Clin. exp. Immunol. (1979) 37, 359–366.

Antigens of the human trophoblast plasma membrane

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(*Accepted for publication* 26 *January* 1979)

SUMMARY

Using purified plasma membranes of human syncytial trophoblast as immunogen, we have raised heterologous antisera which reacted by indirect immunohistological methods with the surface of the trophoblast. This antigenic activity appears to be absent from other adult and foetal tissues. Its absence from maternal sera and its resistance to mild detergent treatment suggest that it is intimately associated with the surface membrane of the syncytial trophoblast. This antigen was found to be immunologically different to a number of proteins, including those known to be produced by the trophoblast. The identification of this antigen may indicate a potential line of research for the immunological regulation of human fertility.

INTRODUCTION

In spite of much research, the expression of antigens by the syncytiotrophoblast, which is the ultimate layer of the placenta in contact with maternal tissue, remains unclear. Investigations on alloantigens have produced conflicting results (Faulk & Temple, 1976; Goodfellow *et al.*, 1976; Goto *et al.*, 1976; Loke & Ballard, 1973; Loke, Joysey & Borland, 1971). There appears to be general agreement, however, that human trophoblastic tissue is sufficiently immunogenic when injected into animals to induce the production of antibodies showing a high degree of tissue specificity (Beer, Billingham & Yang, 1972; Behrman *et al.*, 1974; Rigby & Curzen, 1969). The significance of these findings is clearly dependent on the purity of the trophoblast preparations used as an antigenic source. We have recently devised our own method for obtaining plasma membrane-enriched fractions from human trophoblast. The specificity of the antiserum raised to our preparations differs from that reported by others in that it localizes exclusively on the plasma membrane of the syncytial trophoblast and not in the cytoplasm. We now report studies using this trophoblast membrane antiserum.

MATERIALS AND METHODS

Plasma membrane isolation. Various isolation methods were attempted before the procedure detailed in Fig. 1 was finally adopted. Small groups of chorionic villi, obtained after therapeutic terminations of pregnancy in the first trimester, were dissected free of associated membrane, washed several times with Hanks' balanced salts solution (HBSS), and homogenized in a loose-fitting Dounce glass homogenizer (twenty strokes). Reproducible results were also obtained when an MSE overhead-type homogenizer (two bursts of 5 sec each at half-speed) was used instead. This and subsequent steps in the membrane isolation were performed at 0–4°C. The homogenization medium used was 0·01 M Tris buffer pH 7·4, containing 0·15 M sodium chloride and 0·002 M phenyl-methyl-sulphonyl fluoride (PMSF). Two differential centrifugation steps were used to obtain a crude microsomal fraction (Fig. 1). After centrifugation at 80,000 *g* for 15 hr on a 30–45% continuous sucrose gradient, the microsomal fraction was separated according to the buoyant densities of the membranes. The milky-white middle fraction, which was subsequently shown to be plasma membrane-enriched and which occurred between lighter and heavier bands of particulate material, was harvested and diluted with 4 volumes of 0·01 M Tris/PMSF (without saline). After pelleting, the membranes were again resuspended in 0·01 M tris/PMSF and centrifuged at 20,000 *g* for 1 hr. This was repeated once to yield the final plasma membrane (PM) pellet. For the purposes of enzymatic and chemical assays the

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0099-9104/79/0800-0359$02.00

fractions from the density gradient centrifugation were designated (from the top of the tube): light membrane (M-1), a particulate material band below a red haemoglobin layer; middle membrane (M-2), a milky-white homogeneous suspension occupying the middle portion of the gradient; heavy membrane (M-3), a band of particulate material near the bottom of the gradient; and the pellet (P). Membrane preparations, if not used immediately, were stored for up to 1 month at −70°C in 0·01 M Tris/PMSF buffer. No proteolysis of proteins occurred under these conditions as revealed using electrophoresis in sodium dodecyl sulphate.

Production of antiserum. The purified trophoblast plasma membranes (TrPM) were resuspended in sterile phosphate buffered saline (PBS) at a concentration of approximately 20 mg/ml, emulsified with an equal volume of Freund's complete adjuvant (FCA), and injected i.m. into rabbits. The rabbits were boosted repeatedly by subcutaneous immunization of TrPM in PBS alone, before each bleeding. Control rabbit serum was obtained by bleeding the experimental rabbits before immunization with TrPM. Serum from rabbits injected i.m. with FCA/PBS alone was also used as a control. All sera were heat-inactivated at 56°C for 30 min. Antisera were absorbed with adult human lymphocytes and foetal skin cells. In each case the absorbed antiserum was reacted with trophoblastic tissue derived from the same conceptus from which the skin cells were obtained.

Immunohistology. The TrPM antisera were used in indirect immunoglobulin staining methods with thin-sectioned, fixed material for immunoperoxidase (Taylor, 1976) and with frozen sections of unfixed tissue for immunofluorescence. In the case of the immunoperoxidase method, the TrPM antiserum was used at a 1:400 dilution and the swine anti-rabbit and peroxidase/anti-peroxidase complexes (PAP) (Taylor, 1976) at 1:50 dilutions. In the blocking experiments the listed antisera (Table 1) were used at 1:10 and 1:20 dilutions. In the distribution pattern experiments (Table 1), the antisera were used at 1:50, 1:100 and 1:400 dilutions on sections of trophoblast before treatment according to the method of Taylor (1976). For the immunofluorescent staining, the thin-sectioned frozen trophoblastic villi were stained with TrPM antiserum at 1:40 dilution followed by FITC-conjugated goat anti-rabbit IgG at 1:20 dilution. All dilutions were done with PBS, and incubations with antisera were for 30 min at room temperature.

Immunoelectrophoresis and radioimmunoassay. Crossed and rocket immunoelectrophoresis were performed by application of the methods detailed in Axelsen, Krøll & Weeke (1973). Radioligand-receptor assay for the presence of antibodies to human chorionic gonadotrophin (HCG) involved reaction with sheep anti-rabbit serum and ^{125}I-labelled HCG in saline containing 0·1% bovine serum albumin and 0·05% Tween 80. The precipitate was filtered off after 2 days at 4°C. Binding of HCG was expressed as a percentage of total counts. The anti-membrane serum was tested at dilutions of 1:50, 1:100 and 1:200 with the sheep anti-rabbit serum diluted at 1:10 and 1:20.

Electron microscopy. Membranes were fixed in osmium tetroxide and embedded in Spurr's medium. After sectioning and staining with uranyl acetate they were examined on a Philips EM 300 electron microscope operated at 80 kV. In the case of the immunoferritin labelling, the TrPM antiserum was used at 1:20 dilution and the goat anti-rabbit ferritin-conjugated IgG (Nordic) at 1:40 dilution.

TABLE 1. Proteins tested to determine whether they might be responsible for the trophoblast membrane antigenic activity

Blocking experiments	Distribution pattern	Immunoelectrophoresis	Radioimmunoassay
α_2-macroglobulin (goat)	α_2-macroglobulin		
	Pregnancy associated α_2 glycoprotein		
β_2-microglobulin (swine)	β_2-microglobulin		
IgG, IgA, IgM, IgD and IgE (goat)	IgG, IgA, IgM, IgD and IgE		
Transferrin (goat)	Transferrin		
Albumin (goat)	Albumin		
	SP_1	SP_1	
	α-foetoprotein		
	α_1-glycoprotein		
	carcinoembryonic antigen		
	HCG	HCG	HCG
	HPL	HPL	
	β-lipoprotein		

In the blocking experiments the sections were pre-treated with the listed antisera at 1:10 and 1:20 dilution before treatment with TrPM antiserum at 1:400 dilution in the indirect immunoperoxidase method (Taylor, 1976). None of the antisera reduced binding compared with slides pre-treated with normal swine and goat sera. In the distribution pattern experiments the antisera were tested at 1:50, 1:100 and 1:400 dilutions on sections of trophoblast. Only IgG, SP_1, HCG and HPL were found to stain the trophoblast. In these cases the peroxidase reaction occupied all the cytoplasm of the syncytium. No precipitin arcs were observed in the immunoelectrophoretic experiments, and no binding of HCG occurred in the radioimmunoassay. SP_1 = Pregnancy-specific B_1-glycoprotein; HCG = human chorionic gonadotrophin; HPL = human placental lactogen.

Enzyme and chemical assays. 5′-Nucleotidase (EC 3.1.3.5.) was assayed by the method of Avruch & Wallach (1971) using phenyl disodium orthophosphate (0·01 M) to inhibit non-specific alkaline phosphohydrolyases. Alkaline phosphatase (EC 3.1.3.1.) was assayed using the Boehringer Mannheim GmbH kit. Other enzymes were determined using the authors' instructions: leucine aminopeptidase (EC 3.4.11.1.) (Goldbarg & Rutenburg, 1958); Na^+/K^+-stimulated Mg^{2+}-dependent adenosine triphosphatase (EC 3.6.1.3.) (Wallach & Kamat, 1966); nicotinamide adenine dinucleotide (NADH) oxidase (EC 1.6.99.3.) and succinate cytochrome c reductase (EC 1.3.99.1.) (Avruch & Wallach, 1971). Acid phosphatase (EC 3.1.3.2.), protein, sialic acid, cholesterol, phospholipid, DNA and RNA were determined using the methods described in Allan & Crumpton (1970).

Sources of materials. Swine anti-rabbit serum, PAP, normal swine serum, α-foetoprotein antiserum and carcinoembryonic antigen antiserum were from Dakopatts, Copenhagen. Nordic Laboratories (Maidenhead, England) provided antisera to α_2-macroglobulin, β_2-microglobulin, immunoglobulin series, transferrin and albumin. Behring (Hoechst House, Middlesex, England) provided antisera to α_1-acid glycoprotein, pregnancy-associated α_2-glycoprotein, β-lipoprotein, human placenta lactogen (HPL) and pregnancy-specific β_1-glycoprotein (SP_1). Antisera to HCG were obtained from Serono (Rome) and Miles Laboratories (England). HCG samples were obtained from Serono and Sigma (London). HPL and SP_1 standards were purchased from Behring. 2-[^{3}H]-adenosine monophosphate, ammonium salt (22 Ci/mmol) was obtained from the Radiochemical Centre, Amersham, England.

RESULTS

Plasma membrane isolation

The density gradient centrifugation used in the plasma membrane isolation (Fig. 1) resulted in an heterogeneous distribution of material. The fractions produced were classed in four groups: light membrane (M-1); middle membrane (M-2); heavy membrane (M-3); and pellet (P). The results of various assays performed on these four fractions, in comparison with the original homogenate, are summarized in Table 2. The plasma membrane 'labels' showed enrichment of activities in the M-2 fraction, in comparison with the homogenate, of ×7·0 (5′-nucleotidase), ×5·4 (alkaline phosphatase), ×1·6 (leucine aminopeptidase), ×9·7 (adenosine triphosphatase). Enzyme markers for other subcellular membranes were low in the M-2 fraction, and expressed relative to the homogenate activity they were: ×0·2 (NADH oxidase—endoplasmic reticulum), ×0·1 (succinate cytochrome c reductase—mitochondria), ×0·1 (acid phosphatase—lysosomes). RNA levels in the M-2 fraction fell within the normal range for plasma membranes (Glick, 1976) and DNA could not be detected in the M-2 fraction. Sialic

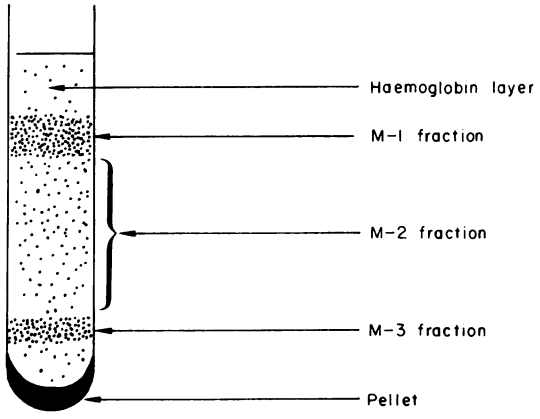

FIG. 1. The method used to prepare purified plasma membranes from the first trimester trophoblasts. (1) Trophoblast villi dissected free of associated membranes and homogenized in 10 mM Tris buffer pH 7·4, containing 0·15 M sodium chloride and 2 mM phenyl methyl sulphonyl fluoride. The homogenate was centrifuged at 300 *g* for 15 min. (2) The resultant supernatant was spun at 4000 *g* for 15 min. The supernatant was again collected and centrifuged at 20,000 *g* for 30 min to yield the microsomal pellet. (3) The microsomal pellet from (2) was resuspended in 10 mM Tris buffer pH 7·4 (without saline) and overlaid onto a 30–45% continuous sucrose gradient in the same buffer. The gradient was spun at 80,000 *g* for 15 hr. (4) The fractions obtained after the density gradient centrifugation are illustrated. The middle (M-2) fraction was collected, diluted with four volumes of 10 mM Tris buffer and centrifuged at 20,000 *g* for 1 hr. The pellet was resuspended in buffer and repelleted at 20,000 *g* for 1 hr. This last stage was repeated once to yield the final (plasma membrane) pellet.

TABLE 2. Enzymatic and chemical determinations on the various subcellular fractions produced from the trophoblast

		Homogenate	M-1 fraction	M-2 fraction	M-3 fraction	Pellet
6	5′nucleotidase	0·053±0·004	0·163±0·002	0·371±0·007	0·150±0·008	0·070±0·013
4	Alkaline phosphatase	4·182±0·038	9·329±0·076	22·412±1·110	6·137±1·021	3.774±0·740
3	Leucine aminopeptidase	0·360±0·009	0·609±0·007	0·559±0·009	0·404±0·005	0·395±0·009
5	Na^+/K^+-Mg^{2+}-ATPase	0·42±0·02	0·95±0·04	4·08±0·06	0·87±0·04	0·62±0·05
4	NADH oxidase	1·08±0·05	0·27±0·05	0·18±0·01	0·93±0·04	5·47±1·30
5	Succinate cytochrome c reductase	0·201±0·030	0·072±0·029	0·012±0·003	0·048±0·015	0·065±0·022
4	Acid phosphatase	2·09±0·05	0·30±0·02	0·20±0·03	0·62±0·02	0·65±0·07
3	Sialic acid	0·036±0·004	0·042±0·002	0·093±0·005	0·031±0·006	0·028±0·004
3	RNA	143·5±4·5	23·2±2·0	19·1±2·4	74·2±4·4	183·0±11·2
1	Cholesterol/phospholipid molar ratio	—	0·34	0·59	0·32	0·29

All enzyme activities are expressed as μmol of substrate produced per hr per mg of protein±s.e. RNA is given in μg/mg protein and sialic acid is in units of μg/ml per mg protein. n = Number of determinations.

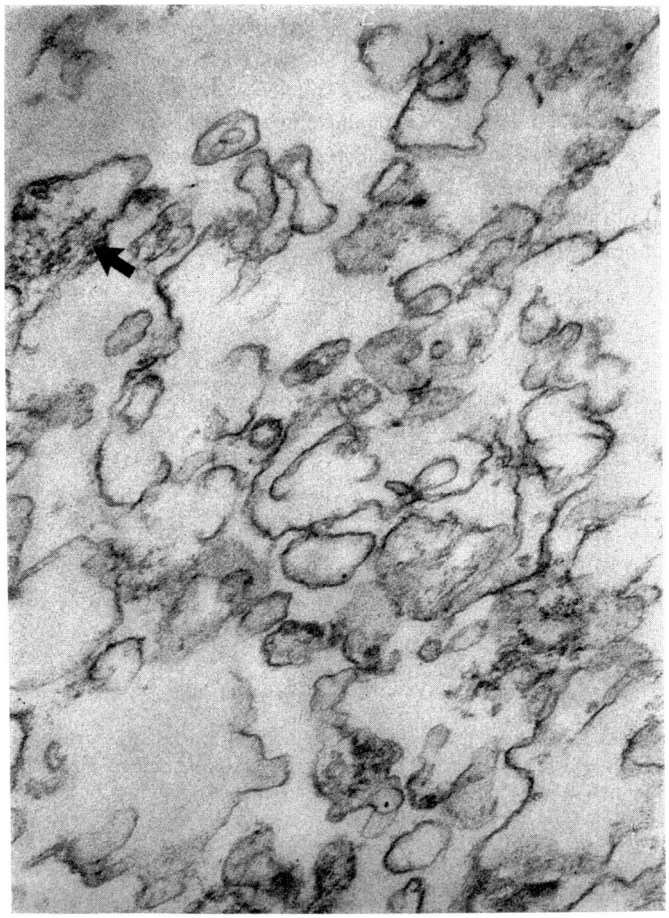

FIG. 2. Transmission electron micrograph of a thin section of the trophoblast plasma membrane preparation. (Arrow) Microfilaments. (Magnification × 82,500.)

acid levels in the M-2 fraction were higher than those of most plasma membranes (Glick, 1976). The cholesterol/phospholipid molar ratio, which appears to be higher in plasma membranes than in other subcellular membranes (Graham, 1975), was greatest in the M-2 band. The M-2 fraction was an homogeneous membrane preparation (Fig. 2) with structures suggestive of microfilaments present (arrow).

On the bases of enzymatic, chemical and morphological characteristics, the M-2 fraction was considered to be the nearest to the generally accepted criteria of pure plasma membranes, and was subsequently used as trophoblast plasma membrane (TrPM). The yield of plasma membrane was about 30% as determined from the total 5′-nucleotidase activity.

Immunohistological studies

The reactions of the heterologous antisera with trophoblastic tissue are illustrated in Fig. 3. Fig. 3a shows the reaction with methanol-fixed placenta. There was a predominant localization of the peroxidase reaction to the surface membrane of the syncytiotrophoblast. No reaction deposits were observed elsewhere within the trophoblast or in the villous mesenchyme. A similar pattern was obtained using indirect immunofluorescence on frozen tissue sections (Fig. 3b). Again, no fluorescence was observed in the syncytio- or cyto-trophoblasts, in the Hofbauer cells, or in foetal blood vessel endothelia. The TrPM

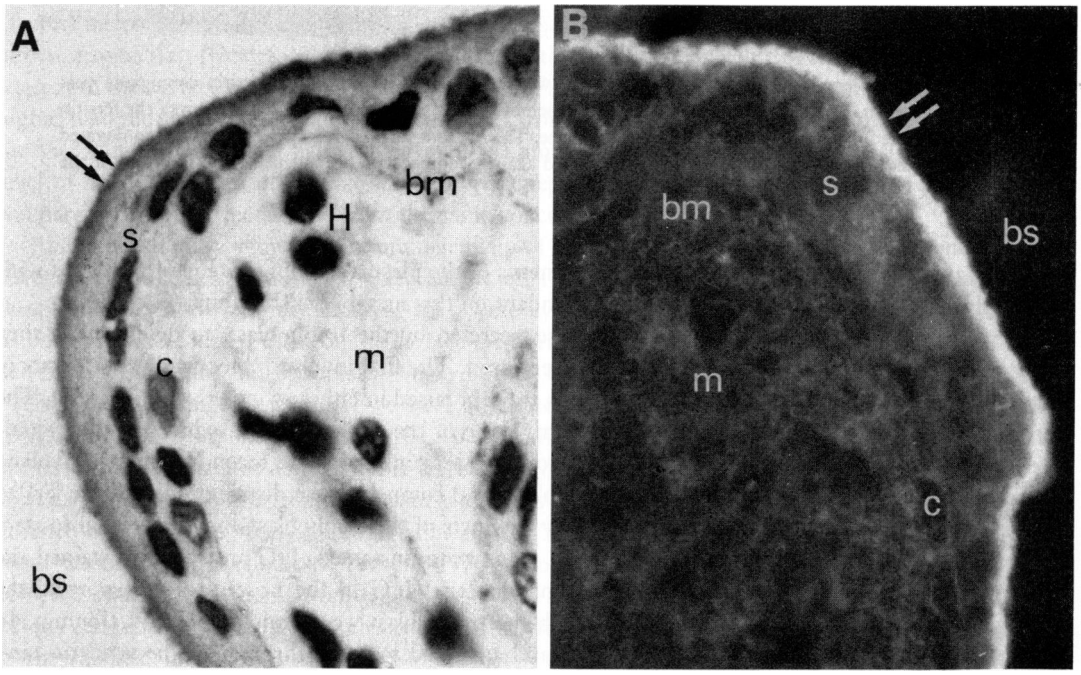

FIG. 3. Distribution of the trophoblast membrane (TrPM) antigen demonstrated using indirect immunohistology. Control tissues (see Results section) were negative. (a) Thin-sectioned, methanol-fixed, trophoblast was stained with TrPM antiserum at 1:400 dilution by the PAP method (Taylor, 1976). There was a marked membrane reaction (arrows), but no reaction in the syncytial cytoplasm (s), cytotrophoblast (c), Hofbauer cells (H), or villous mesenchyme (m). The nuclei were counterstained with Carazzi's haematoxylin. A similar staining pattern was obtained when unabsorbed TrPM antiserum was used, an observation which tends to confirm the data of others that alloantigens are reduced or absent from the syncytial trophoblast. (b) Thin-sectioned frozen trophoblast villus stained with TrPM antiserum at 1:40 dilution followed by FITC-conjugated goat anti-rabbit IgG (Nordic) at 1:20 dilution. The trophoblast explants were maintained for 48 hr in organ culture and during that time the membrane immunofluorescence appeared to increase in intensity. Because the antigen was absent from sera, this increase in membrane fluorescence may represent the replacement of antigen lost during the trauma of evacuation from the uterus. bs = maternal blood space; bm = basement membrane (Magnification × 1300.)

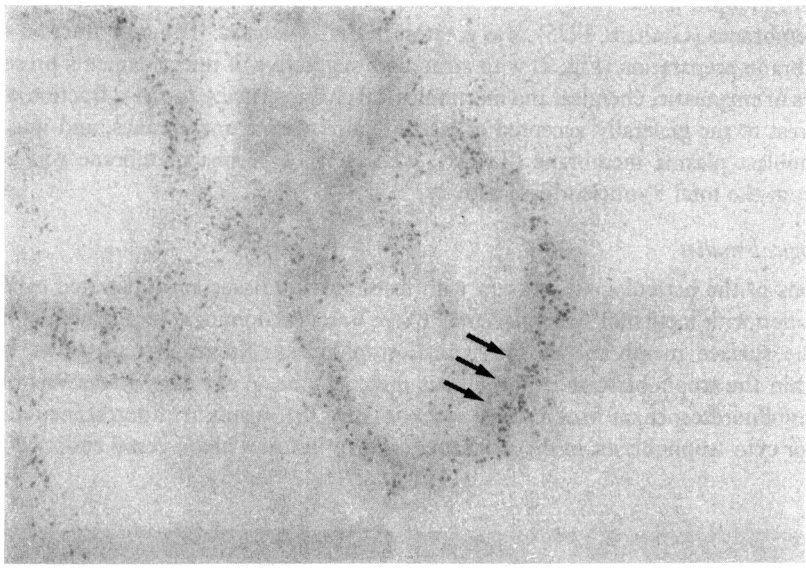

FIG. 4. Thin-sectioned trophoblastic tissue stained with the TrPM antiserum and ferritin-conjugated goat anti-rabbit serum. In those places where the plasma membrane was sectioned transversely (arrows), the ferritin deposit appeared to be separated from it by a clear zone. This pattern was also seen with peroxidase-conjugated antisera. The TrPM antiserum may thus be directed against components present in the outer layers of the 'glycocalyx'. (Magnification × 90,600.)

antiserum appeared to react with the outer components of the plasma membrane (Fig. 4), perhaps with surface glycomolecules which are particularly abundant on this membrane (Bradbury *et al.*, 1970).

We examined many substances, including those secreted by the trophoblast, to determine if they might be the antigen involved. Two approaches were used. The first involved 'blocking' experiments in which the tissue was pre-treated with heterologous antisera raised in either swine or goat (Table 1). The section was then stained with the TrPM antiserum. None of the antisera tested reduced binding compared with slides pre-treated with normal swine or goat sera instead. The second approach involved staining the trophoblast with various antisera (Table 1) and comparing the distribution with the TrPM pattern (Fig. 3). SP_1, HCG and HPL are secretory products of the trophoblast and were found to stain the entire syncytium. The other cell types of the villus were unstained. IgG antisera also stained the whole trophoblast intensely, probably representing maternal IgG in the process of transport to the foetus. The only other antisera which reacted with the trophoblast were against IgM and albumin. In both cases, however, the reaction was very weak and appeared to occur throughout the syncytio- and cytotrophoblasts.

Although the tissue specificity of the TrPM antigen is unestablished, no reaction deposits were observed after immunohistological treatment of sections of normal human rectum, kidney, breast, lung, ovary, liver, skin and cartilage tissues. Treatment of the trophoblast sections with detergent (1% sodium deoxycholate) before staining did not reduce the peroxidase binding.

Immunoelectrophoretic experiments

The samples of HCG, HPL and SP_1 tested by rocket and crossed immunoelectrophoresis did not reveal any precipitin bands with the TrPM antiserum. In addition, the TrPM antiserum did not react by immunoelectrophoretic methods with any components in normal adult sera (both male and female), nor with maternal sera from primiparous and multiparous women at first and third trimesters of gestation.

Radioimmunoassay

No significant binding of HCG was observed at any dilution of the TrPM antiserum.

DISCUSSION

Placental plasma membranes prepared using the methods detailed above showed enrichment of several surface membrane markers (Table 2). 5′-Nucleotidase, alkaline phosphatase and Na^+/K^+-adenosine triphosphatase were increased in the plasma membrane (M-2) fraction. Contamination with endoplasmic reticulum (NADH oxidase), mitochondrial membranes (succinate cytochrome c reductase) and lysosomes (acid phosphatase) was low in each case. Some other features are worthy of note. Firstly, leucine aminopeptidase, which occurs on the plasma membranes of some cell types, e.g. liver, did not appear to be localized uniquely to the surface membrane of the syncytiotrophoblast. Secondly, the cholesterol/phospholipid molar ratio of the membrane (Table 2) was low. Where this ratio is high (0·8 or greater) microvillous borders appear to be rigid, e.g. in intestine (Glick, 1976). The flexible villi of the human trophoblast (Ockleford & Whyte, 1977) may therefore be explicable in terms of a low cholesterol content of the surface membrane. Lastly, the level of sialic acid in the isolated membrane was very high, higher than the levels found in plasma membranes from other cell types (Glick, 1976). The top portion of the M-2 band had, in fact, a sialic acid level of 153 nmol/mg protein. We have previously observed a high level of sialyl extension of trypsin-sensitive glycopeptides of trophoblast cells in tissue culture when compared with other foetal cells from the same conceptus (Whyte & Loke, 1978).

Other workers have shown the immunogenicity of human trophoblastic tissue in xenogeneic systems (Beer *et al.*, 1972; Behrman *et al.*, 1974; Faulk *et al.*, 1978; Rigby & Curzen, 1969). The demonstration of these antigens, however, revealed that they were cytoplasmic in distribution, whilst it was not clear whether they were also present on the surface membrane of the syncytiotrophoblast. The antigen described here did not show this cytoplasmic distribution, but rather was restricted to the plasma membrane.

Using a wide range of antisera, we have attempted to characterise this antigen. Although HCG is thought to be a component of the surface membrane of the syncytiotrophoblast (Whyte, 1978), antibodies to HCG were not demonstrable in the TrPM antiserum by any of the techniques used. Other secretory proteins of the trophoblast such as HPL and SP_1 were also not involved (Table 1). The absence of antibodies to β_2-microglobulin in the TrPM antiserum (Table 1) tends to confirm the observations of others (Faulk & Temple, 1976; Goodfellow *et al.*, 1976) that major histocompatibility complex antigens are absent from trophoblast. The fact that the TrPM antiserum did not stain foetal villous cells either before or after absorption with lymphocytes or foetal skin cells lends further support to the conclusion that alloantigens are absent from trophoblast. Although both α_2-macroglobulin and transferrin have been associated with the apical aspects of the human trophoblast (Johnson & Faulk, 1978), neither protein was responsible for the binding of the TrPM antiserum (Table 1). Similarly, although IgA and IgG have been described on the cell membrane of the trophoblast (Dimmette, 1976), neither these nor any other immunoglobulin classes were involved in the TrPM antiserum binding (Table 1). Wheatgerm lectin, a 'receptor' for which has been described on trophoblast (Whyte, Loke & Stoddart, 1978), also had no blocking effect with the TrPM antiserum.

The nature of this antigen located on the plasma membrane remains obscure. It appears to be trophoblast-specific. In view of its absence from sera and its resistance to detergent extraction, it would also appear to be an 'integral' component of the plasma membrane. It does not resemble the antigens described as trophoblast-specific by Faulk *et al.* (1978) because it is apparently absent from lymphocytes and HEp-2 cells.

The discovery of a membrane-located tissue-specific antigen on the human trophoblast may be of importance as a potential means of fertility regulation in the human female. Immunization against placental antigens is a concept being increasingly considered as a method of birth control (Stevens, 1978). Placental hormones have been particularly well studied in this respect although, so far, no obvious candidate has emerged. Steroids will probably have little practical application (Edwards, 1976), as will HPL and SP_1 (Stevens, 1978). HCG shows most promise although the problems associated with the

production of a non-cross-reacting antiserum are great (Stevens, 1978). Hormonal imbalance will also probably be a serious side-effect of such immunization (Edwards, 1976). Complement-fixing antibodies to an integral membrane component found only in the syncytial trophoblast might be of use in the regulation of fertility, particularly if the antigen is present on the early trophoblast. We do not yet know the stage-specificity of the TrPM antigen except that it is still present in term placentae.

We would like to thank Dr G.T. Rogers (Charing Cross Hospital, London) for performing the HCG radioimmunoassay and Dr C.D. Ockleford (University of Leicester) for his help with the electron microscopy.

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