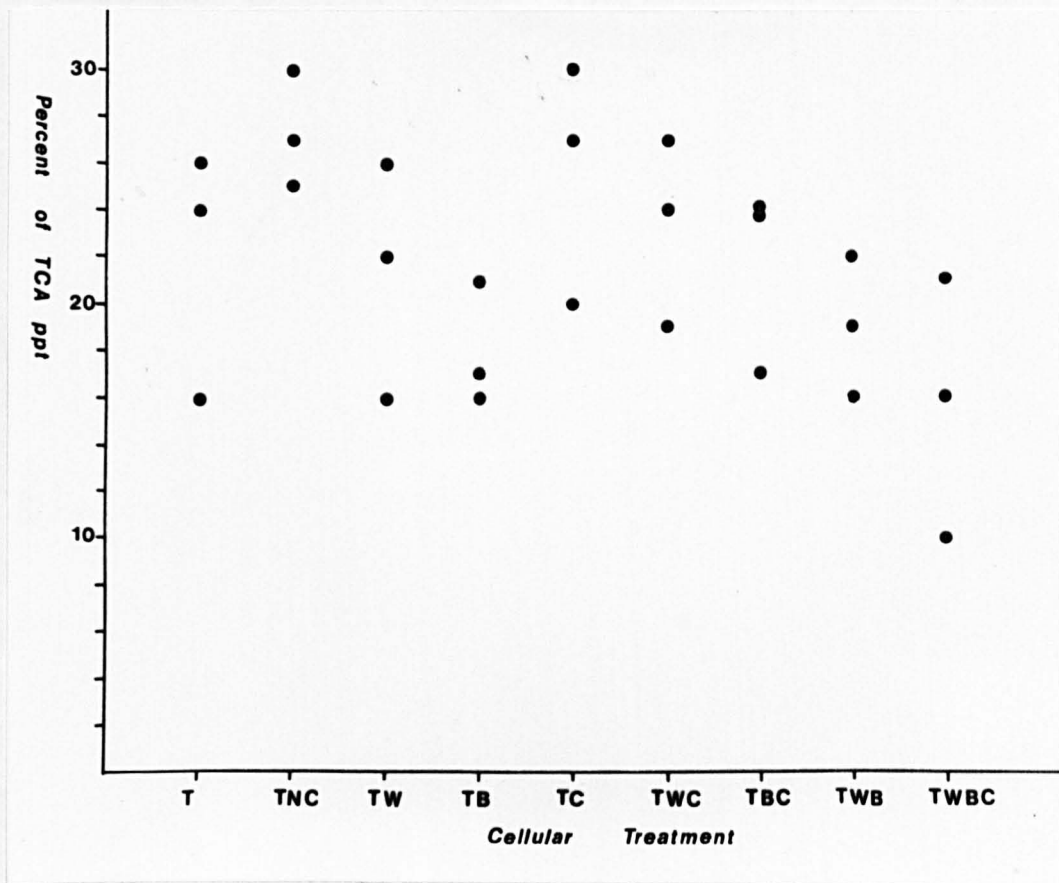


Figure 8. The effect of more than one antibody on trophoblasts in the presence of complement. The cells were set up in triplicates. The data represent the percentage of ^{35}S -methionine incorporation into TCA precipitated proteins. Trophoblasts from the chorionic plate (T) were incubated as follows: normal human serum (NHS) + rabbit complement (TNC), W6/32 (TW), B₂M (TB), rabbit complement (TC), W6/32 + rabbit complement (TWC), B₂M + rabbit complement (TBC), W6/32 + B₂M (TWB), and W6/32 + B₂M + rabbit complement (TWBC).

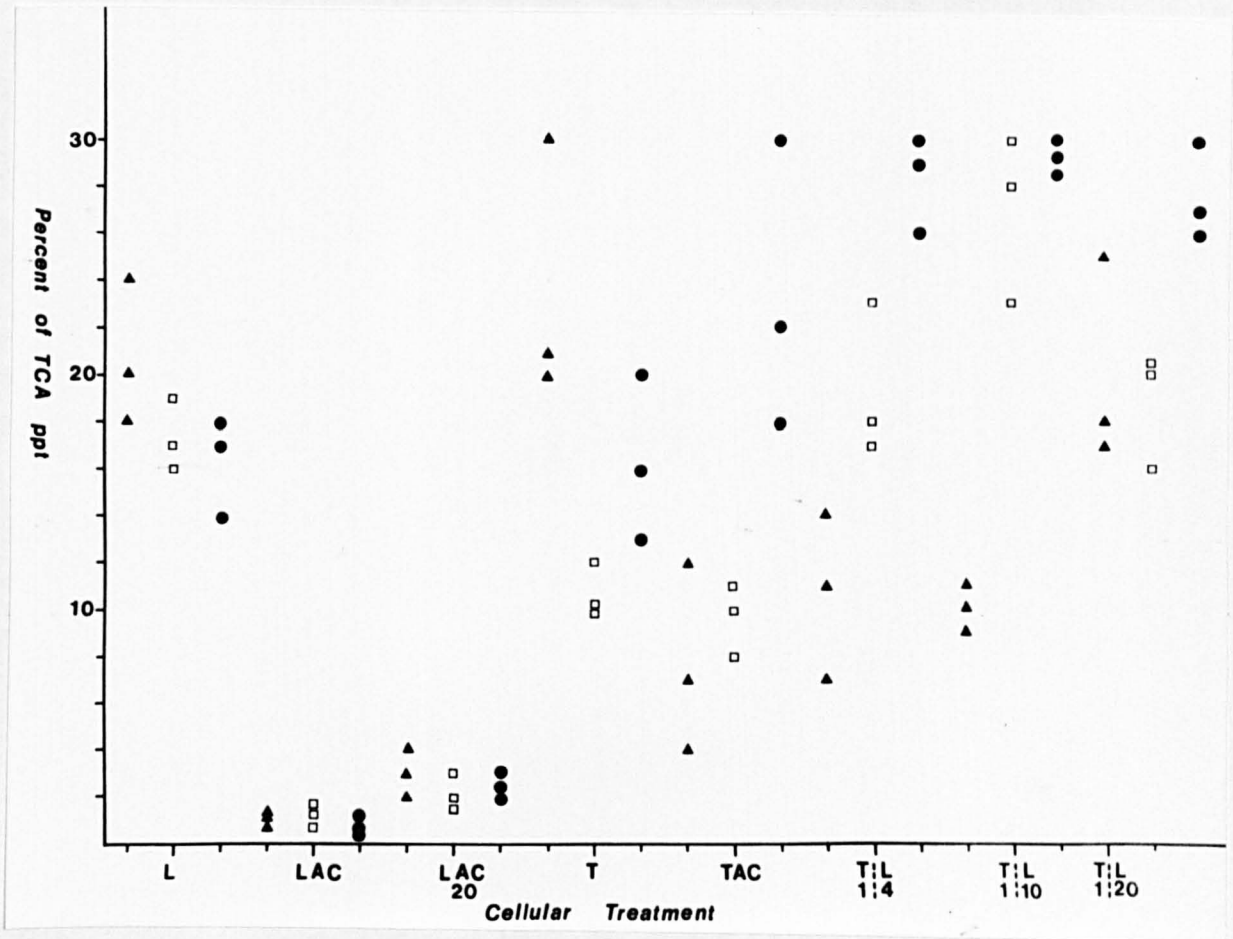


Figure 9. The effect of non-pregnant peripheral blood lymphocytes on the chorionic plate cells after being mixed at the ratios 1:4, 1:10, and 1:20. Three placentae were used (▲, ◻, ●). The cells were set up in triplicates. The data shows the percentage of ^{35}S -methionine incorporation into the TCA precipitated proteins. Treatments are as shown in the graph. (T) Trophoblasts, (L) lymphocytes, and (A) W6/32.

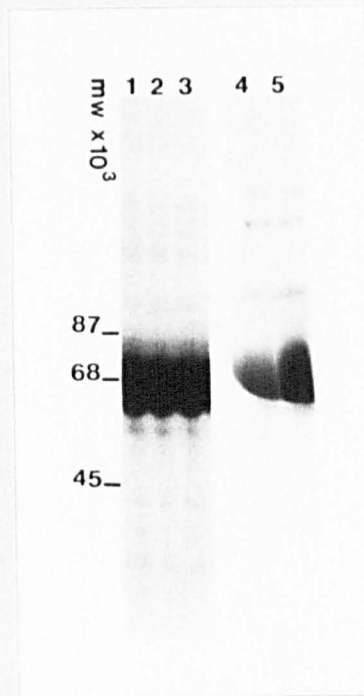


Figure 5. Analysis for complete media supernatant using SDS gels after incubation with ^{35}S -methionine for 48 hrs. Tracks 2 and 3 show the incorporation of ^{35}S -met into mainly bovine serum albumin after treatment with 50ug/ml chloramphenicol and 100ug/ml cycloheximide respectively. Track 1 shows complete media without treatment. Track 4 show ^{35}S -met incorporation into mainly bovine serum albumin after incubation with DTT compared to the control (track 5).

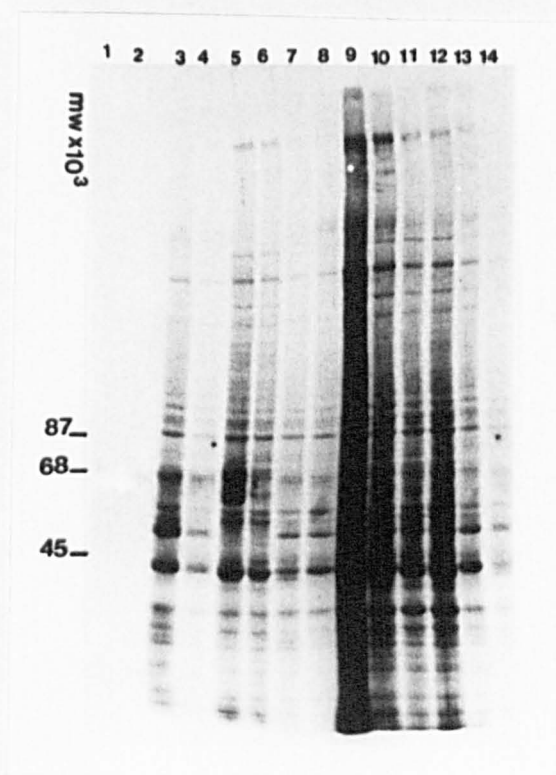


Figure 10. Analysis for trophoblasts and lymphocytes pellets after treatment with 2% SDS using 5-15% SDS electrophoresis gel. Tracks 1 and 2 show no ^{35}S -met radiolabelled polypeptides after incubating lymphocytes with W6/32 + rabbit complement. Tracks 3 and 4 show several ^{35}S -met labelled proteins for trophoblasts alone. Tracks 5 and 6 represent lymphocytes alone; tracks 7 and 8 represent mixed trophoblasts and lymphocytes at the ratio 1:4 ; tracks 9,10 and 11,12 represent mixed trophoblasts and lymphocytes at the ratios 1:10 and 1:20 respectively; tracks 13 and 14 represent trophoblasts treated with W6/32 + rabbit complement.

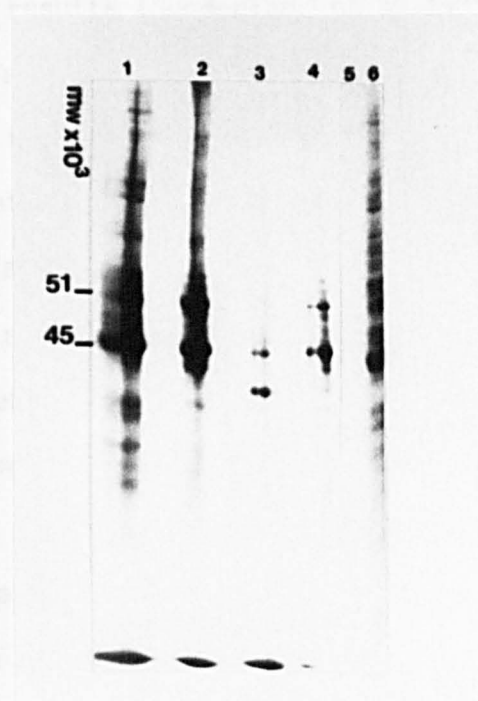


Figure 11. Keratin extraction. The extraction supernatants were analysed on 10% SDS gel electrophoresis. Track 1 shows the ^{35}S -met labelled proteins obtained after trophoblasts treatment with 2% SDS; tracks 2 and 3 show the labelled proteins in the first and last washes respectively. Track 4 shows the final keratin extract. Track 5 shows the keratin extract from the lymphocytes compared to the total cellular lymphocyte extract shown in track 6 after treatment with 2% SDS.

5:4 D I S C U S S I O N

According to the previous results, it was apparent that the placental cells do express class I MHC antigens on their surface, and concomitantly lack the expression of class II MHC. These results are consistent with previously obtained results (Sunderland *et al*, 1981; Sunderland, Redman and Stirrat, 1981a; Faulk and Temple, 1977; Goodfellow *et al*, 1976). In these studies it was found that MHC antigenic determinants are localized in the cytotrophoblast, whereas such antigens are not expressed by the syncytiotrophoblasts. Subsequently, it became interesting to do HLA typing on these cells to study the phenotypes of HLA class I antigens expressed by both fetal and maternal cells.

The results presented in this chapter amply demonstrated that the placental cells when subjected to antibody (W6/32) and rabbit complement failed to lyse, compared to the peripheral blood lymphocytes (PBL). This was assessed by the eosin dye exclusion method. The placental cells looked refractile compared to the killed PBL which were dark red due to their lysis. This resistance to lysis might be due to scattered or few alloantigens on the large surface of these placental cells compared to PBL. If that is the case then these cells are not expected to be lysed when treated with these cytotoxic IgG type antibodies that cause lymphocyte death due to complement fixation. This led me to try and incubate the cells with more than one antibody for different specificities (eg. W6/32 and anti-B₂M) to see if a greater density of antibody on their surface would increase cell lysis. However, no decrease in the number of viable cells was observed (Table 4). From this it could be argued that the antibodies applied did not bind to their

specificities, the complement failed to bind to the antigen-antibody complexes, or that it bound in insufficient quantities to cause lysis.

Further studies using indirect immunofluorescence staining, revealed that both antibodies and the complement do bind to these cells in suspension (Tables 2 and 3, Figures 1 and 4). This would indicate that antigen-antibody complexes are formed, and complement binding to these complexes was shown to occur. These results are in agreement with previous studies. Smith (1983), found that cultures of murine placental cells after isolation from whole placentae at 9 days post-coitum, were normally resistant to immune damage by lymphocytes, cytotoxic antibodies or antigen/ antibody complexes. From this it would be interesting to know why the placental cells are resistant to such attack. From the results mentioned above, it could be possible that the trophoblastic cells behave like the TL thymic leukemia cells (Chatenoud and Bach, 1984); they might become stimulated by the exposure to different antibodies (e.g. W6/32), therefore, they would tend to modify their antigenic determinants by means of antigenic modulation process. After patch formation capping and endocytosis might take place leading to the degradation of the capped immunoglobulins (Chatenoud and Bach, 1984). Additionally, these placental cells might synthesise a special type of glycoprotein that would mask the antigen-antibody complex, thereby preventing them from being lysed by the added complement. The addition of NaN_3 to these cells did not cause any change in the cell viability compared to the control (Tables 5 and 6). This indicated that the addition of this antigenic modulation inhibitor either did not affect or prevent that process displayed by these cells, or these placental cells did not exhibit such phenomenon. Additionally, it was found from studies carried out on mouse RADA1 leukaemia cells and A/J

thymocytes that modulating activity of human C3 , unlike that of mouse serum, was not destroyed by heating, and modulation was achieved with the IgG fraction of TL antiserum and heated human C3 , suggesting a contribution of factor B-like, C3-cleaving activity by the cells, resulting in the deposition of C3 on the cell surface. From this it was concluded that the intercalation of C3 into aggregated TL antigen-antibody complexes might be required to achieve modulation (Stackpole, Jacobson and Galuska, 1978). It could be postulated that the placental cells undergo the latter process, since both antibodies and human C3 were detected on their surface, and the addition of NaN_3 would not affect the antigenic modulation displayed by these cells. The possibility cannot be excluded, however, that the detected C3 on the surface of the placental cells is not bound to an antigen-antibody complex, but to either complement receptors or to endogeneous alloantibody-antigen complexes. This has been shown as mentioned above (Table 3, Figures 3 and 4), since complement and IgG binding was detected in the absence of added antibody. However, the staining intensity and the number of stained cells were increased after the addition of NHS, indicating complement binding . From this it can be concluded that the placental cells are resistant to the complement attack. However, it has been shown recently that placental specific antiserum raised to purified human placental villous plasma membrane were cytotoxic to BeWo choriocarcinoma cells in the presence of complement as determined by trypan blue uptake (Sinha *et al*, 1984). As far as the normal placental cells are concerned, no sign of cell lysis was detected. This could be due to the possibility that these cells behave like tumor cells in resisting or escaping any immune attack. It was found that the resistance of tumor cells to killing was accompanied

by an enhancement in complex lipid synthesis by the cells, due to physical and/or chemical changes in the cell membrane. Therefore, the lipid synthesis might be involved as a mechanism to repair damage to the membrane resulting from complement action. In addition, such lipids might be part of anticomplementary moities on the cell membrane affecting binding and/or activation of C components. They also could be exported from the cell to become anticomplementary to fluid phase C (Schlager, Ohanian and Borsos, 1978; Schlager and Ohanian, 1983; Ohanian, Schlager and Saha, 1981). Bischof, (1981, 1984), claimed that PAPP-A inhibits directly and completely the whole complement system, since it binds to C3. This also might account for the resistance of the trophoblast to complement lysis, since they reacted strongly with anti-PAPP-A. However, other studies did not support Bischof finding, for PAPP-A failed to inhibit complement-lysis of sensitized red cells (Gore and Sutcliffe, 1984).

The trophoblast cytotoxicity results as assessed by dye exclusion method were confirmed using ^{35}S -met as a mean to assess cellular viability by detecting the cellular uptake of this radiolabelled aminoacid using TCA precipitation and SDS polyacrylamide gel electrophoresis. In contrast to PBL, as mentioned in the results section, in some placentae the trophoblasts incubated with antibody and complement showed relatively slight increase in the TCA counts compared to trophoblasts alone (Fig. 6, 7 and 9). The autoradiography of the SDS gels revealed large numbers of polypeptide bands from the trophoblasts under different conditions, indicating their viability (Fig. 10). On the contrary, PBL after treatment with antibody and complement, failed to show radiolabelled bands on the autoradiograph (Fig. 10, tracks 1 and 2), indicating their death. This was consistent with the low

percentage of TCA precipitable counts compared to the lymphocytes alone (Fig. 6,7 and 9).

The primary results of the radiolabelled cytotoxicity assay revealed an unexpected variations among the total ^{35}S -met counts, which might be accounted for by cellular clump formation, cellular viabilities and perhaps other unknown reasons. The TCA precipitable counts were affected by these variations in the corresponding total counts when plotted against them as shown in Figure(7A). Thus it appears difficult to manifest the actual events that took place in those experiments. Accordingly, it became necessary to study the percentages rather than the actual TCA counts in order to normalize the obtained results.

The incubation of the placental cells with lymphocytes caused a relative increase in the TCA precipitation in some placentae. This could be due to recognition of the MHC antigens or other trophoblast antigenic determinants that induced interleukins or growth factor , which consequently resulted in the metabolically active cells (Wegmann,1984). As shown in the autoradiograph (Figure 10), the mixed trophoblasts and lymphocytes as well as trophoblasts alone showed a 51k polypeptide that concurrently distinguished them from lymphocytes. This 51k radiolabelled polypeptide lies within the molecular weight range of cytokeratins (40-58k, Wu and Rheinwald, 1981). Because these trophoblastic cells display a positive reaction with antibodies against cytokeratin such as TR1, PKK1 as shown earlier in Chapter 4, this 51k polypeptide might belong to the cytokeratin family. However, since this polypeptide is absent from the lymphocyte preparations, it still can be used as a marker for these cells.

In conclusion, by understanding the mechanisms by which the placental cells may seek to subvert immunologic attack, it may be possible to

design regimens to overcome the tumor cells subversion, and allow the immune system to play a potent role in tumor defence, concomitantly, to try and help in the establishment of successful organ tissue transplant.

CHAPTER 2

GENERAL DISCUSSION

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From the short term tissue culture experiments which were carried out on placental tissue fragments taken from different areas including the uteroplacental interface (Hitchcock's layer), the placental villi and the chorionic plate, it was apparent that large numbers of polypeptides are synthesized and secreted to the culture supernatant (Chapter 3, Fig. 1). Both fibrinogen and pregnancy associated plasma protein A (PAF-A) were identified. This was confirmed when immunohistochemical localization studies were carried out on wax-embedded and frozen placental sections (Chapter 2, Figures 4 and 5). Synthesis and localization studies showed similar distribution of these proteins in extra-villous placental cells in Hitchcock's layer, placental cytotrophoblast cell islands, and the chorionic plate. Patchy localization was found in the syncytiotrophoblasts for PAF-A only. These results encouraged us to try and isolate these cells in order to identify them and study their immunological aspects. Accordingly, by means of trypsinization, cells were isolated from Hitchcock's layer and the chorionic plate. The isolated cells were highly heterogeneous. Their viability which was assessed by trypan blue exclusion showed variation among the different placentae. The viability percentages ranged between 40-93% (Chapter 3, Figure 1). These cells were cultured successfully for 2-4 months without fibroblast overgrowth using different substrates including glass clot. Although cells from both the chorionic plate and Hitchcock's layer digested the plasma clot substrate, Hitchcock's layer cells were more active in that respect (Chapter 3, Figure 1). Such observation could be due to differences between the trophoblast populations depending on their position (Bord and Garton, 1970). The

GENERAL DISCUSSION

From the short term tissue culture experiments which were carried out on placental tissue fragments taken from different areas including the uteroplacental interface (Nitabuch's layer), the placental villi and the chorionic plate, it was apparent that large numbers of polypeptides are synthesized and secreted to the culture supernatant (Chapter 2 Fig 1). Both fibronectin and pregnancy associated plasma protein A (PAPP-A) were identified. This was confirmed when immunohistochemical localization studies were carried out on wax-embedded full placental sections (Chapter 2 Figures 4 and 5). Synthesis and localization studies showed similar distributions of these proteins in non-villous placental cells in Nitabuch's layer, placental septae, cytotrophoblast cell islands, and the chorionic plate. Patchy localization was found in the syncytiotrophoblasts for PAPP-A only. These results encouraged me to try and isolate these cells in order to identify them and study their immunological aspects. Accordingly, by means of trypsinization, cells were isolated from Nitabuch's layer and the chorionic plate. The isolated cells were highly heterogeneous. Their viability which was assessed by trypan blue exclusion showed variation among the different placentae. The viability percentages ranged between 40-98% (Chapter 3 Figure 1). These cells were cultured successfully for 2-4 months without fibroblast overgrowth using different substrates including plasma clot. Although cells from both the chorionic plate and Nitabuch's layer digested the plasma clot substrate, Nitabuch's layer cells were more active in that respect (Chapter 3 Figure 3). Such observation could be due to differences between the trophoblast populations depending on their position (Boyd and Hamilton, 1970). The

proteolytic activity which was inhibited by the respiratory inhibitor EACA (Chapter 3), might be due to plasminogen activator synthesis (Martin and Arias, 1981) which has also been identified in the postimplantation mouse embryo (Kubo et al, 1982 ; Strickland, Reich and Sherman, 1976) and guinea pig and rat blastocyst (Owers and Blandau, 1971). This synthesis would help the invading cytotrophoblasts, during the implantation process and the establishment of the placenta, to cause local breaching of the decidual spiral arterial walls with the result of multiple openings of the vessels into the intervillous space. This is usually followed by the replacement of the blood vessel endothelium by these trophoblasts forming the so-called endovascular trophoblasts (Pijnenborg et al, 1980; Pijnenborg et al, 1981b; Pijnenborg et al, 1983). Subsequently, Rohr's and Nitabuch's layers would be formed. It has been shown by Sutcliffe et al, (1982) that the uteroplacental fibrinoid is composed of several polypeptides including fibrin/fibrinogen type proteins as well as others with molecular weight over 105,000. Additionally, in my study I managed to locate fibronectin and PAPP-A in that layer (Chapter 2 Figures 4 and 5). From both Sutcliffe's and my study on the fibrinoid layer, it was evident that the origin of fibrin/fibrinogen polypeptides is not placental (Chapter 2), therefore indicating their maternal origin. In my opinion, the formation of fibrinoid layers after trophoblast invasion might be of great significance. This could be a result of endovascular trophoblast invasion and their subsequent opening to the decidual spiral arteries into the intervillous space. The excess of maternal blood that enters the intervillous space might participate in the fibrin deposition at the fibrinoid layer due to its seepage into those regions. This might lead to the cytotrophoblasts in the superficial decidua being embedded by the

fibrinoid material. Such cytotrophoblasts probably do not synthesize plasminogen activator; instead they might be another trophoblast population which synthesize other proteins such as fibronectin and PAPP-A. The process of fibrinoid formation might be gradual; as fibrin is laid down, these cytotrophoblasts would synthesize placental FN in order to anchor themselves to fibrin and perhaps to use fibrin scaffolding for their movement as the placental development proceeds. In addition, since plasminogen binds to fibrin/fibrinogen (Lucas, Fretto and McKee, 1983), PAPP-A synthesis might inhibit any maternal plasmin activity as claimed by Bischof (1979). However, such a finding was inconsistent with the results of Gore and Sutcliffe (1984). After the establishment of the placenta, more fibrinoid might be formed for the regulation of blood flow into the placenta (Brosens, Robertson and Dixon, 1967) leading to more cytotrophoblast embedding. Thus, the formation of fibrinoid and the availability of clotting factors might be very important for the placental establishment. For, it was observed that women with Factor XIII deficiency suffer from recurrent abortions that can be remedied by plasma transfusion (Ikkala, Myllyla and Nevanlinna, 1964).

Further immunohistochemical localization studies were carried out on placental frozen sections and cytospin preparations from Nitabuch's layer and chorionic plate. From these studies it was found that cytotrophoblasts in both layers stained for alpha and beta sub units of the placental protein HCG (Chapter 4 Figures 3 and 4). They also reacted with anti-cytokeratin (marker for epithelial cells) (Chapter 4 Figure 2) and other trophoblast markers including H315, H316 and 7A11 (hybridoma antibody prepared in our lab) (Chapter 4 Figures 5 and 6). Moreover, these cells were stained for B₂M and HLA class I monomorphic

determinants using the mAb W6/32 (Chapter 4 Figure 8). These results are consistent with previous studies as mentioned in Chapter 4. Furthermore, the cytotrophoblasts did not react with anti-LCA, anti-vimentin (PK-V), anti-UEA I Lectin and anti-HLA-DR (Chapter 4 Figure 9). Cells in the mesenchyme of the villous stroma and the subepithelial layer in the chorionic plate were positively stained for HLA-DR which is in accord with previous results (Sunderland et al., 1981; Sutton, Mason and Redman, 1983; Jenkins, O'Neill and Johnson, 1983; Bulmer and Johnson, 1984). In addition, HLA-DR positive cells were reported in the placental bed (Bulmer and Sunderland, 1984; Sutton, Mason and Redman, 1983). These cells were found to be macrophages rather than dendritic cells since they display both the Leu-M₃ tissue macrophage antigen and lysosomal enzyme activities (Bulmer and Johnson, 1984). These macrophages were postulated to serve primarily as (i) antigen presenting cells for the development of forms of immune responses to genetically dissimilar tissue antigens, (ii) part of the host cellular defence system against infection, (iii) phagocytotic and pinocytotic cells removing unwanted protein debris with the help of tissue fibronectin (Yamada and Older, 1978).

On the other hand, the trophoblasts in the cytospin preparations showed two major placental cell populations. One population showed evident staining for placental proteins (FN, PAPP-A, SP, HPL and alpha and beta subunits of HCG), other trophoblast markers including cytokeratin, H315, H316 and the hybridoma antibody 7A11 (Chapter 4 Table 1). In addition, this population showed staining for B₂M and the monomorphic determinants for class I HLA antigens using the mAb W6/32. The minor cell population were stained with anti-UEA I lectin (an endothelial cell marker), anti Vimentin, anti LCA and anti HLA-DR using

the mAb DA6.231. This indicated low contamination of endothelial cells, fibroblasts and leucocytes, therefore confirming the previous culture studies which demonstrated lack of stromal fibroblast overgrowth. As far as the HLA-DR stained cells are concerned, it could be probable that such cells are macrophages since they are smaller than cytotrophoblasts and bigger than lymphocytes. This, therefore, requires further elucidation, perhaps by means of double staining using markers for macrophages, endothelial cells and possibly trophoblast markers such as cytokeratin. A previous study performed by Brami and co-workers (1983) found by using double immunofluorescence techniques on epitheloid trophoblast monolayers, that these cells react with both anti-HLA-DR and anti-HCG antibodies. These results remain uncertain, since it is still agreed that HLA-DR is not detectable on trophoblasts (Redman 1983).

Immunological studies were carried out on the isolated chorionic plate cells, as described in Chapter 5. This was performed as an attempt to type those cells for HLA antigens using the standard Terasaki microcytotoxicity test to study whether the expected alleles are expressed. Cytotoxicity was assessed using eosin dye exclusion and ^{35}S -met incorporation. A range of antibodies were used for this purpose. Human peripheral blood lymphocytes were used as positive control. Trophoblastic cells were shown to be relatively resistant to this sort of attack; this was confirmed by the incorporation of ^{35}S -met into several polypeptide bands, which were not seen with the lymphocytes (Chapter 5 Figure 10). More recently, it has been shown that antibodies against purified human term placental villous plasma membrane were cytotoxic to BeWo choriocarcinoma cells in the presence of complement as determined by trypan blue uptake (Sinha *et al*, 1984). Immunofluorescent studies revealed the binding of anti-HLA class I monomorphic

determinants (W6/32) and C3 complement component. Previous studies demonstrated bound IgG and complement on placental villous trophoblasts (Faulk and Johnson 1977), but not non-villous trophoblasts. Incubation with mono- and polyclonal antibodies as well as with NaN_3 did not reveal any change in the cellular viability compared to the controls (Chapter 5 Tables 5 and 6). From this, it seems unlikely that trophoblast resistance to complement lysis was due to antigenic modulation (Chatenoud and Bach, 1984), unless their modulation is insensitive to NaN_3 , for which there is no precedent. Recently, studies showed that human C3 possesses modulating activity which was not destroyed by heating. It was shown that the acquisition of lytic resistance of mouse RADA1 leukaemia cells was due to the modulation which was achieved with IgG and heated human C3, resulting in the intercalation of C3 into the aggregated antigen-antibody complexes (Stackpole, Jacobson and Galuska, 1978).

In addition to this humoral attack, incubation with non-pregnant peripheral blood lymphocytes followed by the addition of W6/32 and rabbit complement in the presence of ^{35}S -met was studied. In some placentae, mixed trophoblasts and lymphocytes showed higher ^{35}S -met incorporation compared to trophoblasts alone (Chapter 5 Figure 9). The autoradiographic films revealed large numbers of labelled polypeptides in the trophoblasts compared to the lymphocytes which did not show any (Chapter 5 Figure 10). These results are consistent with a previous study performed on mouse trophoblasts which were shown to be resistant to immune cell lysis (Jenkinson and Billington, 1974). Such behaviour of these trophoblastic cells seems to be similar to that revealed by certain tumor cells. Previous studies (Schlager, Dhanian and Borsos, 1978; Ohanian, Schlager and Saha 1982; Schlager and Ohanian 1983) showed

that the ability of tumor cells to resist or escape from immune attack depends on their metabolic and physical properties. It was found that the susceptibility of the cells to Ab-C killing correlates with their ability to synthesize complex cellular lipids. Their ability to resist CTL killing correlates with the synthesis and composition of specific cellular phospholipids in order to retain the selective semipermeable properties of their membrane; however, such resistance does not correlate with their ability to express antigen (Schlager and Ohanian, 1983). The significance of these lipid complexes might be involved in repairing membrane damage due to complement action. In addition, the synthesized cellular lipids might become either (i) part of anticomplementary moieties on the cell membrane affecting binding and/or activation of complement components, or(ii) be exported from the cell to become anticomplementary to fluid phase complement. In conclusion, these properties of the target cell might enable the cell to "respond" to immune attack, thus preventing or repairing CTL or C-induced damage.

From these studies, it is apparent that the non-villous trophoblastic cells that come into direct contact with the genetically alien maternal tissue express class I MHC antigens. These antigens usually are sufficient for tissue rejection (Klien, 1975). They carry haplotype-specific determinants which are unique to the strain or the individual human involved, and elicit the destructive immune response. Although the trophoblasts carry such antigens, when they encounter the maternal immune system in the context of the placenta implanted into the uterus, there is no such rejection. It was also reported by Gill (1984), that the histocompatibility relationship between the organ donor and the recipient and between mother and fetus which can incite an immune response are quite different. Misra, Kunz and Gill (1983), reported that

class I cell surface antigens consist of families of homologous molecules bearing different combinations of antigenic determinants. Only a few of these molecules carry the haplotype-specific determinants, and the rest carry broadly shared antigenic determinants. These findings suggest that the loci encoding class I molecules have multiple duplications and mutations, and that only a few of these molecules carry the determinants responsible for the uniqueness of the inbred strain or of the individual human. Studies were carried out to examine the types of the molecules expressed on the placental surface that incite the major immune response in the mother. It was found that these molecules are class I MHC antigens which do not have the serological properties of the classical class I transplantation antigens. Redman *et al* (1984), also pointed out that the class I MHC antigens expressed by the extravillous cytotrophoblasts in the human, are not recognized by antisera reactive with polymorphic rather than monomorphic determinants. From this, it was suggested that the class I MHC antigens expressed only by these extravillous trophoblast populations may constitute a unique group, possibly involving either the genetic deletion of the terminal alpha 1 domain of the HLA-A, B heavy chain structures, or, alternatively, the expression of a non-HLA class I MHC differentiation antigen analogous to those encoded from the less polymorphic murine Qa complex. Such a possibility might be interesting since blocking antibody activity in human placental clutes was associated with alloantibodies reactive with Qa-like antigens expressed on activated lymphocytes. Other additional candidates for contributors to blocking antibody activity as suggested by Bulmer and Johnson (1985b), include the involvement of the TLX system (Faulk and McIntyre, 1983), of non-cytotoxic antibodies to an MHC-linked antigen detected by the blocking of the Fc receptor activity on B cells,

and of auto-anti-idiotypic antibodies reactive with maternal T cell receptors for paternal alloantigens (Bulmer and Johnson, 1985b). The origin and nature of the MHC-associated antigens expressed by human trophoblasts may be determined by the additional application of the molecular genetics and the generation of specific antisera (Bulmer and Johnson, 1985b). Others suggested the necessity for other antigens such as the TA of the TLX type in signalling maternal recognition to mount a protecting response that is instrumental in deflecting rejection of the blastocyst (McIntyre and Faulk, 1982; Faulk, 1984). Maternal recognition of such antigens has been shown to be essential for fetal survival. Maternal response would be by antibody production and immunosuppression. It has been found that antibodies directed against the placental antigens would bind to these cells, where it would be digested intracellularly and then be released as fragments back into the mother circulation. Accordingly, the placenta would react as an immunoadsorbent removing and destroying these antibodies without being damaged (Chaouat, Kolb and Wegmann, 1983; Gill, 1984). However, others reported that the adsorbed antibodies would form a layer of immune complexes at the uteroplacental interface, where they would not pass into the fetus, avoiding possible detrimental consequences. Moreover, being fixed on placental antigens, they would mask them from cytotoxic lymphocytes and perhaps activate suppressor cells and inactivate cytotoxic lymphocytes (Voisin, 1984). Further, the maternal immune recognition induces the formation of suppressor cells within the placenta. Clark *et al* (1984) described a set of suppressor cells present in the placentae during normal pregnancy which release a non-specific suppressor substance. Recruitment of suppressor cells by trophoblast may suppress the generation of cytotoxic cells in the normal pregnancy. The trophoblasts

recruit or signal the migration into the uterine lymphatics and uterine decidua of lymphocytes which suppress reactivity or are functionally hypo-responsive to paternal and non-paternal antigens (Clark and McDermott, 1984). Further, these trophoblasts produce progesterone and other hormones in local concentrations. Progesterone, in particular, is anti-inflammatory in that it allows the trophoblast to heal-in and anchor the placenta securely to the decidua without inciting granulomatous, inflammatory or antigenic responses which could interfere with this process (Stites and Siiteri, 1983). Additionally, placental proteins such as hCG, the various SP, PP, PAPP and others might induce local immunosuppression. These may have several suppressive properties: on immune recognition of alloantigens by lymphocytes; on lymphocyte activation as in mixed lymphocyte reaction; acting directly or through induced suppressor cells; and possibly acting on the decidua and helping it to be a reciprocal protective barrier. The overall result is that the fetoplacental unit appears weakly immunogenic (Voisin, 1984). Moreover, Voisin (1984) reported the possibility of local suppressor mechanisms of effector agents that involve suppressor cells and placenta inhibitory factors. Their main targets seem to be cytotoxic T cells, killer cells, natural killer cells (NK), and even complement (thus inhibiting C-dependent antibody cytotoxicity).

Chaouat, Kolb and Riviere (1984) mentioned that the trophoblasts might regulate natural killer (NK), K cells and cytolytic T-lymphocytes (CTL) homing and lytic functions, as well as protecting themselves from cytotoxic antibodies by anti-complementary factors.

In addition, the decidual cells might be very important in this respect. These decidual cells are uniquely positioned in the uterine endometrium to serve as the bed for the developing placenta. They arise

as the immediate progeny of endometrial-stromal cells that divide and differentiate in response to ovarian hormones and the implantation of the blastocyst during pregnancy (Lala et al, 1983). Beer and Billingham (1974) reported that the trophoblast- decidual interactions might suppress local alloreactivity of lymphocytes, preventing the generation of cytotoxic cells. Further, Kirkwood and Bell (1981) mentioned the suppression of mixed lymphocyte reactivity with decidual-cell culture supernatants. Slapsys and Clark (1983) and Clark et al(1983, 1984) also described an antigen-non-specific non-T suppressor-cell class in the pregnant-murine endometrium which can strongly abrogate the cytotoxic T-cell generation. Limiting dilution studies showed that suppression does not affect the frequency of CTL precursors, but inhibits their development into mature CTLs. Suppressor cells capable of inhibiting CTL generation peak at the time of implantation, and again during the second half of pregnancy (Chaouat, Kolb and Wegmann, 1983).

All these reactions, as well as the absence of class II HLA-DR antigens and the resistance to immune damage by lymphocytes, cytotoxic antibodies or antigen/antibody complexes, might be very important in protecting the tissue-specific antigens. However, in case of MHC compatibility or in case of their absence, the tissue specific antigens might become recognized by the infiltrating maternal lymphoid cells. This would lead to low suppression, and maturation of the CTL precursors, which therefore become associated with the subsequent abortion. Several studies revealed that women who have undergone repeated spontaneous abortions can subsequently deliver live offspring if subcutaneously injected with either paternal or pooled allogeneic lymphocytes in order to develop serum blocking factors (Taylor and Faulk, 1981; Beer et al, 1981). Mowbray et al(1985) reported that

successful pregnancies were also obtained when women were injected with their husbands' blood lymphocytes. Another cause for spontaneous abortion is the presence of recessive lethal genes which are linked to the major histocompatibility complex (MHC). These have been demonstrated in the mouse (t haplotypes), in the rat (grc), and there is some evidence that they also exist in man (Gill, 1983).

As mentioned earlier, the maternal immune stimulation leads to a larger and more viable feto-placental unit. This might be similar to the tumor immunity since anti-tumor immunity can promote rather than hinder tumor growth (Prehn, 1972; Prehn, 1983).

From this, it seems that solving the riddle of the fetal allograft might have important fallouts in transplantation biology, cancer immunotherapy, fertility control and eventual treatment of immunological abortions and pregnancy losses (Voisin, 1984).

Because of the importance of the MHC antigens on the surface of these trophoblasts, it would be of great interest to know the detailed structure of such antigens. This can be established in the future by using these cells as a source of mRNA for the establishment of cDNA library. Such a library can be probed with clones of human class I genes in order to study the type and nature of MHC genes expressed in human trophoblasts.

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APPENDIX

PLACENTAE	A		B		C		D	E	F	G
ANTIBODIES	NL	CP	NL	CP	NL	CP	CP	CP	CP	CP
TR1	41-60 5	40-62 6	53-69 5	48-67 5	63-87 5	66-89 7	-	-	-	-
PKK1	54-71 5	59-77 6	45-61 6	58-88 5	66-84 8	75-92 9	56-73 6	58-72 6	74-95 5	66-84 6
anti-vimentin (PK-V)	- -	0-7 7	- -	2-8 5	- -	4-15 8	0-6 6	3-11 8	6-14 7	3-10 8
anti-LCA	- -	0-3 8	- -	1-6 9	- -	0-6 8	1-7 9	3-10 9	2-8 9	3-9 8
anti-Lectin	3-12 7	4-15 7	4-15 7	3-17 8	7-19 6	4-17 7	0-4 6	3-14 6	6-16 6	2-10 6
W6/32 (Class I)	50-69 5	43-65 5	49-67 6	54-70 6	69-94 6	65-86 6	50-68 6	77-91 6	78-95 5	69-86 5
Beta-2- microglobulin	46-62 6	54-68 6	58-75 6	59-77 5	65-85 6	62-84 6	50-69 7	56-65 5	63-81 6	63-77 6
DA6.231 (Class II)	10-19 9	8-18 9	11-34 10	12-23 9	4-15 9	1-9 9	- -	- -	- -	- -
INN-hCG-17 (anti-alpha-hCG)	12-37 7	13-26 6	10-30 6	17-37 7	13-34 7	16-26 7	8-16 7	4-14 7	17-26 6	14-27 6
INN-hCG-2 (anti-beta-hCG)	8-24 7	7-16 7	5-13 6	5-17 7	1-8 7	3-11 8	0-3 7	2-13 6	3-12 7	5-13 8
anti-Fibronectin	46-67 5	48-63 6	59-70 5	66-78 5	69-84 6	73-85 6	- -	- -	- -	- -
anti-PAPP-A	51-60 6	55-63 6	58-67 7	66-74 5	64-73 6	65-78 5	- -	- -	- -	- -
anti-SP1	22-34 6	26-54 6	33-51 5	40-56 6	47-54 6	53-62 6	- -	- -	- -	- -
anti-hPL	34-45 6	21-44 6	32-57 6	45-57 7	48-55 5	49-60 5	- -	- -	- -	- -
H316	- -	23-44 6	- -	36-56 7	- -	43-65 6	- -	- -	- -	- -
H315	- -	10-21 6	- -	11-23 7	- -	15-29 7	- -	- -	- -	- -
7A11	- -	- -	- -	- -	- -	- -	48-60 6	55-67 7	56-70 6	63-81 6

Table 1. The range for the percentage of stained chorionic plate cells and the number of observations.



MONOCLONAL ANTIBODY	SPECIFICITY	SOURCE
INN-hCG-17	Alpha-hCG	Serotec Ltd (England)
INN-hCG-2	Bata-hCG	Serotec Ltd (England)
W6/32	Class I HLA antigens	Sera-lab (Sussex-England)
Beta-2-microglobulin	Class I HLA antigens	Sera-lab (Sussex-England)
19/9	Class II HLA antigens	Sera-lab (Sussex-England)
PKK1	Cytokeratin	Labsystems (Finland)
PK-V	Vimentin	Labsystems (Finland)
TR1	Cytoskeletal element	Dr.Christopher Sunderland (Bristol)
H315	Trophoblasts	Dr.Peter Johnson (Liverpool)
H316	Trophoblasts	Dr.Peter Johnson (Liverpool)
DA6.231	Class II HLA antigens	Dr.Keith Guy (Edinburgh)

List for the monoclonal antibodies used in the staining experiments (Chapter 4, materials section).