IMMUNOCYTOCHEMICAL IDENTIFICATION OF CYTOTROPHOBLAST FROM OTHER MONONUCLEAR CELL POPULATIONS ISOLATED FROM FIRST-TRIMESTER HUMAN CHORIONIC VILLI

BRIDGET H. BUTTERWORTH AND Y. W. LOKE
Division of Experimental and Cellular Pathology, Department of Pathology, Tennis Court Road, Cambridge CB2 1QF, U.K.

SUMMARY

Trophoblast biologists are often uncertain as to what cell types they are investigating because the mononuclear cell populations prepared from trypsinization of human first-trimester chorionic villi are morphologically very similar. In the present study, immunocytochemical and phagocytic markers have been used to distinguish cytotrophoblast populations from cell types derived from the mesenchyme of the chorionic villus.

Two anti-trophoblast monoclonal antibodies generated in our own laboratory (18B/A5 and 18A/C4) were found to be very efficient in identifying cytotrophoblast, which made up 35–40% of the cells in a smear. Most cytotrophoblast cells did not stain with a monoclonal anti-HLA-A,B,C antibody but a few cells (5%) were found to express both trophoblast and HLA-A,B,C antigens by a double-labelling technique.

Endothelial cells from villous capillaries could be identified by a rabbit anti-factor VIII antibody. These cells formed 28% of the population in a cytospin smear.

Macrophages from the villous mesenchyme were less readily separable as neither specific monoclonal antibodies nor localization of enzymes were found to be effective. However, these cells could be identified by their ability to phagocytose carmine. About 15% of the cells in a smear consisted of macrophages.

The procedure described should prove useful in judging the efficiency of isolation methods from human placental cells.

INTRODUCTION

The human placenta is a composite organ made up of a variety of cell types. These different populations are readily recognizable in histological sections by their location and topographical relationship to each other. However, for detailed functional studies and for tissue culture, it is frequently necessary to use cells that have been disaggregated from chorionic tissues. It is in this situation that it becomes very difficult to discriminate between the cells, as they are morphologically rather similar when viewed in suspension or in a smear. In particular, the identification of cytotrophoblast from other mononuclear cells presents a problem because of the lack of a reliable marker. We have recently raised two monoclonal antibodies against first-trimester human trophoblast plasma membranes, one of which (18B/A5) reacted only with cytotrophoblast (Loke & Day, 1984) while the other (18A/C4) was directed at both...

Key words: human cytotrophoblast, immunocytochemistry, isolated cells.
cytotrophoblast and syncytiotrophoblast (unpublished) when tested on sections of chorionic villi. In the present study, we have used these two anti-trophoblast antibodies in conjunction with markers for other cells in an attempt to identify and to quantify the cell populations in cytospin smears prepared from trypsinization of first-trimester chorionic villi.

**MATERIALS AND METHODS**

**Preparation of cell suspensions and smears**

First-trimester placental tissue was obtained from vaginal termination of pregnancy. The tissue was thoroughly washed in cold phosphate-buffered saline (PBS), pH 7.4. Adherent decidua, which can be recognized as pale solid strips of tissue attached to the tips of some chorionic villi, was carefully removed using a dissecting microscope. The remaining pieces of villi were washed once more, minced between scalpel blades and then treated with 20 ml of 0.25% trypsin (Difco) containing 0.02% EDTA and 2200 Kurz units/ml of DNase I (Sigma) at 37°C while on a magnetic stirrer (Loke, 1983). After 30 min the resultant suspension was filtered through two layers of muslin and the filtrate centrifuged at 400 g for 5 min. The supernatant was discarded and the pellet was resuspended in PBS at a concentration of 1 × 10⁶ cells/ml. A sample (2 ml) of the cell suspension was carefully layered over a similar volume of Lymphoprep (Gibco). Following centrifugation at 400 g for 10 min to remove erythrocytes, the cells at the interface were collected, diluted five times with medium 199 containing 10% foetal calf serum (FCS) (Flow Labs; 199/FCS) and centrifuged at 400 g for 10 min. The cells were then resuspended at a concentration of 10⁶ cells/ml in PBS and smears prepared using a cytocentrifuge (Shandon).

The tissue debris remaining after 5, 10 and 30 min of trypsinization was fixed in formalin-saline and processed in the usual way for histological examination in order to ascertain the degree of structural disruption.

**Phagocytosis with carmine**

A sample (2 ml) of placental cell suspension together with one drop of a 1% carmine solution made up with 199/FCS were placed in a wide-bodied culture bottle. These were incubated at 37°C in a roller apparatus. After 2 h, the suspension was centrifuged at 400 g for 5 min and resuspended in 2 ml of 199/FCS. Extracellular carmine particles were removed by separation on Lymphoprep as already described. The cells in the interface were then resuspended in fresh 199/FCS and reincubated in the roller apparatus at 37°C for a further 15 min to allow attached particles to be ingested. After determining the viability of the cells by Trypan Blue exclusion (over 95% viable), smears were prepared in a cytocentrifuge as described above.

**Immunocytochemistry of frozen sections and cell smears**

First-trimester placental tissue selected for areas rich in chorionic villi was washed in PBS and snap-frozen in liquid nitrogen. Cryostat sections (8 μm) were air dried and fixed in cold acetone at 4°C for 10 min. Cytospin smears were similarly fixed in cold acetone at 4°C for 10 min.

The antibodies used, their specificities, source and working dilutions are detailed in Table 1. All are monoclonal antibodies except the rabbit polyclonal antiserum to factor VIII.

For single immunocytochemical staining of section or smear, the biotin-avidin immunoperoxidase method described previously by Loke & Day (1984) was used. The procedure was performed at room temperature throughout. The section or smear was incubated with the appropriate antibody for 30 min followed by biotin-conjugated horse anti-mouse immunoglobulin G (IgG) (Sera-Lab) diluted 1 in 100 for 30 min and then by avidin–horseradish peroxidase (Sera-Lab) diluted 1 in 400 for a further 30 min. Each step was preceded by a wash in PBS for 5 min. The peroxidase was developed with diaminobenzidine (DAB) (Sigma) for 5 min, giving a brown reaction product or with 4-chloro-1-napthol (4C1N) to give a blue reaction product and then counterstained with Carazzi's haematoxylin. Rabbit antiserum to factor VIII was detected by a similar technique but the biotin conjugate was goat anti-rabbit IgG (Sera-Lab) diluted 1 in 100.
Table 1. Antibodies used in the study

<table>
<thead>
<tr>
<th>Designation</th>
<th>Specificity</th>
<th>Source</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monoclonal 92A3</td>
<td>Schistosomulae (for control)</td>
<td>Dr Taylor</td>
<td>Culture supernatant undiluted</td>
</tr>
<tr>
<td>Monoclonal 18A/C4</td>
<td>Cytotrophoblast and syncytiotrophoblast</td>
<td>Dr Loke</td>
<td>Culture supernatant undiluted</td>
</tr>
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<td>Monoclonal 18B/A5</td>
<td>Cytotrophoblast</td>
<td>Dr Loke</td>
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<tr>
<td>Monoclonal W6/32</td>
<td>HLA-A,B,C shared determinant</td>
<td>Sera-lab</td>
<td>Ascites fluid 1 in 100</td>
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<td>Becton Dickinson</td>
<td>Purified immunoglobulin 1 in 50</td>
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<tr>
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<td>Becton Dickinson</td>
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<tr>
<td>Monoclonal C3bR</td>
<td>C3b receptor</td>
<td>Dakopatts</td>
<td>Culture supernatant undiluted</td>
</tr>
<tr>
<td>Monoclonal FMC 32</td>
<td>Monocyte/macrophage</td>
<td>Dr Volsen</td>
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</tr>
<tr>
<td>Polyclonal factor VIII</td>
<td>Factor VIII</td>
<td>Sera-lab</td>
<td>Serum 1 in 100</td>
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Table 2. Percentage of cells expressing different characteristics in cytospin smears obtained from immature human placentae

<table>
<thead>
<tr>
<th></th>
<th>92A3</th>
<th>18B/A5</th>
<th>18A/C4</th>
<th>W6/32</th>
<th>Esterase</th>
<th>Factor VIII</th>
<th>HLA-DR</th>
<th>Leu-M3</th>
<th>C3bR</th>
<th>FMC 32</th>
<th>Phagocytosis</th>
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<tbody>
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<td>-</td>
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<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
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<tr>
<td>Cytotrophoblast</td>
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<td>Cytotrophoblast</td>
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<td>Macrophages</td>
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<td>Endothelial cells</td>
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<td>+</td>
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| %                    | 0    | 35     | 40     | 69    | 28      | 2           | 0      | 0      | 0    | 0      | 15           |
For double-labelling the primary monoclonal antibody was localized by the biotin–avidin
immunoperoxidase method and developed with 4-chloro-1-napthol (4C1N) to give a blue reaction
product; 40 mg of 4C1N (Sigma) were dissolved in 0.5 ml ethanol and mixed with 100 ml 0.05M-
Tris (pH 7.4) containing 0.001 % H2O2. A precipitate formed, which was removed by filtration.
The slides were incubated in the filtrate in Coplin jars for 30 min and washed in PBS. The second
monoclonal antibody was then applied and the biotin–avidin–peroxidase localization was repeated
but 3-amino-9-ethyl carbazole (AEC) was used as the chromogen to give a red reaction product (Van
Noorden & Polak, 1983); 120 mg of AEC (Sigma) was dissolved in 15 ml of N,N-dimethyl-
formamide (Sigma); 0.5 ml of this AEC solution was added to 9.5 ml of 0.1 ml of 0.1 m acetate buffer
(pH 5.2) and 50 μl of 3 % H2O2. The solution was immediately filtered onto the sections or smears.
After 10 min, the slides were rinsed and washed in PBS. After counterstaining, the double-labelled
sections or smears were mounted in glycerol–gelatin because the reaction products of the
chromogens are soluble in ethanol.

Control reactions were performed by substituting 92A3 for either the primary or the secondary
sequence monoclonal antibody.

Histochemical demonstration of non-specific esterase on frozen sections and cell
smears

Non-specific esterase was localized on unfixed cryostat sections and cytospin smears by a histo-
chemical method as described by Stuart, Habeshaw & Davidson (1978). A stock solution of sub-
strate was prepared, which contained 1 g of α-napthyl acetate (Sigma), 50 ml distilled water and
50 ml of acetone. The working substrate was prepared as follows: 2 ml α-napthyl acetate stock
solution, 15 ml 0.1 M phosphate buffer (pH 7.3), 15 ml distilled water and 20 mg Fast Red TR salt
(Sigma), and was filtered immediately before use. The sections were incubated in the substrate at
room temperature for 40 min. Subsequently, they were washed in tap water, counterstained with
Carazzi’s haematoxylin and mounted in glycerol–gelatin. Control sections were treated in the same
manner, but with omission of substrate from the incubation medium.

RESULTS

Histological examination of the fixed tissue debris remaining after trypsinization
revealed that the syncytial layer was just lifting off after 5 min. After 10 min the villi
were all denuded of syncytiun and only a thin layer of cytotrophoblast remained.
There was complete disruption of villous architecture after 30 min in trypsin.

The staining patterns of anti-trophoblast monoclonal antibodies 18A/C4 and
18B/A5 on frozen section of first-trimester chorionic villi are shown in Figs 1 and 2.
A double-staining procedure was used in conjunction with an anti-HLA-A,B,C
monoclonal antibody (W6/32). The trophoblast antigens were defined by the red
reaction product while the HLA-A,B,C antigens stained dark blue. It can be seen that
18A/C4 reacted with both the cytotrophoblast and syncytiotrophoblast layers sur-
rounding the chorionic villous but staining by 18B/A5 was restricted to
cytotrophoblast. Only the mesenchymal cells stained with W6/32. The results ob-
tained on cytospin smears are summarized in Table 2. The percentages represent
mean values from three specimens. Both anti-trophoblast antibodies reacted very
strongly with some cells but not with others (Fig. 3), with 18A/C4 staining a slightly
higher percentage of cells than 18B/A5 (40 % to 35 %). Using a similar double-
staining procedure as that used for sections, the majority of cells were found to express
either the trophoblast antigens (red) or HLA-A,B,C antigens (blue) (Fig. 4).
Approximately 69 % of the cells were HLA-A,B,C positive. There were, however, a
few cells (5%) that stained with a mixture of the two colours denoting coexpression of antigens.

Factor VIII was localized on frozen sections of first-trimester chorionic villi to the endothelial lining of foetal capillaries in the villous mesenchyme (Fig. 5). About 28% of the cells in cytospin smears stained with this antibody in a granular pattern similar to that seen on sections (Fig. 6). These endothelial cells tended to aggregate in clumps that did not stain with anti-trophoblast antibody 18A/C4 or 18B/A5 (Fig. 7).

A monoclonal antibody to HLA-DR (Warnke & Levy, 1980) was found to react with only a very few mesenchymal cells on sections of first-trimester chorionic villi while a monoclonal antibody to C3b receptors (Gerdes, Naiem, Mason & Stein, 1982) did not react at all, although both these antibodies stained a significant number of cells in the villous mesenchyme of term placentae. These two surface molecules therefore are not very useful markers for placental macrophages during the early stages of gestation. Only about 2% of cells in cytospin smears were HLA-DR positive, and none stained with the anti-C3b receptor antibody. This is in accord with the findings on sections. Two further monoclonal antibodies Leu-M3 (Dimitriu-Bona, Burmester, Waters & Winchester, 1983) and FMC32 (Brooks et al. 1983) against monocyte/macrophage cell surface components were found to be reactive with a large number of stellate cells in the villous mesenchyme of both first-trimester and term placentae. Surprisingly, no cells in cytospin smears were stained by these antibodies.

Enzyme histochemical staining for non-specific esterase on sections of first trimester chorionic villi revealed that this enzyme was present mainly in the two layers of trophoblast but was not detectable in any of the mesenchymal cells (Fig. 8). A population of cells in cytospin smears (38%) was found to contain this enzyme (Fig. 9).

The red carmine particles that had been ingested by phagocytic cells were very conspicuous against the background nuclear counterstain. All phagocytic cells stained with the anti-HLA-A,B,C monoclonal antibody W6/32 (Fig. 10). A few of these phagocytic cells were HLA-DR positive (Fig. 11) but the majority, which made up 15% of the total cell populations of the smear, were not stained by antibody against this determinant. None of the phagocytic cells reacted with anti-trophoblast or with anti-factor VIII antibodies, confirming they are not trophoblast or endothelial cells (Fig. 12).

**DISCUSSION**

In this paper, we have described procedures that could be used to identify the various cell populations, particularly trophoblast, obtained by enzymic disaggregation of human first-trimester chorionic villi. The two anti-trophoblast monoclonal antibodies (18A/C4 and 18B/A5) have proved to be very effective in defining one population of cells clearly from the others in the smears, and we are confident that these positively stained cells are trophoblast. Although 18B/A5 (Loke, Day, Butterworth & Potter, 1984) and 18A/C4 (unpublished) have been shown to cross-react with certain other foetal epithelial surfaces, on sections of first-trimester chorionic villi
they are directed only at the trophoblast and are totally unreactive with any of the mesenchymal components as can be seen in Figs 1 and 2. Therefore, as markers for trophoblast as against mesenchymal cells in the present study, they may be regarded as sufficiently specific. Of the two antibodies, 18A/C4 reacts with both cytotrophoblast and syncytiotrophoblast while 18B/A5 is directed at cytotrophoblast alone. It is interesting to note that 18A/C4 regularly stains a larger percentage of mononuclear trophoblast cells in a smear than 18B/A5 (40% as against 35%). This percentage

Fig. 1. Double biotin-avidin-peroxidase technique on frozen section of human first-trimester chorionic villus. Anti-HLA-A,B,C monoclonal antibody W6/32 stains only the villous mesenchymal core (blue) while the anti-trophoblast monoclonal antibody, 18A/C4, stains both the cytotrophoblast and the syncytiotrophoblast. \( \times 1250 \).

Fig. 2. Double biotin-avidin-peroxidase technique on frozen section of human first-trimester chorionic villus. W6/32 stains the villous mesenchymal core (blue) while 18B/A5 reacts only with the single layer of cytotrophoblast (red). \( \times 1250 \).

Fig. 3. Cytospin smear of cells from human first-trimester placenta stained with the anti-trophoblast monoclonal antibody 18A/C4 and localized by a biotin-avidin-peroxidase technique. The brown reaction product of the chromogen, DAB, is clearly seen on two cells. \( \times 3125 \).

Fig. 4. Double biotin-avidin-peroxidase technique on cytospin smear of cells from human first-trimester placenta. A trophoblast cell is distinguished by 18A/C4 staining (red) and HLA-A,B,C positive cells are shown by W6/32 staining (blue). \( \times 3125 \).

Fig. 5. Frozen section of first-trimester placenta stained with rabbit anti-factor VIII in a biotin-avidin-peroxidase technique with DAB as chromogen. Endothelial cells lining the villous capillaries are stained. \( \times 1250 \).

Fig. 6. Cytospin smear of cells from human first-trimester placenta stained with rabbit anti-factor VIII in a biotin-avidin-peroxidase technique with DAB as chromogen. Cells aggregated in a clump are stained in a granular pattern. Two cells at the periphery of the clump are not stained. \( \times 3125 \).

Fig. 7. Cytospin smear of cells from human first-trimester placenta stained with the anti-trophoblast monoclonal antibody 18A/C4, and localized by the biotin-avidin-peroxidase technique. The brown DAB reaction product is found only on cells at the periphery of the clump. \( \times 3125 \).

Fig. 8. Localization of esterase on frozen section of human first-trimester chorionic villus. The red reaction product is confined to the trophoblast, while the mesenchyme is unreactive. \( \times 1250 \).

Fig. 9. Cytospin smear of cells from human first-trimester placenta stained for esterase. The red reaction product denotes enzyme activity. \( \times 1250 \).

Fig. 10. Double biotin-avidin-peroxidase technique on cytospin smear of cells from first-trimester human placenta. The phagocytic cell that has ingested red carmine particles is stained only by the anti-HLA-A,B,C monoclonal antibody W6/32 (blue). None of the phagocytic cells were stained with the anti-trophoblast monoclonal antibody 18A/C4 (red). \( \times 3125 \).

Fig. 11. Biotin-avidin-peroxidase technique on cytospin smear of cells from human first-trimester placenta stained with anti-HLA-DR monoclonal antibody. The blue reaction product of the chromogen, 4C1N, is seen on the phagocytic cell. The adjacent non-phagocytic cell is not stained. \( \times 3125 \).

Fig. 12. Cytospin smear of cells from first-trimester human placenta stained with the anti-trophoblast monoclonal antibody 18B/A5. A biotin-avidin-peroxidase technique was used with DAB as chromogen. The phagocytic cell that has ingested carmine is unreactive while three non-phagocytic cells are stained. \( \times 3125 \).
difference may be accounted for by the presence of 'intermediate' cells (Boyd & Hamilton, 1976), which are half way along the differentiation path towards becoming syncytiotrophoblast. Thus, besides their ability to distinguish between trophoblast cells and other mononuclear populations derived from the villous mesenchyme, these two monoclonal antibodies also appear to be capable of discriminating between the different trophoblast subpopulations according to their degree of differentiation.

Using a double immunocytochemical procedure with anti-trophoblast monoclonal antibodies combined with an anti-HLA,B,C monoclonal antibody (W6/32), the vast majority of cells are found to stain with either one or the other. This further confirms that the cells in a smear that react with 18A/C4 and 18B/A5 but not with W6/32 are trophoblast, as it is now widely accepted that the two layers of trophoblast enveloping chorionic villi do not express HLA-A,B,C antigens. This can be seen in Figs 1 and 2 where only mesenchymal cells are stained with W6/32. However, there are recent reports that Class I HLA antigens are demonstrable on extra-villous trophoblast (Sunderland, Redman & Stirrat, 1981; Redman et al. 1984). We have recently observed that even some villous cytotrophoblast cells that are proliferating to form new primary villi do indeed stain with anti-trophoblast as well as anti-HLA-A,B,C monoclonal antibodies (Butterworth, Khong, Loke & Robertson, unpublished data). The HLA-A,B,C positive trophoblast cells found in cytospin smears, therefore, are likely to be derived from this population.

A polyclonal rabbit antibody to factor VIII is found to stain 28% of the cells in a cytospin smear with a similar granular pattern as that seen when the antibody reacts with the lining of chorionic villous capillaries on frozen sections. We conclude that these cells are therefore endothelial cells derived from disruption of foetal capillaries in the villous mesenchyme.

The identification of macrophages in cytospin smears by the use of specific antibodies has been less successful. While a significant number of mesenchymal cells in sections of term placentae are found to be reactive with monoclonal antibodies to HLA-DR and to C3b receptors, very few cells do so in first-trimester material. Similar findings were reported by Bulmer & Johnson (1984). This may be because these surface components are insufficiently developed at early stages of gestation. It is therefore not surprising that only 2% of the cells in smears are found to be HLA-DR positive in the present study. We possess two further monoclonal antibodies (Leu-M3 and FMC 32) that were found to react with a large number of stellate mesenchymal cells on sections of first-trimester chorionic villi and we had hoped that these could provide us with the necessary markers for early placental macrophages. Unfortunately, none of the cells in cytospin smears were stained by these monoclonal antibodies. It seems unlikely that the relevant cells have not been released by the enzymic procedure because histological examination of a piece of tissue that had been treated in this way showed there was complete disruption of villous architecture after 30 min of trypsinization. This confirms our previous observations (Loke, 1983). The alternative explanation is that the epitopes recognized by Leu-M3 and by FMC 32 antibodies were trypsin-sensitive and therefore are no longer detectable on cell smears that have
been obtained by disaggregation using this enzyme. We have tried using collagenase but it was also found to denature these determinants (unpublished).

Although the presence of non-specific esterase is said to be a good marker for placental macrophages (Fox & Kharkongor, 1970), we have not managed to confirm this. Indeed, our studies on the distribution of this enzyme on sections of first-trimester chorionic villi by a histochemical procedure have shown that the enzyme is present mainly in trophoblast, a finding that is more in accord with that reported by Boyd & Hamilton (1970) and by Bulmer & Johnson (1984). There is no staining at all among first-trimester mesenchymal cells. Therefore, the cell population in a cytopin smear that contains non-specific esterase is more likely to be of trophoblast lineage rather than macrophages.

The ability to phagocytose particles superimposed on immunocytochemical staining seems to offer the best way of identifying placental macrophages early in gestation. We have used latex beads and steam-killed *Staphylococcus aureus* in the past (Loke, Eremin, Ashby & Day, 1982), but for this study we find that carmine is more appropriate because the red particles are readily seen against both the haematoxylin counter-stain of the cell nuclei and the different coloured peroxidase reaction products when an immunocytochemical staining procedure is used at the same time. We feel that the 15% phagocytic cells in cytopin smears that are HLA-A,B,C (W6/32) positive but do not stain with antibodies to trophoblast (18A/C4 and 18B/A5), factor VIII and HLA-DR represent the major macrophage population derived from first-trimester chorionic villi. This percentage is somewhat lower than previous estimates (Wood, 1980; Loke *et al.* 1982; Wilson, Haas & Weaver, 1983), but different placental samples, variations in disaggregation techniques and the kinds of particles used for phagocytosis will undoubtedly account for the discrepancies noted.

The absolute numbers of each cell type present are perhaps not too relevant as they are bound to vary from specimen to specimen. What our study has shown is that, with conventional enzymic methods for obtaining disaggregated cells from immature human placentae, trophoblast cells are contaminated to a large extent by other cell types like mesenchymal macrophages and capillary endothelial cells, all of which are morphologically indistinguishable. With the procedures just described, we can now at least identify these cells with a reasonable degree of confidence. This will enable us to judge between different isolation techniques for cytotrophoblast by the purity of the cells obtained.

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Cytotrophoblast identification


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