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Characterization and Localization of Human Placental Ferritin

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Ferritin has been purified from normal full-term human placentae and its antigenic and molecular characteristics compared with adult liver ferritin. Placental ferritin is composed predominantly of a single subunit type, co-migrating with a liver ferritin standard on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. Comparison of dose–response curves in an immunoradiometric assay indicated some tissue-specific antigenicity for placental ferritin. This was supported by immunofluorescence studies on cryostat sections of human placentae by using antibodies to placental and spleen ferritin. Specific staining for placental ferritin was demonstrated within placental syncytiotrophoblast, particularly localized towards the microvillus plasma membrane. Ferritin has also been shown by electrophoretic and antigenic analysis to be present in protein fractions solubilized from isolated human syncytiotrophoblast microvillus plasma-membrane preparations, suggesting that ferritin may play an active role in the transfer of iron from maternal transferrin across the syncytiotrophoblast plasma membrane.

Ferritin is an iron-containing protein of wide distribution among eukaryotic cells, and has been most extensively studied in rat, horse and man (Crichton, 1973; Harrison *et al.*, 1974). In these species, ferritin has been isolated from many tissues, including liver, spleen, lung, heart, kidney, bone marrow, gastrointestinal mucosa and placenta (Crichton *et al.*, 1975; Drysdale & Singer, 1974; Munro *et al.*, 1975). The protein moiety of ferritin has a molecular weight of approx. 450000, and consists of 24 subunits (Harrison *et al.*, 1974) surrounding a central core of iron in the form of ferric hydroxyphosphate (Granick & Hahn, 1944). Multiple molecular forms of ferritin, termed isoferritins, exist and may represent hybrid molecules built up of various proportions of two subunit types of different size and charge (Drysdale, 1977; Drysdale *et al.*, 1977). Adult human heart, kidney and pancreatic ferritins, as well as ferritin derived from several neoplasms, show more acidic isoferritins on isoelectric focusing than liver and spleen ferritins (for review see Drysdale, 1977; Drysdale *et al.*, 1977). Natural apoferritin, and liver or spleen apoferritin produced in iron-overload disease, are the most basic isoferritins (Drysdale, 1977; Drysdale *et al.*, 1977).

Human placental ferritin has previously been isolated by Bohn (1973), and the term carcinofoetal isoferritins has been used by Drysdale & Singer (1974) to describe two acidic isoferritins isolated from human placenta that appeared to correspond to isoferritins from HeLa cells. The present study describes the further characterization of human placental ferritin in terms of molecular and antigenic properties, as well as its localization within placental tissue. The results indicate that human placental ferritin may consist of only one subunit type, and could have a function in direct acceptance of iron from the trophoblast plasma membrane during transplacental iron passage.

Methods and Materials

Purification of human ferritin

Normal full-term human placental tissue from uncomplicated pregnancies was obtained immediately after delivery. Normal adult human liver, spleen and heart tissue was obtained *post mortem*. Tissue was homogenized in a Moulinex blender with water and tissue debris removed by centrifugation at 1500*g* for 10min. The supernatant was subjected to carefully controlled heating at 70°C for 10min, and then quickly cooled in ice. Heat-denatured protein was removed by centrifugation at 1500*g* for 10min. The supernatant was made 0.05M with respect to sodium acetate, adjusted to pH5.5 with acetic acid and brought to 60% saturation with respect to $(NH_4)_2SO_4$. The protein precipitate was allowed to form overnight at 4°C, collected, dissolved in 50ml of 0.9% NaCl solution and centrifuged at 100000*g* for 2h. The resultant pellet was re-dissolved in 0.05M-sodium barbitone buffer, pH8.0, containing 0.15M-NaCl plus 0.01% NaN_3 and subjected to column chromatography on Sepharose 6B (Pharmacia A.B., Uppsala, Sweden). Contaminating albumin was usually

removed from tissue ferritin preparations by affinity chromatography on Affi-Gel Blue (Bio-Rad Laboratories), but in the case of the placenta albumin did not bind to the column in sufficient quantities as assessed by double-immunodiffusion studies with rabbit anti-(human albumin) sera (Behringwerke A.-G., Marburg, Germany). Subsequently, placental ferritin preparations were further purified by preparative polyacrylamide-gel electrophoresis by using a cell as described by Brown *et al.* (1977). Protein concentrations were determined by the method of Lowry *et al.* (1951) with bovine serum albumin as standard.

Purity of ferritin preparations and gel-electrophoretic analysis

Purity of tissue ferritin preparations was confirmed by polyacrylamide-gel disc electrophoresis in 7% gels with a 3% stacking gel and a discontinuous buffer system (Davis & Ornstein, 1968). Gels were stained for protein with Coomassie Brilliant Blue R250 and for iron with acidified $K_4Fe(CN)_6$.

Sodium dodecyl sulphate/polyacrylamide-gel disc electrophoresis was performed with the discontinuous buffer system of King & Laemmli (1971) and 15% gels with a 3% stacking gel prepared by the method of Segrest & Jackson (1972). Protein samples had first been reduced by addition of 5% 2-mercaptoethanol and the mixture immersed for 3min in a boiling-water bath. A variety of protein standards were used in sodium dodecyl sulphate/polyacrylamide-gel disc electrophoresis to calibrate apparent molecular weights.

Preparation of ferritin antisera

Dunkin Hartley guinea pigs were immunized with either purified human spleen or placental ferritin in Freund's complete adjuvant. Each guinea pig received 100μg of protein in multiple intramuscular sites three times at 2 week intervals. Blood was collected after a further 2 weeks and serum portions were stored at -20°C with 0.01% NaN_3. Activity of the anti-ferritin sera was confirmed by double-immunodiffusion analysis in 1% agarose gels in barbitone buffer, pH 8.6 (I 0.05), with as antigens human liver, placenta, spleen and heart ferritins prepared by the method described above. Antisera were adsorbed by passage over normal human serum immobilized on CNBr-activated Sepharose 4B (Pharmacia A.B.).

Ferritin assay

Ferritin was determined by using the immunoradiometric assay of Addison *et al.* (1972). Antiserum to human spleen ferritin was adsorbed with a cellulose-based horse spleen ferritin (Koch–Light, Colnbrook, Bucks., U.K.) immunoadsorbent (Miles & Hales, 1968). Anti-ferritin antibodies purified in this manner were labelled with ^{125}I by the chloramine-T method (Hunter & Greenwood, 1962). Human spleen, heart and placental ferritin preparations of known protein concentration were used to construct standard curves. Ferritin in placental membrane preparations was determined by using a placental ferritin standard.

Human placental syncytiotrophoblast microvillus plasma-membrane preparations

Full-term human placentae from uncomplicated pregnancies were collected immediately after delivery, stripped of chorionic membrane and umbilical-cord tissue, and syncytiotrophoblast microvillus plasma-membrane preparations obtained from villus tissue by cold iso-osmotic NaCl extraction and differential ultracentrifugation as described by Smith *et al.* (1974). Some syncytiotrophoblast microvillus plasma-membrane preparations were further purified by centrifugation on a two-phase system of dextran T500 and poly(ethylene glycol) 6000 as described by Hourani *et al.* (1973). Purity of syncytiotrophoblast microvillus plasma-membrane preparations was confirmed by electron microscopy.

Syncytiotrophoblast microvillus plasma-membrane preparations were solubilized in 3M-KCl, 1% sodium deoxycholate or 0.5% Nonidet NP40. There was no difference in the results described in the present study on the basis of the solubilizing agent employed. Resultant syncytiotrophoblast microvillus plasma-membrane preparations contained 6–9 units of alkaline phosphatase/mg of total protein and <30ng of β_2-microglobulin/mg of total protein, confirming the trophoblastic source of the plasma-membrane preparations (Carlson *et al.*, 1976; Faulk & Johnson, 1977; Faulk & Temple, 1976). The molecular and morphological characteristics of human full-term placental syncytiotrophoblast microvillus plasma-membrane preparations isolated by these techniques are reported elsewhere (Ogbimi *et al.*, 1979).

Immunofluorescent studies

Small pieces of fresh full-term human placental villus tissue were washed in iso-osmotic NaCl and quick-frozen. Cryostat 4μm sections were studied by indirect immunofluorescence as described in detail elsewhere (Faulk & Johnson, 1977; Johnson *et al.*, 1977; Johnson & Faulk, 1978). In some experiments small pieces of tissue were held in organ culture for 5 days (Faulk & Temple, 1976) before quick-freezing for immunofluorescent studies. Tissue sections were first washed in phosphate-buffered iso-osmotic NaCl, pH 7.2, for 30min or, in some experiments, pre-washed in 0.5M-NH_4SCN at 4°C for 10min to remove any adsorbed protein. The fluorescein-conjugated rabbit anti-(guinea-pig immunoglobulin) serum (Miles Research Products, Slough, Berks., U.K.) was adsorbed before use by passage over normal human serum immobilized on CNBr-activated Sepharose 4B.

Titration experiments with unlabelled antisera, control non-immune sera and fluorescein-labelled conjugates were performed by the method of Faulk & Johnson (1977). Stained sections were examined by using a Leitz Dialux 20EB microscope with a Ploemopak 2.4 epi-illuminator and a 50W high-pressure mercury lamp. Photomicrographs were taken with a Leica camera on Kodak Ektachrome film.

Results

Immunodiffusion analysis

Guinea-pig antisera raised against human spleen ferritin gave single precipitin lines of apparent identity in double-radial-immunodiffusion analysis against human spleen, liver, heart and placental ferritin, as well as against human full-term syncytiotrophoblast microvillus plasma-membrane preparations. These precipitin lines stained strongly for iron with $K_4Fe(CN)_6$. Antisera to human placental ferritin gave single precipitin lines of apparent identity with liver (Fig. 1), spleen and heart ferritins, but showed two precipitin lines against both placental ferritin (Fig. 1) and syncytiotrophoblast microvillus plasma-membrane preparations. The second non-iron-staining band gave a precipitin line of apparent identity with that obtained between rabbit anti-(human albumin) sera and human placental ferritin, indicating some residual albumin capable of being recognized immunogenically in placental ferritin preparations. Anti-albumin activity was completely removed from anti-(placental ferritin) sera by adsorption with immobilized normal human serum. The reaction of adsorbed and nonadsorbed anti-(placental ferritin) sera with isolated human liver and placental ferritin is shown in Fig. 1. After further adsorption of the anti-(placental ferritin) sera with excess human liver ferritin, no precipitin line could be observed against either purified ferritin or syncytiotrophoblast microvillus plasma-membrane preparations. The above results refer to syncytiotrophoblast microvillus plasma-membrane preparations purified by the method of Smith *et al.* (1974). After phase centrifugation of syncytiotrophoblast microvillus plasma-membrane preparations on dextran/poly(ethylene glycol), no precipitin line against anti-(placental ferritin) sera that had been adsorbed with immobilized normal human serum was observed either directly or after staining for protein or iron.

Immunoradiometric assay for ferritin

The dose–response curves for human placental ferritin compared with adult liver and heart ferritin in an immunoradiometric assay by using ^{125}I-labelled antibodies to human spleen ferritin are shown in Fig. 2. Antibody binding to liver ferritin was greater than to placental ferritin or heart ferritin. Determination of ferritin content in solubilized human full-term syncytiotrophoblast microvillus plasma-membrane preparations, by using isolated placental ferritin to construct a standard curve, demonstrated 5–15 μg of ferritin/mg of total protein before, and

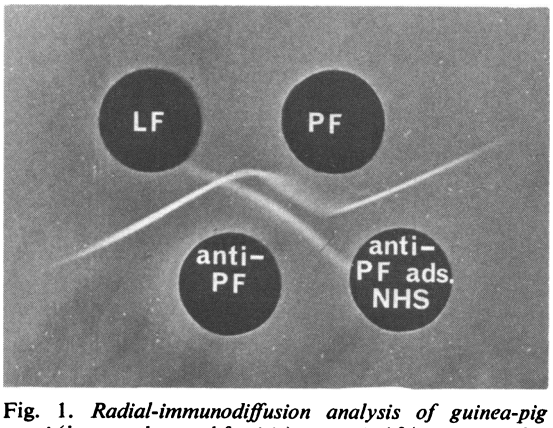

Fig. 1. *Radial-immunodiffusion analysis of guinea-pig anti-(human placental ferritin) serum in 1% agarose gels*
Abbreviations used: LF, adult liver ferritin; PF, placental ferritin; anti-PF, anti-(placental ferritin) serum; anti-PF ads. NHS, anti-(placental ferritin) serum adsorbed with immobilized normal human serum.

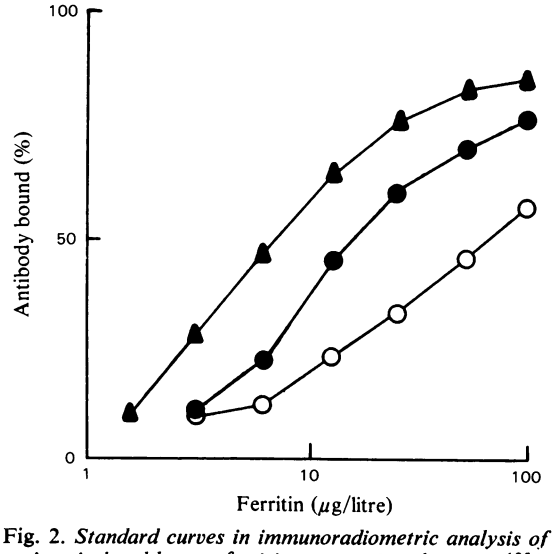

Fig. 2. *Standard curves in immunoradiometric analysis of various isolated human ferritin preparations by using ^{125}I-labelled anti-(spleen ferritin) antibodies*
Symbols: ▲, liver ferritin; ●, placental ferritin; ○, heart ferritin.

1–3 μg/mg of total protein after the phase centrifugation employed in the isolation of syncytiotrophoblast microvillus plasma-membrane preparations.

Electrophoretic analyses

In protein-subunit analysis by sodium dodecyl sulphate/polyacrylamide-gel disc electrophoresis in 15% polyacrylamide gels, both human placental and adult liver ferritin appeared to consist of a single subunit type (Fig. 3). The molecular weight of the ferritin subunit was between 18000 and 20000. Contamination of both preparations with another protein subunit of mol.wt. approx. 65000, which may represent albumin, was demonstrated. Unlike the liver ferritin preparation, the placental ferritin preparation had been further subjected to preparative polyacrylamide-gel disc electrophoresis and contained less contamination than the liver preparation. In neither case were contaminant proteins detected by polyacrylamide-gel disc electrophoresis, and ferritin preparations were also unreactive in double-immunodiffusion studies to a wide variety of antisera against normal human plasma and tissue proteins (antisera as documented by Faulk & Johnson, 1977; Johnson & Faulk, 1978). A single protein subunit band with identical migration with that of isolated placental ferritin was also observed on sodium dodecyl sulphate/polyacrylamide-gel disc electrophoresis of solubilized syncytiotrophoblast microvillus plasma-membrane preparations before further treatment by phase centrifugation.

The polyacrylamide-gel disc electrophoresis pattern for solubilized syncytiotrophoblast microvillus

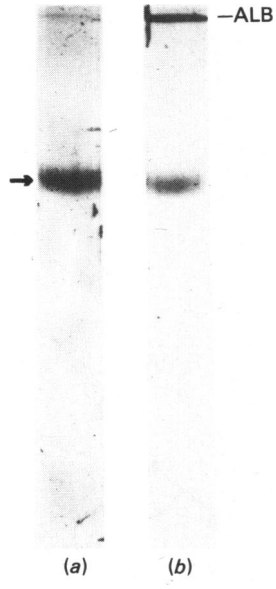

Fig. 3. *Sodium dodecyl sulphate/polyacrylamide-gel disc electrophoresis in* 15% *polyacrylamide gels for a placental ferritin preparation* (*a*) *and an adult liver ferritin preparation* (*b*)

The arrow indicates the ferritin subunit bands. Residual albumin contamination can be seen in both ferritin preparations. Abbreviation: ALB, albumin.

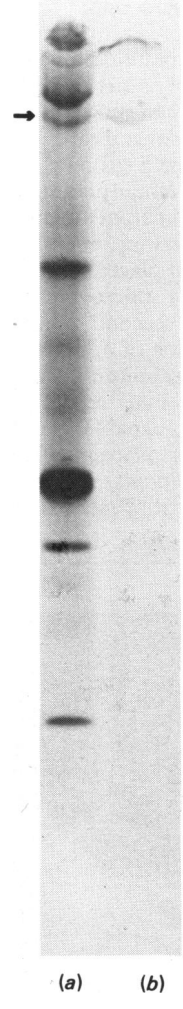

Fig. 4. *Polyacrylamide-gel disc electrophoresis of a full-term human syncytiotrophoblast microvillus plasma-membrane preparation that had been solubilized in* 1% *sodium deoxycholate*

Gels had been stained for protein (*a*) and for iron (*b*). The arrow marks the position for co-migration of isolated placental or liver ferritin preparations.

plasma-membrane preparations stained for protein and iron is shown in Fig. 4. Polyacrylamide-gel disc electrophoresis shows multiple protein bands, one of which co-migrates with purified placental and liver ferritin standards and stains strongly for iron. No iron-staining band was observed after phase centrifugation, but a protein-staining band was still visible at the same point of co-migration with the ferritin standards.

An unsolubilized syncytiotrophoblast microvillus plasma-membrane preparation that had not undergone phase centrifugation was washed thoroughly in phosphate-buffered iso-osmotic NaCl, pH 7.4, and subjected directly to polyacrylamide-gel disc electrophoresis. Substantial protein was desorbed into the gel, including a ferritin band demonstrated by iron staining and co-migration with isolated ferritins. The syncytiotrophoblast microvillus plasma-membrane preparation was reclaimed from the surface of the gel and solubilized in 1% sodium deoxycholate. No iron-containing band could be observed after repeated polyacrylamide-gel disc electrophoresis of this preparation.

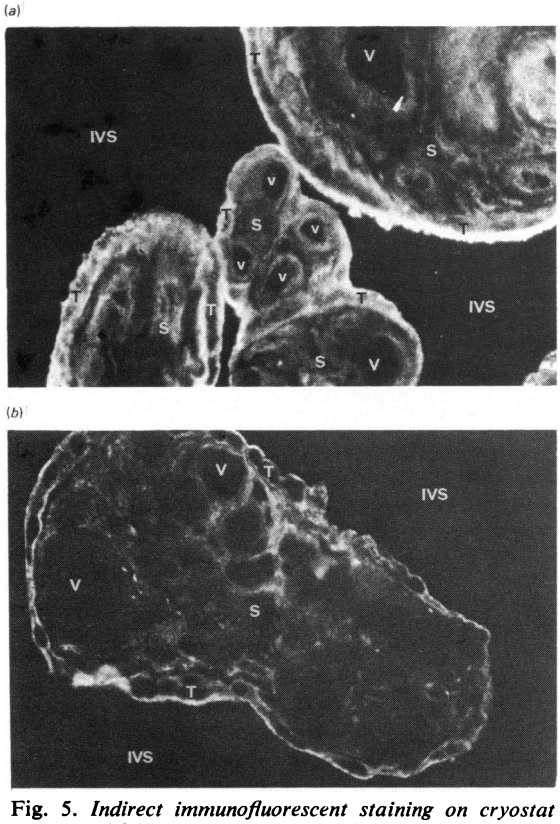

Fig. 5. *Indirect immunofluorescent staining on cryostat sections of full-term human placental villus tissue by using guinea-pig anti-(human placental ferritin) sera that had been adsorbed with immobilized normal human serum*

(*a*) shows normal placental tissue; (*b*) shows 5 day-old organ-culture placental tissue. Trophoblast staining can be seen in both (*a*) and (*b*) particularly in the apical region. Abbreviations: T, trophoblast; IVS, intervillus spaces; S, villus stroma; V, foetal stem vessel.

Immunofluorescence studies

Guinea-pig anti-(placental ferritin) sera that had been adsorbed by passage over immobilized normal human serum clearly stained cryostat sections of normal full-term human placentae in indirect immunofluorescence. Syncytiotrophoblast was stained, particularly towards the apical surface, and there was also staining within the mesenchyme of chorionic villi, notably the cytoplasm of stromal cells (Fig. 5*a*). The degree of trophoblastic staining varied from villus to villus, and often could appear markedly granular in appearance. Endothelial cells commonly gave no significant reaction. Areas of fibrinoid necrosis gave weak, but definite, staining. The optimal dilution of anti-(placental ferritin) sera for indirect immunofluorescence was 1 : 500. This was decreased approx. 10-fold if the antisera had been absorbed previously with excess liver ferritin, although the characteristic trophoblastic-staining pattern was retained. The staining reaction with anti-(placental ferritin) sera was completely removed by absorption with excess placental ferritin apart from a weak diffuse staining of the mesenchymal stroma. Guinea-pig anti-(spleen ferritin) sera, in contrast, stained stromal cells within human placental chorionic villi, but gave little, if any, specific staining of trophoblast.

Prior chaotrope treatment of placental tissue sections removed much of the stromal staining with anti-(placental ferritin) sera, whereas staining of trophoblast and areas of fibrinoid necrosis was clearly retained. Similarly, organ culture of placental tissue for 5 days before immunofluorescence studies did not remove the trophoblastic staining for placental ferritin, whereas most of the stromal staining was removed (Fig. 5*b*).

Discussion

Ferritin is a major iron-storage protein of the body and is synthesized in all tissues, but particularly high concentrations are found in the liver, spleen and bone marrow. Human placental ferritin preparations have been investigated previously after isolation by two different methods (Bohn, 1973; Drysdale & Singer, 1974). Drysdale & Singer (1974) showed that placental ferritin preparations have acidic isoferritins similar to those found in HeLa cells, suggesting some oncofoetal protein characteristics.

In the present report we have purified ferritin from

normal full-term human placentae. Placental ferritin has been shown to contain a single subunit species co-migrating with purified adult liver ferritin on sodium dodecyl sulphate/polyacrylamide-gel disc electrophoresis under reducing conditions. Dose–response curves for purified placental ferritin have been compared with liver and heart ferritin in an immunoradiometric assay by using ^{125}I-labelled antibodies to human spleen ferritin. The results showed a decreased affinity of anti-(spleen ferritin) antibodies for placental ferritin compared with liver ferritin, although this difference was not as marked as for heart ferritin compared with liver ferritin. Nevertheless, these results would appear to indicate some tissue-specific antigenicity for placental ferritin. However, such immunoradiometric analyses may not be precisely quantitative, since it would appear that isolated placental and liver ferritin preparations may contain some contamination that could not be detected by polyacrylamide-gel disc electrophoresis and could not be removed by either preparative polyacrylamide-gel disc electrophoresis or affinity chromatography on immobilized Cibracon Blue (Affi-Gel Blue; Bio-Rad Laboratories). The presence of albumin could only be detected by its immunogenicity and also presumptively identified under dissociating conditions in sodium dodecyl sulphate/polyacrylamide-gel disc electrophoresis analysis. Furthermore, the use of bovine serum albumin as a standard in the determination of ferritin by the Lowry method (Lowry *et al.*, 1951) might give erroneous results (Stauffer & Greenham, 1976), and it may be that an apoferritin standard would be more accurate. Confirmation of specific antigenicity of placental ferritin in immunoradiometric assays must await studies assaying tissue ferritins with antibodies to human placental or heart ferritin.

Indirect immunofluorescence studies on cryostat sections of human placentae by using antisera raised against both human placental and spleen ferritin preparations also indicated some tissue-specific antigenicity for placental ferritin. Specific staining for placental ferritin, not given by anti-(spleen ferritin) sera, was observed within syncytiotrophoblast and was particularly localized towards the microvillus plasma membrane. This staining would appear to be membrane-bound, since it was not removed by prior chaotrope treatment of the tissue sections, and to represent locally synthesized ferritin, since this staining was retained in tissues that had been held in organ culture. Previous immunofluorescence studies have also demonstrated a pronounced localization of transferrin at the apical surface of syncytiotrophoblast and an absence of significant amounts of albumin at this site (Faulk & Johnson, 1977).

During human pregnancy, iron transfer across the placenta from mother to foetus proceeds rapidly and unidirectionally against a concentration gradient. The amount of iron transferred can reach 5mg per day near term (Fletcher & Suter, 1969). The immediate source of iron is maternal plasma transferrin, which binds strongly to receptors on the placental syncytiotrophoblast microvillus plasma membrane (Faulk & Johnson, 1977; Johnson & Faulk, 1978; Faulk & Galbraith, 1979). Several reports (Fletcher & Huehns, 1968; Fletcher & Suter, 1969; Ockleford & Menon, 1977) support a selective uptake of one iron atom from the membrane-bound maternal transferrin and an active process of iron transport through the trophoblast plasma membrane from iron-loaded transferrin to an intracellular iron-acceptor protein. Electron-dense particles have been observed lying immediately below the membrane, and it has been suggested that this may represent accumulation of an intracellular iron-carrier protein (Ockleford & Menon, 1977). Iron transfer from receptor-bound transferrin has been shown in rats to continue even when the foetus had been removed and that iron then accumulates in placental membranes (Mansour *et al.*, 1972). More recently, Nunez *et al.* (1978) have shown that ferritin is capable of directly mobilizing iron from receptor-bound transferrin on mouse reticulocyte membranes, and suggest that this may reflect a pathway of iron uptake in which ferritin plays an active role.

Ferritin has been demonstrated by immunodiffusion and immunoradiometric analysis, as well as by polyacrylamide-gel disc electrophoresis, to be associated with isolated pre-phase centrifugation syncytiotrophoblast plasma-membrane preparations. Ferritin was not demonstrated in post-phase centrifugation syncytiotrophoblast microvillus plasma-membrane preparations after polyacrylamide-gel disc electrophoresis or immunodiffusion analysis of the solubilized proteins. Immunoradiometric analysis indicated a 5-fold decrease in ferritin content after phase centrifugation on dextran/poly(ethylene glycol). Thus a significant amount of ferritin, along with some other membrane-associated proteins including transferrin (Ogbimi *et al.*, 1979), would appear to become dissociated from syncytiotrophoblast microvillus plasma-membrane preparations during the phase-centrifugation procedure. This could reflect a loose association or receptor-binding nature of ferritin with syncytiotrophoblast microvillus plasma-membrane preparations.

It is tempting to speculate that placental ferritin may be synthesized in syncytiotrophoblast in response to iron accumulation on the maternal side of its plasma membrane through interaction of maternal plasma transferrin with a membrane receptor. An apparently loose association with the membrane may reflect molecules that, on becoming progressively saturated with iron, become less membrane-associated before transport into the cell cytoplasm. Such a concept would predict that placental ferritin may have some apoferritin characteristics, i.e. a

predominantly liver-like ferritin subunit and a basic behaviour on isoelectric focusing (Drysdale, 1977). The present results have shown placental ferritin is composed predominantly of a liver-like ferritin subunit type, but our studies of the isoelectric-focusing profile have been inconclusive in that placental ferritin preparations may contain variable amounts of both more acidic and more basic isoferritins than those of liver ferritin. It is, however, quite conceivable that several isoferritin populations exist within the human placenta, one synthesized in response to an iron-overload at the syncytiotrophoblast plasma membrane and at least one other that Drysdale & Singer (1974) report as being carcinofoetal and containing acidic isoferritins.

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