PINOCYTOSIS AND INTRACELLULAR DIGESTION OF 125I-LABELLED HAEMOGLOBIN BY TROPHOBLASTIC CELLS IN TISSUE CULTURE IN THE PRESENCE AND ABSENCE OF SERUM

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SUMMARY

One aspect of human placental function which has not hitherto been studied is the ability of the placenta to digest proteins intracellularly and use the products of hydrolysis to supply its own and foetal needs for nutrition and protein synthesis. Placental lysosomes have a full complement of hydrolytic enzymes, including the acid proteases cathepsin C and D. We have used trophoblast cells in monolayer tissue culture as a model for the study of endocytosis and intracellular digestion of 125I-haemoglobin. The normal use of serum in tissue culture medium has shown up differences from the pattern observed with other phagocytic cells such as macrophages, in that serum allows endocytosis but prevents intracellular digestion of 125I-haemoglobin. Replacement of serum by lactalbumin hydrolysate enables both endocytosis and intracellular digestion of 125I-haemoglobin to occur as in other phagocytic cells. Digestion is followed by release into the medium of acid-soluble, lower-molecular-weight compounds. The reasons for this major difference between trophoblast and other cells are discussed in the light of our results and their possible relevance to placental function.

INTRODUCTION

Biochemical studies have shown the human placenta to perform such diverse tasks as transporting gases and metabolites, synthesizing and secreting protein and steroid hormones, regulating the appropriate glucose concentration in foetal blood and possibly serving as an important organ of lipid and protein metabolism for the developing foetus (Assali, 1968). This paper is concerned with the nutritive and digestive roles that the human placenta can play in the foeto-placental relationship.

Electron-microscopic studies (Boyd & Hamilton, 1970) have lent support to our proposal that pinocytosis may play a role in the transport functions of the trophoblast. The maternal surface of the trophoblast has been shown to be covered in many areas by elaborate microvilli and their endocytosis-associated coated pits, tubules and vesicles (Ashley, 1965). Transport of molecules by pinocytosis frequently involves digestion of the vacuolar contents by lysosomal enzymes.

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Uptake and digestion of molecules by the syncytiotrophoblast may function as an invasive mechanism, as a defence mechanism or a nutritive mechanism. Hofbauer cells of the stroma are believed to be tissue macrophages because of their ultrastructural appearance and abundant acid-phosphatase-positive cytoplasmic granules (Lewis, 1924; Geller, 1957; Fox, 1967; Boyd & Hamilton, 1970; Fox & Kharkongor, 1970). These cells have not been studied in detail, but if they are macrophages, they may function in the same way as Kupffer cells do for liver or mesangial cells for kidney. One can postulate, therefore, that with their endocytic-lysosomal apparatus these cells may clean up villous stroma of unwanted or deleterious materials such as immune complexes, bacteria or viruses, and thereby protect the foetus from injury. In addition, materials may be processed by trophoblastic cells for nutritional use of the foetus or the placenta; for example, degradation of protein could provide amino acids necessary as building blocks of foetal protein.

To investigate the role of pinocytosis and subsequent lysosomal digestion in trophoblast we studied the uptake and digestion of $^{125}$Iodine-labelled haemoglobin using trophoblastic cells in tissue culture as a model. These studies showed that in the presence of serum, uptake but no digestion was detectable in these cells. In serum-free medium, however, digestion of this substrate was readily demonstrated.

**MATERIALS AND METHODS**

Chemicals used in these studies were A.R. grade obtained from B.D.H. Limited, Poole, Dorset, with the following exceptions: Dulbecco A—Oxoid Limited, Basingstoke, Hants.; Bactotryptsin—Difco Laboratories, West Molesey, Surrey; Newborn Calf Serum—Flow Laboratories, Irvine, Ayrshire; Medium 199 and Hanks' Salt Solution—Wellcome Laboratories, Beckenham, Kent; $^{125}$I, Radiochemical Centre, Amersham, Bucks.; Sephadex, DEAE and Dextran-Pharmacia Limited; Triton X-100, RNA and Lactalbumin Hydrolysate—Sigma Limited, Kingston-on-Thames, Surrey.

**Culturing of trophoblast.** The method for culturing trophoblast was modified from that of Fox & Kharkongor (1970). Human placentae were obtained within 15 min of normal term deliveries. Suitable, non-infarcted and non-calcified areas of cotyledons were cauterized by singeing with a hot plasterer's knife. Wedge-shaped sections of tissue from within the margins of these sterilized areas were removed and placed in sterile balanced salt solution, Dulbecco A, free of Ca$^{++}$ and Mg$^{++}$ ions (BSS). The burnt surface was discarded and the remaining fragments were shredded further with a pair of tweezers to make a suspension of finely divided villous tissue. This suspension was washed by repeated decantation with BSS until all blood was removed, resuspended in 0.5 % Bactotryptsin and incubated at 37 °C for 40–60 min. Trypsinization was stopped by addition of an equal volume of Hanks' salt solution containing newborn calf serum (NCS) to a final concentration of 1 %. Following filtration through a double layer of surgical cotton gauze, the cells were centrifuged (190 g, 10 min), washed once with Hanks' solution and resuspended in 10 % NCS/Medium 199 (M199) to give a concentration of 1 x 10$^6$ cells/ml; 3.0 ml of cells were usually placed in 50-mm disposable Petri dishes (Sterilin) and cells were grown overnight at 37 °C in an atmosphere of 5 % CO$_2$ in air before being used. This initial period of growth in the presence of serum was necessary to ensure that the maximum number of cells adhered to the dishes. In some experiments cells were also grown on flying coverslips in Leighton tubes. Only 1.0 ml of cells was added to these tubes, but otherwise they were treated identically to cells in Petri dishes. Four cell types were seen in cultures of human placenta. These have been histochemically related to their counterparts in the intact tissue (Fox & Kharkongor, 1970) and are: (1) multinucleated cells, derived from the syncytiotrophoblast; (2) epitheloid or medium-sized, round cells derived from cytotrophoblast; (3) large ovoid cells derived from Hofbauer cells, and (4) fibroblasts,
either spindle- or stellate-shaped, which may be of either stromal or endothelial origin. All these cells were easily discernible in the first few days of culture by phase-contrast microscopy. Fibroblasts usually made up 30–50 % of the cell types, with round cells and syncytial cells comprising the major part of the rest of the population. A few ovoid cells were always present. Cells were used on days 1 to 3 because the population of cells started to decline on day 4.

All cell types have been shown to contain cathepsin D by immunofluorescent staining (Contractor & Krakauer, unpublished observations).

Viability of cells was tested by incubation in 10 % NCS/M199 containing 0.05 % trypan blue for 5 min immediately before they were counted. In all experiments viability was greater than 95 % at this point. During culture, viability was tested by addition of trypan blue (0.05 %) to the medium, and counting all cells in several fields to a total of at least 100 cells, or by assaying for lactate dehydrogenase (LDH) in the medium and cells by the method of Bergmeyer, Bernt & Hess (1963). Units were expressed as average E₄₅₀ per min × 10⁴ per ml enzyme solution.

Induction of haemoglobin (Hb).

The chloramine T method of iodination was used for labelling with ¹²⁵I. The procedure was modified from Freeman (1967), Hunter (1967) and Stapleton (personal communication). A Seltron Automatic Gamma Counting System (Packard Instruments) was used for detecting ¹²⁵I. Samples (1–0 ml) were placed in 2–5 ml plastic vials which were placed in 10 ml polyethylene tubes specially designed for counting. All samples were counted for a net 10⁴ counts. Two similar routine iodination procedures were used for the preparation of ¹²⁵I-labelled Hb. They differed only in resultant specific activity. All reagents were sterilized either by autoclaving or ultrafiltration.

Preparation A. Reagents were added in the following order: 20 μl Na ¹³¹I (100 mCi/ml); 20 μl dilute sodium hydroxide (pH 9.0); 250 μl 0.5 M sodium phosphate buffer, pH 7.5; 250 μl chloramine T (4 mg/ml in 0.5 M sodium phosphate buffer, pH 7.5); and 250 μl Hb (10 mg/ml in 0.15 M sodium phosphate buffer, pH 7.5). After 4 min at room temperature the reaction was stopped by the addition of five 50-μl aliquots of sodium metabisulphite (10 mg/ml in 0.05 M sodium phosphate buffer, pH 7.5). All reagents were added with disposable Microcap pipettes (V. A. Howe Ltd, London).

Preparation B. The reagents were added in the following order: 5 μl Na ¹³¹I (100 mCi/ml); 5 μl 0.5 M sodium phosphate buffer, pH 7.5; 20 μl chloramine T (10 mg/ml chloramine T in 0.05 M sodium phosphate buffer, pH 7.5); and 20 μl Hb (8 mg/ml Hb in 0.15 M sodium phosphate buffer, pH 7.5). After 4 min incubation at room temperature, the reaction was stopped by addition of four 5-μl aliquots of sodium metabisulphite (40 mg/ml in 0.05 M sodium phosphate buffer, pH 7.5).

After following these procedures, the incubation mixtures were immediately placed on a column (10 ml bed volume) of Sephadex G-50 prepared in 0.15 M sodium phosphate buffer, pH 7.5 and eluted with the same buffer. Both buffers and Sephadex had been sterilized before use by autoclaving for 15 min at 68–95 kN m⁻² (10 lbf in⁻¹); 1–0 ml samples were collected and counted. ¹²⁵I-labelled Hb appeared in the first peak of radioactivity, while free salt was eluted in the following peak. Overnight incubation of this protein at 37 °C and subsequent chromatography on a Sephadex G-25 column (10 ml bed volume) reduced the trichloroacetic acid (TCA)-soluble radioactivity in these preparations and was therefore performed immediately before their use.

Culturing and harvesting of cells after incubation with ¹²⁵I-Hb. Following the initial growth period in serum, the medium was removed from these cells and replaced with 1–2 ml of (a) 10 % NCS/M199 containing ¹²⁵I-Hb, or (b) 25 % LAH/M199 containing ¹²⁵I-Hb. In all experiments ¹²⁵I-Hb was sterilized before use by ultrafiltration (pore size = 0.45 nm). Incubation with ¹²⁵I-Hb was continued at 37 °C or 4 °C as stated in the individual experiments in 5 % CO₂ in air. Samples which were to be incubated at 4 °C were gassed first for 12 min at 37 °C and then placed in an airtight sandwich box (also gassed) in the refrigerator at 4 °C. Following incubation at the 2 temperatures, the radioactive medium was removed, adjusted to its original volume, counted and precipitated by a final concentration of 3 % TCA (w/v). To ensure complete precipitation, 0.1 ml of 10 mg/ml Hb solution was added after TCA and this mixture was incubated overnight at 4 °C. Following centrifugation (20 min, 1200 g, room temperature) supernatants were removed and counted for TCA-soluble digestion products. After removal of radioactive medium, the cells were washed a minimum of 5 times.
with 1o ml of BSS each time, for 3-5 min per wash. Less than 1 % of the total radioactivity added to the cells was present in the fifth wash of the cells. Cells were removed from the dishes with the help of a rubber policeman and 10 ml of 0·1 % Triton X-100. They were re-suspended by vigorous agitation and counted.

Samples at 37 °C were run in triplicate unless otherwise stated and samples at 4 °C were run in duplicate. The results for each of these conditions were expressed as the mean and for the 37 °C samples the standard deviation (s.d.) was determined.

Controls used for non-specific breakdown of substrate were media containing substrate incubated at 37 °C in the absence of cells, followed by TCA precipitation.

The protein content of cells in culture was determined by the method of Lowry, Rosebrough, Farr & Randall (1951).

Identification of TCA-soluble digestion products. Supernatants obtained after TCA precipitation of media were chromatographed on Sephadex G-50. One-millilitre samples of medium were extracted 3 times with equal volumes of peroxide-free (alumina-treated) ether which removed TCA. No radioactivity was lost during this extraction. Samples (0·5 ml) of the aqueous phases were applied to 10-ml Sephadex G-50 columns (10 x 1·1 cm) in BSS. Columns were eluted with BSS and 1·ml fractions were collected. The same column was used for all preparations with thorough washing of Sephadex with BSS after each run. Before the initial use of this column, 1·0 ml of a 10 mg/ml Hb solution in BSS was washed through it to saturate non-specific binding sites of peptides or amino acids. Standards of Na123I were used to calibrate the column.

RESULTS

Uptake and digestion of 125I-Hb in 10 % NCS/M199 over time. 1·9 x 10⁶ cells were incubated for 6, 16 and 41 h at 37 °C and 4 °C in 10% NCS/M199 containing 5·12 μg 125I-Hb (16600 cpm). Table 1 shows uptake of Hb increased over time at both 37° and 4 °C but the 4 °C value was very much lower over the entire time period. After 6 h incubation there was a very slight increase of TCA-soluble counts in the presence of cells compared to their absence and by 41 h this difference was no longer apparent. Viability of cells at 41 h was 85 % by trypan blue exclusion test.

<table>
<thead>
<tr>
<th>Time, h</th>
<th>Temperature, °C</th>
<th>In cells (cpm ± s.d.)</th>
<th>TCA-soluble (cpm ± s.d.)</th>
<th>TCA-soluble, no cells (cpm ± s.d.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>37</td>
<td>3·38 ± 0·09</td>
<td>8·40 ± 0·30</td>
<td>6·24</td>
</tr>
<tr>
<td>4</td>
<td>37</td>
<td>0·83 ± 0·16</td>
<td>6·54 ± 0·36</td>
<td>—</td>
</tr>
<tr>
<td>16</td>
<td>37</td>
<td>5·68 ± 0·09</td>
<td>11·0 ± 0·29</td>
<td>—</td>
</tr>
<tr>
<td>4</td>
<td>37</td>
<td>1·09 ± 0·10</td>
<td>7·28 ± 0·37</td>
<td>—</td>
</tr>
<tr>
<td>41</td>
<td>37</td>
<td>21·1 ± 1·04</td>
<td>15·3 ± 0·46</td>
<td>15·4</td>
</tr>
<tr>
<td>4</td>
<td>37</td>
<td>4·15 ± 0·04</td>
<td>9·38 ± 0·28</td>
<td>—</td>
</tr>
</tbody>
</table>

1·9 x 10⁶ cells (see text) were incubated for 6, 16 and 41 h at 37° and 4 °C in 10 % NCS/ M199 containing 5·12 μg 125I-Hb (16600 cpm). At the end of the incubation period the medium was removed, adjusted to its original volume, and counted. TCA at a final concentration of 3 % (w/v) was added to the medium, and the mixture centrifuged. The supernatant was counted and recorded as TCA-soluble cpm as a percentage of the total cpm. The cells were washed 5 times with BSS and removed from the dishes with 0·1 % Triton X-100 and counted. The cpm in cells were recorded as a percentage of the total cpm.
Effect of cell concentration on uptake of $^{125}$I-Hb. Increasing numbers of cells ranging from $1 \times 10^8$ to $8 \times 10^8$ were incubated for 24 h in 10% NCS/M199 containing 6.85 $\mu$g $^{125}$I-Hb (18000 cpm). Fig. 1 shows uptake of Hb at 37 °C. It can be seen that uptake was linear from $1 \times 10^8$ to $4 \times 10^8$ cells and began to drop slightly at greater cell concentrations. Values of percentage TCA-soluble counts were similar in all cultures, indicating that digestion had not taken place.

![Graph](image)

Fig. 1. Uptake of $^{125}$I-Hb with increasing cell population. $1 \times 10^6$ to $8 \times 10^8$ cells were grown for 18 h in 10% NCS/M199. The media were then changed to 10% NCS/M199 containing 6.85 $\mu$g $^{125}$I-Hb (18000 cpm) and incubation continued for 24 h before harvesting. Bars represent S.D. from the mean of triplicate measurements.

Effects of $^{125}$I-Hb concentration on uptake and digestion. Concentrations of 5 $\mu$g (15500 cpm), 10 $\mu$g (31000 cpm) and 20 $\mu$g (62000 cpm) of $^{125}$I-Hb were incubated with $2 \times 10^6$ cells for 20 h at 37 °C and 4 °C. It can be seen from Fig. 2A that uptake of Hb was directly proportional to the amount of Hb present when expressed as cpm. There was only a slight increase in uptake of Hb at 4 °C. Fig. 2B demonstrates, however, that percentage Hb taken up by cells at 37 °C was identical at all concentrations. In the experiments illustrated in Fig. 2A, B no enzymic breakdown of Hb was detected when percentage TCA-soluble counts in cell-free medium was compared to that in the presence of cells.

Intracellular digestion of $^{125}$I-Hb in the presence and absence of serum. To ensure nutrition of the cells, LAH in M199 was substituted for serum-free conditions.
during $^{125}$I-Hb uptake and digestion. Table 2 shows an experiment where $3 \times 10^6$ cells were grown for the initial 18 h in 10% NCS/M199 after which the medium was changed to either 0·25% LAH/M199 or 10% NCS/M199 for another 18 h. This medium was then changed to either 0·25% LAH/M199 or 10% NCS/M199,

![Graph](image)

Table 2. Uptake and digestion of $^{151}$I-Hb in presence of
0·25% LAH or 10% NCS

<table>
<thead>
<tr>
<th>Medium</th>
<th>Temperature, °C</th>
<th>% total cpm In cells</th>
<th>TCA-soluble</th>
</tr>
</thead>
<tbody>
<tr>
<td>0·25% LAH:</td>
<td>37</td>
<td>7·64</td>
<td>52·8</td>
</tr>
<tr>
<td>+ cells</td>
<td>4</td>
<td>2·50</td>
<td>10·2</td>
</tr>
<tr>
<td>- cells</td>
<td>37</td>
<td>—</td>
<td>13·4</td>
</tr>
<tr>
<td>10% NCS:</td>
<td>37</td>
<td>5·51</td>
<td>18·8</td>
</tr>
<tr>
<td>+ cells</td>
<td>4</td>
<td>0·88</td>
<td>9·43</td>
</tr>
<tr>
<td>- cells</td>
<td>37</td>
<td>—</td>
<td>11·2</td>
</tr>
</tbody>
</table>

$3 \times 10^6$ cells were cultured for initial 18 h in 10% NCS/M199. The medium was then replaced by either 0·25% LAH/M199 or 10% NCS/M199 and the cells incubated for a further 18 h. The media were again replaced with ones containing 7·2 µg $^{125}$I-Hb (7000 cpm). Incubation was continued for a further 18 h.

both of which contained $^{151}$I-Hb. Incubation was continued for an additional 20 h. This experiment clearly demonstrated that there was a much greater breakdown of labelled protein (52·8% of total) when the cells were incubated in the absence of serum as compared to its presence (18·8%).
Fig. 3. Uptake of $^{125}$I-Hb over time in the presence of LAH. $3 \times 10^8$ cells in 10% NCS/M199 were grown overnight in Leighton tubes. On day 1 the medium was replaced by 0.25% LAH/M199 and incubated for 3 h at 37°C. It was then replaced by 0.25% LAH/M199 containing 2.28 μg $^{125}$I-Hb (20000 cpm). Cultures were incubated in duplicate at 37° and 4°C (● and ○, respectively) for 1, 2, 4, 8 and 18 h and harvested. Points represent means of duplicate measurements.

Fig. 4. Digestion of $^{125}$I-Hb by cells in the presence of LAH. Medium from cells cultured as shown in Fig. 3 was precipitated with TCA and the non-precipitable supernatant was counted as explained in text. The counts were called TCA-soluble and were used as a measure of intracellular digestion and release. Points represent means of duplicate measurements. △, 37 °C + cells; ×, 37 °C − cells; ▲, 4 °C + cells.

Uptake and digestion of $^{125}$I-Hb over time in the presence of LAH. Term tissue was studied for uptake and digestion of $^{125}$I-Hb in 0.25% LAH/M199 over 18 h at 37° and 4°C. $3 \times 10^6$ cells in 10% NCS/M199 were grown overnight in Leighton tubes and on day 1 this medium was changed to 0.25% LAH/M199 for 3 h at 37°C. Finally, this was replaced by 0.25% LAH containing 2.28 μg $^{125}$I-Hb (20000 cpm). Cultures were incubated in duplicates at 37° and 4°C for 1, 2, 4, 8 and 18 h and harvested. Fig. 3 shows the percentage total uptake of $^{125}$I-Hb over 18 h. Term cells took up a maximum amount of Hb between 2 and 4 h at 37°C, and this was followed by a loss of radioactivity from cells. Fig. 4 shows that there was a much greater increase in the release of TCA-soluble $^{125}$I in the presence of cells at 37°C than in their absence from 2 h of incubation onwards.

A large number of experiments were performed to ensure the validity of this digestion. Fig. 5 shows an average release of TCA-soluble radioactivity from cells...
Fig. 5. Digestion of $^{131}$I-Hb by several different cell cultures in the presence of LAH. Data for release of TCA-soluble material from several different cultures from term and early specimens. All values corrected for absence of cells used as controls for each specimen. Cell concentration in each case was maintained at $3\times10^4$ cells. The line fits the equation $y = 2.95x - 3.45; r = 0.81, n = 24$.

Fig. 6. Elution from Sephadex G-50 columns of TCA-soluble radioactivity obtained from medium after digestion of $^{131}$I-Hb by cells. Media obtained for experiments illustrated by Figs. 3 and 4 were precipitated by TCA (see text). The TCA-soluble material was chromatographed on Sephadex G-50 columns; 0.5 ml of sample volume applied to each column. A, 18 h, 37°C, no cells; B, 18 h, 4°C, with cells; C, 8 h, 37°C, with cells; D, 18 h, 37°C, with cells. ■, incubated medium; □, sodium $^{131}$iodide.
over time when corrected for background in the absence of cells. Linearity of digestion was maintained for at least 12 h.

Estimation of the nature of TCA-soluble digestion products. Sephadex was used to grade, by molecular weights, the digestion products released into the medium in the presence of cells at 37 °C or 4 °C and in their absence. Media from cells shown in Figs. 3 and 4 were used for the estimation of the nature of digestion products. Samples selected were media from 18 h of incubation at 37 °C with and without cells, and with cells at 4 °C. A sample of cells incubated at 37 °C in the presence of Hb for 8 h only was also studied. The results in Fig. 6 show that in the presence of cells at 37 °C there was a shift of radioactivity to the left – towards intermediate-sized peptides. In the absence of cells, primarily lower-molecular-weight material was eluted (possibly mono- and di-iodotyrosine and some Na\(^{125}\)I).

DISCUSSION

Protein uptake and digestion in the presence and absence of serum was investigated in monolayer tissue culture of trophoblastic cells. Using phase-contrast microscopy we were able to distinguish 4 distinct cell types as described by Fox & Kharkongor (1970). These authors have histochemically characterized the cell types which fall into 4 distinct groups and can be related to their counterparts in the intact tissue. Using immunofluorescent techniques we have localized cathepsin D in each of these 4 cell types. We believe this endopeptidase to be responsible for the initial breakdown of labelled haemoglobin, though at this stage no attempt was made to distinguish the cultured cell types responsible for intracellular digestion. The amount of \(^{125}\)I-Hb taken up by trophoblastic cells in 10 % NCS/M199 was directly related to incubation time, cell concentration and Hb concentration. Although the percentage of total Hb taken up by cells at 37 °C was linear over 48 h, the rate of uptake decreased with time. Over the first few hours of incubation, however, this rate was fairly constant from one experiment to another. The average rate of pinocytosis in 4 experiments in which the first 4–8 h of incubation were studied was 0.303 ± 0.058 % total Hb/10\(^6\) cells/h (0.229–0.379 % range) or 1.58 ± 0.319 total Hb/mg cell protein/h (1.19–1.97 % range). Correction for cell-associated Hb at 4 °C reduces these values by approximately 20 %. This range of uptake was unaffected by the concentration of serum present.

In contrast to these results, when serum was substituted by LAH, Hb uptake reached a plateau after 2–4 h of incubation. Similar plateaus were noted for soluble proteins by Dingle, Poole, Lazarus & Barrett (1973) using \(^3\)H-Hb uptake by macrophages and by Williams, Kidston, Beck & Lloyd (1975) for \(^{125}\)I-labelled albumin uptake by rat yolk sac in vitro. The plateaus demonstrated for pinocytosis of proteins could be the result of constant uptake, digestion and exocytosis. In the presence of serum, this plateau was absent for trophoblast cells, because digestion was not taking place. The range of the rate of \(^{125}\)I-Hb uptake in the absence of serum, assuming it was linear, was estimated for the first 6 h of incubation to be very similar to that in the presence of serum: 0.170–0.308 % of total Hb/10\(^6\) cells/h or 1.07–1.94 %
total Hb/mg cell protein/h (both corrected for 4 °C value). This rate of pinocytosis was comparable to the rate of uptake of radioactive gold by mouse macrophages under conditions of high stimulation of pinocytosis, i.e. in 50% NCS at 0.132–0.157% load/10^6 cells/h, i.e. 2.21–2.62% load/mg cell protein/h (Davies, Allison & Haswell, 1973). This high rate of pinocytosis probably involved both macro- and micropinocytosis. Although little adsorption of gold to macrophage plasma membrane was reported (Davies et al. 1973), uptake of adsorbed ^125^I-Hb could not be ruled out for our studies on trophoblast. It was interesting, however, that the presence of serum had no effect on the rate of pinocytosis of Hb and, if membrane adsorption were important for uptake, competition between Hb and serum proteins might have been expected.

Preliminary results with pinocytic inhibitors such as KCN and 2,4-dinitrophenol showed them to be without effect on uptake of ^125^I-Hb, although the lower temperature of 4 °C inhibited uptake by up to 80%. These results were different from those of Cohn (1970) who showed that not only 4 °C but both these compounds were potent inhibitors of macro-pinocytosis in macrophages. On the other hand, Ryser (1968) had similar results in cultured sarcoma cells when he showed that the uptake of ^131^-albumin was inhibited only by 4 °C. Recent results with L cells in culture (Steinman, Silver & Cohn, 1974) showed that uptake of sHRP was only partially prevented by inhibitors of both respiration and glycolysis and a combination of the 2 types of inhibitors gave a maximum of 80% inhibition of pinocytosis. There is some dispute in the literature as to the effect of cold on pinocytosis. Most of the data indicate that 4 °C causes a near 100% inhibition of macro- and micropinocytosis (Cohn, 1970; Ryser, 1968; Davies et al. 1973; Steinman et al. 1974). However, Casley-Smith (1969) and Schmidtke & Unanue (1971) demonstrated that uptake of carbon particles, thorium dioxide, ferritin and ^131^-labelled albumin was not inhibited at 4 °C. It was concluded, therefore, that the ^125^I-Hb which was associated with trophoblastic cells at 4 °C was derived from 3 possible sources: (1) rapidly pinocytosed during the initial 12-min gassing period and before the temperature of the medium had reached 4 °C, (2) taken up by the cells through a mechanism which was not temperature-dependent, and (3) adsorbed to the cell surface.

The mechanism of inhibition of ^125^I-Hb digestion in the presence of serum was not elucidated. Because uptake itself was not inhibited, either enzyme inhibition following fusion of pinosomes and lysosomes, or lack of fusion of these 2 organelles, may have been responsible. Enzyme inhibition could have been produced by such serum components as α-2-macroglobulin (Barrett & Starkey, 1973), haptoglobin (Sato, Sasazuki, Tsuuno & Nakajima, 1973) or plasma proteinase inhibitors such as α-1-antitrypsin (Cohen, 1973; Sandhaus & Janoff, 1974).

Digestion of ^125^I-Hb in the absence of serum was detectable after what appeared to be a maximal uptake and then continued linearly for at least 18 h. The rate of digestion varied from placenta to placenta and this probably depended as much on the different ^125^I-Hb preparations as on differences in the cells themselves. Williams et al. (1975) have shown that the rate of uptake of ^125^I-albumin by rat yolk sac varied with each albumin preparation and that this was directly related to
Pinocytosis in cultured trophoblast cells

the degree of denaturation of the protein (Moore, Williams & Lloyd, 1974). If the rate of uptake varied with the state of the substrate, then the experimental conditions used in our work would affect the rate of digestion. Differences in the cells would most likely involve postmature tissue (42-44 weeks' gestation) when compared to term tissue (39-40 weeks' gestation) as the proportion of degenerating cells in the former cultures is likely to be higher (Longo, 1972). Of 19 different experimental points, each in duplicate or triplicate, and where 3-4 x 10^6 cells were cultured, the range of the rate of breakdown of ^{125}I-Hb was 0.075-0.833 % total Hb hydrolysed/10^6 cells/h (corrected for TCA-soluble counts released in the absence of cells). The average of these values was 0.340 ± 0.236 % (S.D.) total Hb/10^6 cells/h. It can be seen that the average rate of digestion was very close to the estimated rate of uptake discussed earlier. If the rate of pinocytosis is corrected for the rate of digestion, then it becomes 0.44 ± 0.19 % total Hb/10^6 cells/h or 2.3 % ± 0.99 total Hb/mg protein/h (calculations performed on 19 individual experimental points). These results, therefore, point to how a plateau of uptake was produced, and the decrease in cell-associated radioactivity seen was in these cells where digestion had continued following a decrease in the rate of pinocytosis.

Sephadex chromatography of TCA-precipitated medium showed that in the presence of cells there was a shift in the elution pattern when compared with absence of cells or from cells incubated at 4 °C. These results indicated the presence of ^{125}I-labelled Hb fragments with slightly higher molecular weights than those present in the controls. Because control markers other than Na ^{125}I were not put on these columns, it was not possible to tell what these fragments might be. Other reports of similar studies, however, have shown that the digestion products were primarily single amino acids and small peptides (Ehrenreich & Cohn, 1967, 1968; Ryser, 1968a; Williams et al. 1975). The small peak in radioactivity seen in Fig. 6 where the cells were incubated for 18 h at 4 °C could be explained by uptake and digestion by the cells during the initial gassing period of 12 min at 37 °C.

Because digestion was inhibited in the presence of serum, one has to be careful in the interpretation of these results and their significance in the in vivo situation. It is possible that the plasma membrane of mid-gestation to term trophoblast is utilized for endocytic uptake which serves primarily for transcellular transport, rather than for intracellular utilization of the vesicle contents. IgG transport to the foetus has been shown to increase from 22 weeks' gestation onwards and this molecule appeared to be more readily transferred than other serum proteins (Morphis & Gitlin, 1970; Gitlin, Kumate, Urrusti & Morales, 1964). From this period of foetal growth until birth, transcellular transfer of, at least, IgG appears to increase. This may be the consequence of induction of a specific type of vesicle (Rodewald, 1973), of specific IgG receptors, of decreased lysosomal catheptic activity or decreased rate of lysosome fusion with pinosomes. Inhibition of Hb digestion in the presence of serum in term tissue may, therefore, reflect a change in the needs of the foetus and in the role of the placenta as a whole. The nutritional demands of the foetus on the mother may be greatest in early pregnancy, when the fastest growth and development are taking place. It is perhaps during this period that lysosomal activities
of the trophoblast make a significant contribution. This also is emphasized by the
fact that teratogenic compounds are effective primarily in early gestation only. They
may either directly inhibit lysosomal enzymes or, as has recently been shown for
trypan blue, directly inhibit pinocytosis (Lloyd, Davies, Williams & Beck, 1971).

Lysosomal proteolysis in trophoblast may play an important role in supplying
amino acids for the synthesis of protein hormones. The placenta has an important
synthetic role throughout gestation, and this activity would demand a large and
constant supply of amino acids. These may be partially supplied by lysosomal
digestion of endocytosed proteins. It is also possible that lysosomal enzymes may
modify protein hormones before release into the maternal circulation.

It appears that digestion of endocytosed molecules by trophoblast lysosomes in
term tissue is probably unimportant for foetal nutrition, under conditions of an
adequate supply of nutrients to the foetus. If, however, there is an inadequate supply
of amino acids, lysosomal digestion of endocytosed materials or autolytic digestion of
its own cellular components may help to supply the necessary nutrients, either to the
foetus or to those parts of the placenta which are active in supporting foetal life. In
early gestation, lysosomal digestion is likely to supply nutrients to the trophoblast
itself, but under healthy conditions would probably not significantly contribute to
foetal nutrition.

The resemblance in appearance of Hofbauer cells to macrophages (Boyd & Hamilton,
1970) implies that they may also perform similar functions. Like mesothelial cells
in the kidney (Farquhar & Palade, 1961), Hofbauer cells may function to endocytose
and destroy molecules which have managed to pass through the trophoblast cells or
which have crossed the endothelial cells of the foetal capillaries. The most important
of these, because they could presumably be very damaging, may be immunoglobulins
and immune complexes. Other debris may also accumulate and macrophages in
the villous stroma would help to destroy them to maintain an efficient transfer of
essential molecules between mother and foetus.

REFERENCES

ASHLEY, C. A. (1965). Study of the human placenta with the electron microscope; functional
implications of a canal system in the trophoblast. Archs Path. 80, 377–390.

Press.

BARNETT, A. J. & STARKEY, P. M. (1973). The interaction of α₁-macroglobulin with proteinases;
characteristics and specificity of the reaction, and a hypothesis concerning its molecular


of particles by small and large vesicles into peritoneal macrophages. J. Microscopy 90,
15–30.

COHEN, A. B. (1973). Interrelationships between the human alveolar macrophages and alpha-

Blackwell.
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