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Class I antigens of the major histocompatibility complex on cytotrophoblast of human chorion laeve

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Summary. Twenty amniochorions from normal, term pregnancies were studied immunohistologically with the use of well-characterized monoclonal antibodies to beta-2-microglobulin (β2M) and to common determinant of HLA-A, -B, -C. In the same study, polyclonal antiserum to trophoblast antigens (TA) were employed with double fluorochrome labels to determine if cytotrophoblast in the chorion laeve expresed TA, HLA or both. The results showed that, in the majority of cases, HLA was not identified on trophoblast, but TA were. However, some cytotrophoblast within the amniochorionic mantle were non-reactive with anti-TA serum and were positive with monoclonal antibodies to HLA and β2M antigens. Since these structures were identified as cytotrophoblast by a battery of techniques, in this report they are tentatively designated as metatrophoblast. The role of these cells in the materno-trophoblastic relationship in normal human pregnancy has yet to be determined. This is not envisaged as a simple matter, for metatrophoblast can be recognized by anti-β2M and anti-HLA (W6/32), but not by anti-HLA (61D2), suggesting that their expression of class I histocompatibility complex (MHC) antigens may be incomplete, or that there may be a contribution of antigens from an extra-embryonic MHC.

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INTRODUCTION

At term, contact between human extra-embryonic membranes and maternal endometrium is formed by cytotrophoblast of the chorion laeve (Bourne, 1962) as well as by interstitial cytotrophoblast in connective tissues of the placental bed (Pijnenborg *et al*., 1981), while contact between chorion and maternal blood is formed by villous syncytiotrophoblast of the placental chorionic villi (Boyd & Hamilton, 1970) and by the endovascular trophoblast of the spiral or utero-placental arteries (Brosens, Robertson & Dixon, 1967; De Wolf *et al*., 1980). Certain immunological aspects of human pregnancy can be interpreted as being analogous to a successful allograft (Faulk & McIntyre, 1981), and this interpretation has prompted interest in immunological mapping of transplantation antigens at the materno-trophoblastic interfaces. Faulk & Temple (1976) first reported immunohistological results to show that the syncytiotrophoblast of term human placentae lack both beta-2-microglobulin (β2M) and class I antigens of the major histocompatibility complex (MHC). This observation has been confirmed by many investigators with the use of both monoclonal and polyclonal antibodies to transplantation antigens (Sunderland *et al*., 1981; Hsi, Yeh & Faulk, 1982), and it has been extended to include the cytotrophoblast of chorion laeve, and the amniotic epithelium of human amnion (Hsi *et al*., 1982; Yeh, Hsi & Faulk, 1983). On the other hand, some investigators have reported that HLA-A,-B,-C antigens are

expressed in non-villous trophoblast of early human placentae (Sunderland, Redman & Stirrat, 1981).

The identification of cytotrophoblast is difficult, particularly at the deciduo-chorial junction (Hsi, 1983). We have thus identified cytotrophoblast at this materno-trophoblast interface by using the following four techniques: (i) traditional morphological criteria (Bourne, 1962); (ii) immunohistology employing specific anti-trophoblast serum that recognizes trophoblast antigens 1 (TA1) (Faulk *et al.*, 1978) to localize trophoblast plasma membranes; (iii) cytoplasmic plasminogen binding (Burgos *et al.*, 1982; Jenkins *et al.*, 1983); and (iv) propidium-iodide nuclear staining (Ockleford *et al.*, 1981; Faulk & McIntyre, 1983). The expression of class I MHC antigens by cytotrophoblast of chorion laeve was studied with the use of two different monoclonal antibodies to a common determinant of HLA (clones W6/32 and 61D2). During the course of examining larger numbers of normal human amniochorions, some morphologically identifible cytotrophoblast in chorion laeve were found to react with anti-MHC reagents (Hsi, 1983). The present study revealed that a small but definite population of cytotrophoblast expressed class I MHC antigens. However, these were only recognized by monoclonal anti-HLA (W6/32) and not by anti-HLA (61D2). These findings form the basis of the following report.

MATERIALS AND METHODS

Tissues

Twenty full-term human amniochorions (AC 1–20) were collected from normal pregnancies during caesarean section at Crawley Hospital, Crawley, Sussex, England; Pembury Hospital, Pembury, Kent, England; and St Roch Hospital, Nice, France. All tissues were judged to be normal by gross and microscopical examination. One-cm^2 pieces of reflected amniochorions were layered between rectangular pieces of fresh rat liver before snap-freezing in liquid nitrogen-cooled isopentane according to the method described by Hsi *et al.* (1982). The frozen tissues were kept at $-20°C$ until use, at which time 4·5 μm sections were prepared with the use of a cryostat (Bright Instruments, Huntingdon, England), mounted on glass slides and air-dried at room temperature.

Antibodies, conjugates and controls

Polyclonal antibodies. Rabbit anti-TA1 serum was prepared according to the technique of Faulk *et al.* (1978). This antiserum was absorbed three times with pooled human red blood cells, once with solid-phase normal human serum, twice with human liver powder and once with amnion powder as described by Hsi *et al.* (1982). These absorptions resulted in an antiserum which reacted strongly and selectively with trophoblast, and the specificity of this antiserum for trophoblast was documented by showing that its reactivity was completely removed by absorption with ultracentrifuge-prepared pellets of syncytiotrophoblastic microvilli (Smith, Brush & Luckett, 1974). Fluorescein isothiocyanate (FITC)-conjugated goat anti-human plasminogen was obtained from Atlantic Antibodies, Scarborough, Maine, U.S.A.

Monoclonal antibodies. Mouse monoclonal antibodies to human β2M, clone 26/114 HLK (Trucco, Stocker & Ceppellini, 1978) and to the shared determinant of HLA-A,-B,-C (clone W6/32) (Barnstable *et al.*, 1978) were purchased from Sera-Lab Ltd, Crawley Down, Sussex, England. A second mouse monoclonal antibody to HLA common determinant (clone 61D2) (Ugolini *et al.*, 1980) was obtained from Bethesda Research Laboratories, Inc., Gaithersburg, Maryland, U.S.A.

Conjugated antisera against animal immunoglobulin (Ig). FITC-conjugated sheep anti-rabbit Ig was purchased from Wellcome Reagents (Beckenham, Kent, England). Tetramethyl rhodamine isothiocyanate (TRITC)-conjugated swine anti-rabbit IgG was purchased from Dakopatts A/S, Copenhagen, Denmark, and FITC-conjugated goat anti-mouse IgG (H+L chains) purified by solid-phase immunoadsorption was obtained from Bionetics, Kensington, Maryland, U.S.A.

Control sera. Sera from rabbits inoculated with phosphate buffered saline (PBS), pH 7·2, 0·15 M, or with saline, pH 7·2, 0·15 M (Hsi *et al.*, 1982) were used as controls for the rabbit antisera. Mouse ascites fluids (Bethesda Research Laboratories) were used as controls for mouse monoclonal antibodies.

Working dilutions of the antibodies. The working dilution for each antibody was determined according to Faulk & Johnson (1977) as follows: anti-TA1, 1/40; FITC-conjugated anti-plasminogen, 1/100; anti-β2M, ascites fluid, 1/10; anti-HLA (W6/32), supernatant, 1/1; anti-HLA (61D2),, ascites fluid, 1/20; FITC-conjugated anti-rabbit Ig, 1/40; TRITC-conjugated anti-

rabbit IgG, 1/40; FITC-conjugated anti-mouse IgG, 1/30; control rabbit antisera, 1/40; and control ascites fluid, 1/20.

Plasminogen binding assay

Sections were incubated with a 1:5 dilution of normal human plasma (NHP) or human plasminogen (5 casein units/ml in PBS) obtained from Kabi Vitrum, London, England, following which they were washed twice in PBS and reacted with FITC-labelled anti-plasminogen as described by Burgos *et al.* (1982).

Propidium-iodide staining

A stock solution was made by dissolving 5 mg of propidium iodide (Sigma London Chemical Co., Poole, England) in 100 ml of 0·1% tri-sodium citrate ($Na_3C_6H_5O_7 \cdot 2H_2O$). This was diluted 1:3 in PBS and 50 μl was applied directly to the tissue sections following the first wash of the conjugate, and this mixture was left to incubate for 5 min at 4°C. The slides were then washed twice in an excess of PBS, as detailed by Ockleford *et al.* (1981).

Immunofluorescence techniques

The basic procedure is described as follows (i) tissue sections were washed once before the first incubation with antibody; (ii) each incubation was with 50 μl of a predetermined dilution of reagent for 20 min; (iii) following incubation, the tissue sections were washed in PBS; (iv) all procedures were carried out at 4°C and (v) sections were mounted in PBS-buffered glycerol.

Two systems of double labelling experiments were performed: the first system simultaneously identified plasminogen binding and trophoblast antigens, and to do this, rabbit anti-TA1 serum was followed by TRITC-conjugated swine anti-rabbit IgG, and plasminogen binding was identified by incubating the tissues with plasminogen or NHP (as a plasminogen source) followed by incubation with FITC-labelled goat anti-plasminogen. The second system involved the simultaneous identification of histocompatibility and trophoblast antigens, and to do this, rabbit anti-TA1 serum and TRITC-conjugated swine anti-rabbit IgG were used in tandem with mouse monoclonal anti-HLA (W6/32 or 61D2) or with mouse monoclonal anti-β2M (36/114 HLK) followed by FITC-conjugated goat anti-mouse IgG. All microscope slides were examined by using a Zeiss Universal microscope (Ockleford *et al.*, 1981).

RESULTS

Morphology

Haematoxylin and eosin (H+E) and propidium-iodide staining revealed the typical structures of cytotrophoblast in human amniochorion (Bourne, 1962; Faulk & Hsi, 1983). The nuclear pattern of staining with propidium iodide also revealed a characteristic doughnut pattern of staining in most cytotrophoblast as described by Faulk & Hsi (1984) and Wells *et al.* (1984).

Plasminogen binding

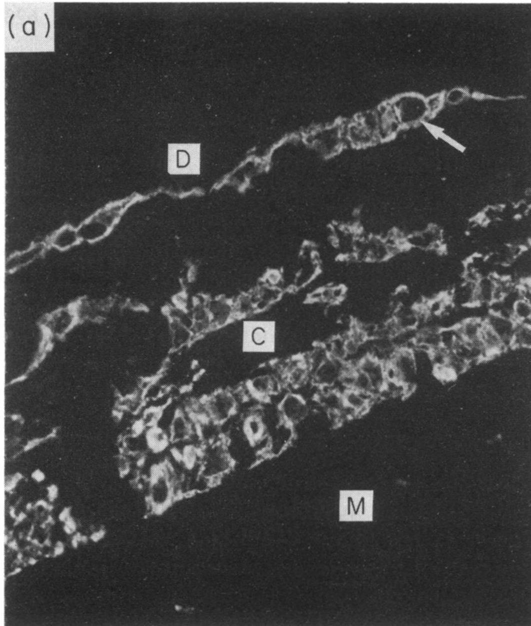
When cryostat sections of amniochorion were studied by direct immunofluorescence with anti-plasminogen sera, only a few amniotic epithelial cells were reactive. However, all amniotic epithelium and cytotrophoblast became strongly positive after the sections were pre-incubated with plasminogen or NHP, washed in PBS and reacted with FITC-conjugated anti-plasminogen serum (Figs 1a and 2a). The cytotrophoblast identified by intracellular binding of plasminogen was the same as those which could be identified by traditional H+E staining of parallel sections.

Trophoblast antigens

Antiserum to TA1 reacted brilliantly with cytotrophoblast, but this reagent was negative with amniotic epithelium, fetal mesenchymal and maternal decidual cells. The intensity of this immunohistological reaction on cytotrophoblast varied from region to region in the amniochorions (Figs 1b and 3b), and some reactive areas could be identified deep in the decidua. The reactive cytotrophoblastic layer was extremely thin in two of the twenty normal amniochorions (AC 4 and 5), and these layers were often discontinuous and disrupted (Figs 2b and 4b). Clearly, in these two perfectly normal amniochorions, some cells which were thought to be cytotrophoblast by their location, morphology and nuclear detail as judged by propidium-iodide staining were found to be negative with anti-TA1 serum.

Histocompatibility antigens

The results of experiments with monoclonal antibodies to β2M (clone 26/114 HLK) and HLA (W6/32) revealed identical patterns of reactivity, thus these findings are recorded together. Fetal cells in the



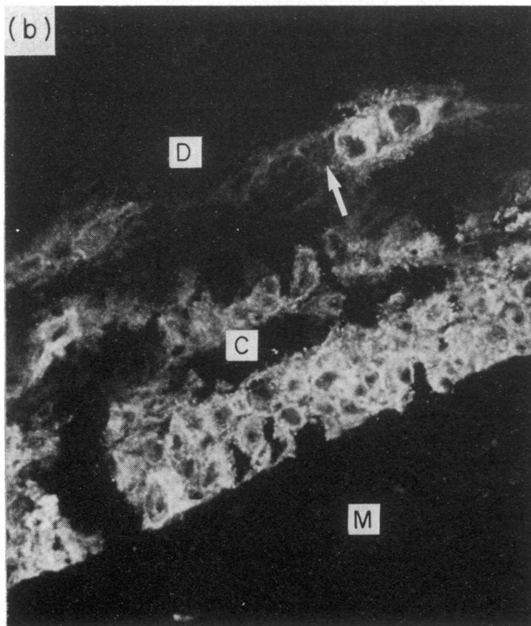
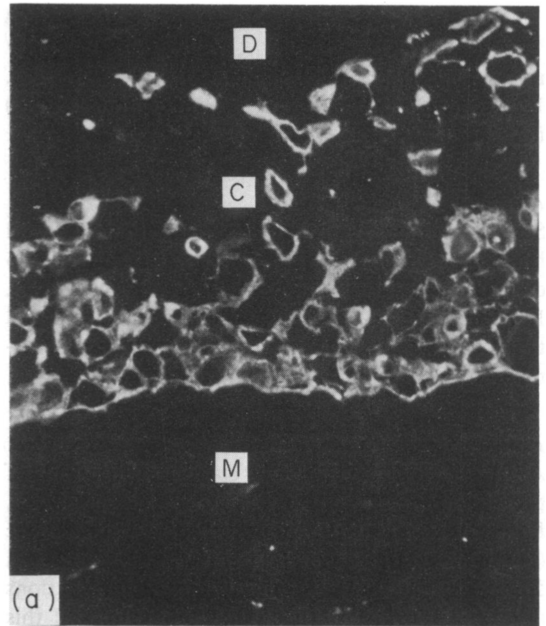


Fig. 1. Plasminogen binding and TA1 expression by cytotrophoblast. A cryostat section of human amniochorion (AC2) incubated with NHP followed with FITC anti-plasminogen and TRITC anti-TA1. (a) Results with FITC filters showed that the chorionic cytotrophoblastic layer (C) was three to five cells thick, and that this technique identified some cytotrophoblast (arrow) in adherent decidual tissue (D). Cells within mesenchymal tissue (M) of amniochorion and decidua (D) were non-reactive. (b) Results with TRITC filters showed that tissues within the cytotrophoblastic mantle (C) reacted with anti-TA1, some more strongly than others (arrow). Cells within extra-embryonic mesenchyme (M) and decidua (D) were negative. (×240.)



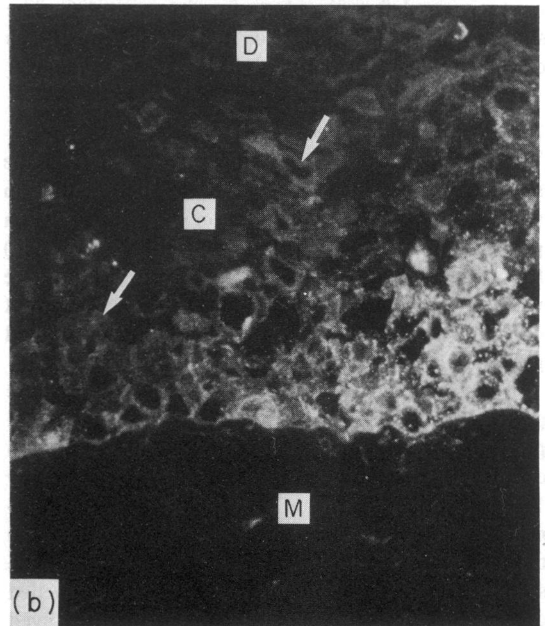
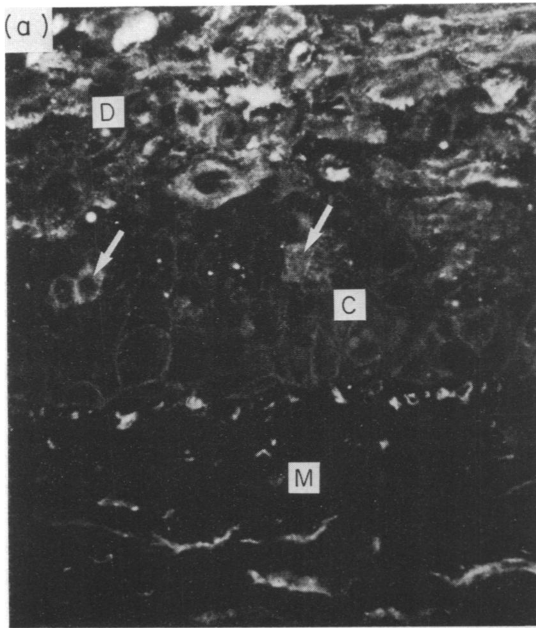


Fig. 2. Plasminogen binding and TA1 expression by cytotrophoblast. A cryostat section of human amniochorion (AC5) incubated with NHP followed with FITC anti-plasminogen and TRITC anti-TA1. (a) Results with FITC filters showed that the chorionic cytotrophoblastic layer (C) was three to five cells thick, as in Fig. 1a, and that cells in both adherent decidual tissue (D) and amniochorion mesenchyme (M) were non-reactive, again as in Fig. 1a. (b) Results with TRITC filters showed that many of the plasminogen-positive cytotrophoblast (C) were TA1-negative or only weakly reactive (arrows), although TA1-positive cytotrophoblast were observed. (×240.)



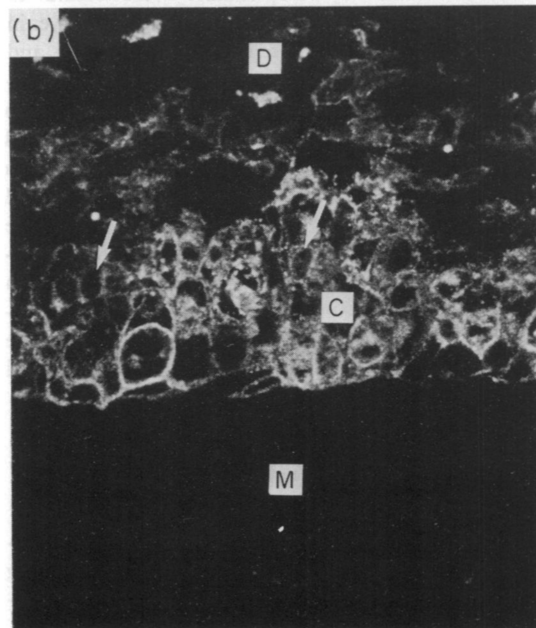
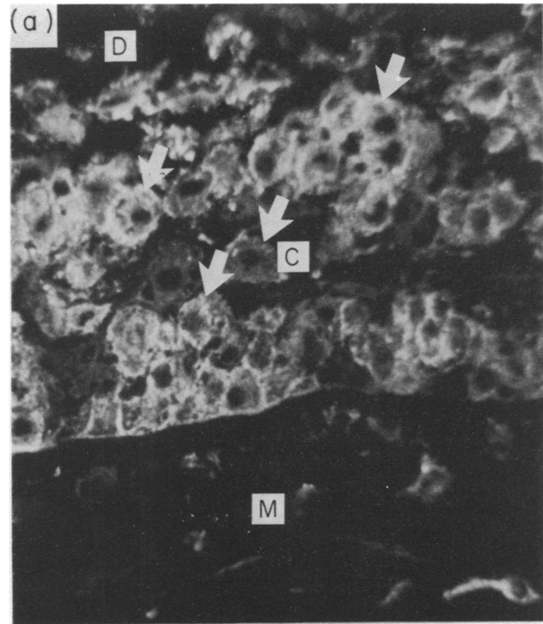


Fig. 3. HLA and TA1 expression by cytotrophoblast. A cryostat section of human amniochorion (AC2) reacted with FITC anti-HLA (W6/32) and TRITC anti-TA1. (a) Results with FITC filters showed mainly HLA-negative cytotrophoblast (C) with occasional positive cells (arrows). Cells within extra-embryonic mesenchyme (M) and decidua (D) were positive. (b) Results with TRITC filters showed mainly TA1-positive cytotrophoblast (C) with occasional TA1-positive cells that also reacted with anti-HLA (arrows). Note negativity in extra-embryonic mesenchyme (M) and scattered TA1 reactivity in decidua (D). (×240.)



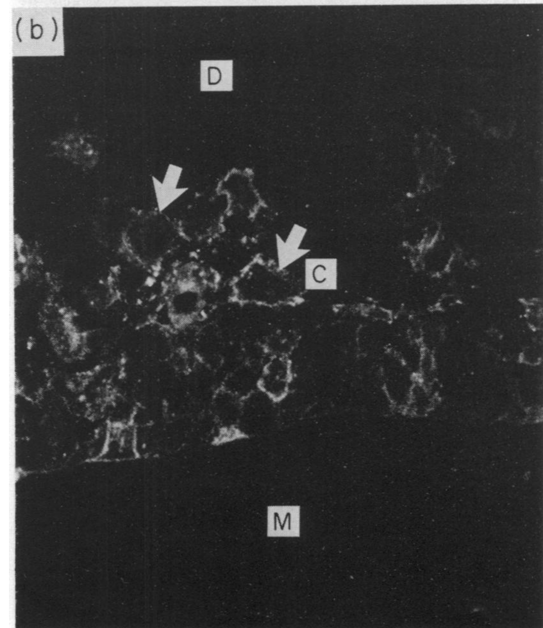


Fig. 4. HLA and TA1 expression by cytotrophoblast. A cryostat section of human amniochorion (AC4) reacted with FITC anti-HLA (W6/32) and TRITC anti-TA1. (a) Results with FITC filters showed that the majority of cytotrophoblast (C) were reactive with the W6/32 anti-HLA (arrows), as were cells in the extra-embryonic mesenchyme (M) and decidua (D). (b) Results with TRITC filters showed negative cytotrophoblast within the chorionic mantle (C), although many TA1-positive cytotrophoblast (arrows) were noted. Occasional cytotrophoblast were positive for both HLA and TA1. Note lack of reactivity in extra-embryonic mesenchyme (M) and scattered positivity in decidua (D). (×240.)

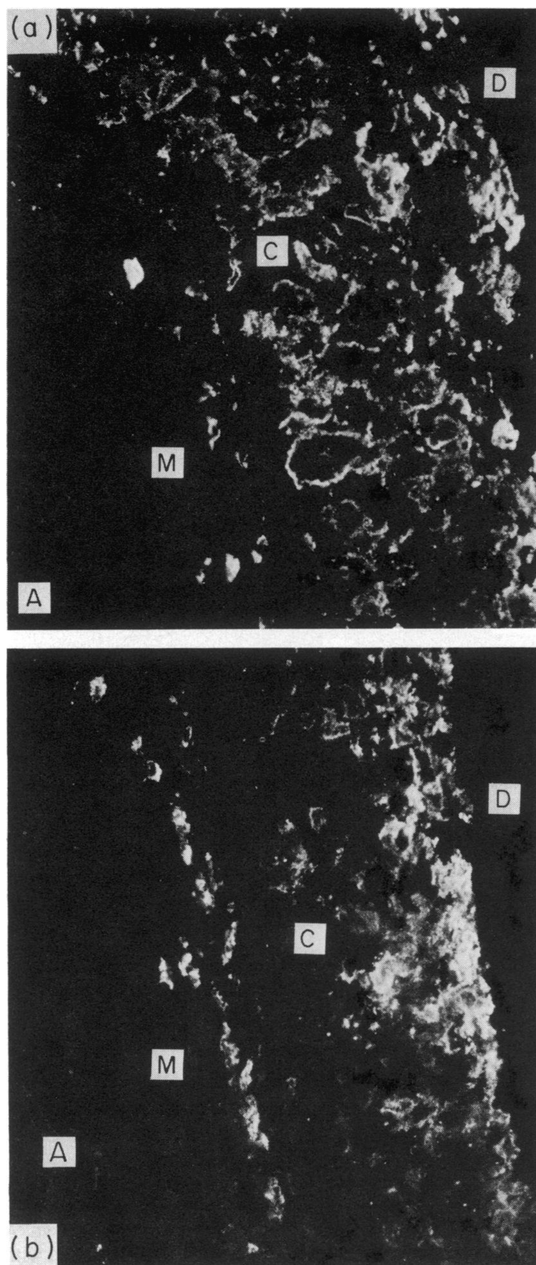


Fig. 5. Expression of HLA (W6/32) and HLA (61D2) by cytotrophoblast. (a) A cryostat section of human amniochorion (AC4) reacted with FITC monoclonal anti-HLA (W6/32) showed HLA-negative amniotic epithelium (A), positive cells in extra-embryonic mesenchyme (M) and decidua (D), and a plurality of positive cytotrophoblast within the chorionic mantle (C). (b) A parallel cryostat section of AC4 reacted with FITC monoclonal anti-HLA (61D2) also showed negative amniotic epithelium (A) and positive cells in extra-embryonic mesenchyme and decidua, as in Fig. 5a. However, structures within the cytotrophoblastic mantle (C) were almost completely negative. (×240.)

mesenchymal aspect of amniochorions as well as cells in the maternal decidua reacted positively with antibodies to β2M and HLA (W6/32). The reactive fetal cells were elongated or polygonal, and they were mostly located near the pseudobasement membrane of the chorionic cytotrophoblast. Several maternal cell types could be identified in the decidua. These included small, round, brightly reactive cells deep in the mothers' uterine tissues, elongated cells less deeply situated which usually aligned themselves parallel with amniotic epitheliun, and endothelium of maternal blood vessels. Also noted were large, oval weakly reactive cells close to the cytotrophoblastic mantle. These cells resembled cytotrophoblast as identified by their morphology in H+E and propidium-iodide staining as well as by the plasminogen-binding assay.

In most instances, the HLA-negative cytotrophoblast layer was about three to five cells thick (Fig. 3a), but in normal amniochorions 4 and 5, anti-HLA (W6/32) reactive cytotrophoblast were found to account for almost all of the cytotrophoblastic layer in the chorionic aspect of these two amniochorions, and it was not uncommonly observed that the HLA-negative cytotrophoblast were sparsely represented within the chorion (Fig. 4a). The results with monoclonal antibodies to HLA (61D2) were quite different from those obtained with the use of HLA (W6/32). It should be highlighted that anti-HLA (61D2) was negative with all cytotrophoblast including those in amniochorions 4 and 5 which were previously positive with anti-HLA (W6/32) (Fig. 5a, b). Other maternal decidual and fetal mesenchymal cells reacted with the anti-HLA (61D2) antibody, and amniotic epithelial cells were negative.

Double labelling experiments

Plasminogen binding and trophoblast antigens

The cytotrophoblastic mantle morphologically identified with the use of H+E, propidium-iodide staining and cytoplasmic plasminogen binding in 20 amniochorions were about three to five cells thick (Fig. 1a, b). However, not all of the cytotrophoblast identified by cytoplasmic plasminogen binding also reacted with anti-TA1 serum. This finding was again most evident in amniochorions 4 and 5 where HLA-positive cells

were abundant and in which only 10% of the plasminogen-binding-positive trophoblast were anti-TA1 reactive (Fig. 2a, b). These trophoblast antigen-positive trophoblast were situated for the most part on the trophoblastic basement membrane, and they were surrounded by trophoblast antigen-negative cytotrophoblast whose cytoplasms were positive for plasminogen binding.

Trophoblast antigens and histocompatibility antigens
Cytotrophoblast which reacted brilliantly with anti-TA1 were reproducibly observed to be non-reactive with antibodies to β2M and HLA (W6/32). In addition, cells which could easily be identified as maternal blood vessel endothelium or uterine fibroblasts reacted strongly with anti-β2M and with both anti-HLA (W6/32), and anti-HLA (61D2), but not with anti-TA1 serum. Some unclassified small round cells which reacted with antibodies to HLA (W6/32) and β2M but not with anti-TA1 serum were found to be scattered throughout the decidual tissues as well as being found deeply within the cytotrophoblastic layer. At the junction between cytotrophoblast and decidua, a population of cells could be identified and classified as trophoblast by morphological and plasminogen-binding criteria, but these did not react with anti-TA1 serum, and they did react weakly with monoclonal antibodies to β2M and HLA (W6/32). If these structures can be classified as a unique type of trophoblast, it also should be pointed out that they could be identified in every amniochorion we have studied, although the quantity varied from specimen to specimen (Fig. 3a, b). These trophoblasts were two to three cells thick in all amniochorions, including amniochotions 4 and 5 in which the mantle of anti-TA1 reactive cytotrophoblast was very thin (Fig. 4a, b). However, it must be emphasized that cytotrophoblast which reacted with anti-HLA (W6/32) were totally negative for both anti-HLA (61D2) and anti-TA1 serum.

At the junction between the anti-HLA (W6/32) reactive trophoblast and anti-TA1-positive, anti-HLA (W6/32)-negative cytotrophoblast, cells which reacted with both anti-HLA (W6/32) and anti-TA1 serum could normally be found (Fig. 3a, b). The reaction pattern of these trophoblast for both anti-HLA (W6/32) and anti-TA1 was granular but weak. It must however be recorded that cells could infrequently be identified in the cytotrophoblastic mantle which were negative for both TA1 and HLA.

DISCUSSION

The results embodied in this report show that the majority of cytotrophoblast in human amniochorion have the following characteristics: (i) typical morphology of cytotrophoblast as shown by H+E and propidium-iodide staining; (ii) cytoplasmic plasminogen binding; (iii) expression of TA1; and (iv) lack of β2M and class I MHC antigens. However, there is a minority of trophoblast within human amniochorion which has the following properties: (i) identical morphology as cytotrophoblast as shown by H+E and propidium-iodide staining; (ii) cytoplasmic plasminogen binding; (iii) lack of TA1 expression; and (iv) expression of HLA and β2M antigens, the former of which can be recognized by anti-HLA (W6/32) but not by anti-HLA (61D2). Since the antigenic profile of this minor group of trophoblast is different from the other trophoblast cells and subsets in placenta and amniochorion (Faulk & McIntyre, 1983), this group has tentatively been designated as metatrophoblast (Hsi, 1983). Metatrophoblast seems to be found in the chorion laeve and to some extent, in the basal plate and placental bed (Hsi, 1983; Wells, Hsi & Faulk, 1984). On the contrary, the syncytiotrophoblast of chorion frondosum and cytotrophoblast of chorion laeve in most cases are predominantly populated by trophoblast which express TA1 but not class I MHC antigens. These trophoblastic tissues for the operational interface between mother and extra-embryonic membranes, and a good deal of information has accrued during the past several years to indicate that these tissues are of central importance in the materno-trophoblastic allogeneic co-existence in the host–parasite relationship of human pregnancy (for reviews, see Faulk & Hsi, 1983, 1984).

It is not presently known how the various sets and subsets of trophoblast described by Faulk & McIntyre (1983) moderate their apparently selective abilities to insert MHC or trophoblast antigens into their plasma membranes, but it has been reckoned that this is associated with their ability to survive in an allogeneic environment. One aspect of this phenomenon may be to insert only those antigens which are compatible with their microenvironment, and one way that this might be accomplished could be to insert an incomplete or extra-embryonic type of MHC antigen. Within this context, it is interesting to note that the metatrophoblast manifests a type of HLA that can be discriminated by anti-HLA (W6/32) but not by anti-HLA (61D2).

The immunogen for the monoclonal anti-HLA (W6/32) was human tonsillar lymphocytes (Barnstable *et al.*, 1978), and the immunogen used for the production of anti-HLA (61D2) was human monocytes (Ugolini *et al.*, 1980). Both antibodies have been shown to have a common specificity on class I MHC antigen-positive cells, and they do not react with class I MHC antigen-negative Daudi cells (Parham *et al.*, 1979). It has been shown that anti-HLA (W6/32) have a high coefficient of binding and specificity for conformationally intact HLA bound to β2M (Barnstable *et al.*, 1978). The structure recognized by 61D2 may be located nearer to the distal end of the allotypic region of the class I MHC antigen heavy chains than that recognized by W6/32. In this case, it is possible that the metatrophoblast not only lacks the HLA structure recognized by 61D2, but that it also does not possess allotypic determinants of class I MHC antigens, a circumstance which would probably augment the survival of metatrophoblast in its allogeneic microenvironment. This is not however mutually exclusive with the possibility that metatrophoblast may manifest extra-embryonic allotypic antigens, for antisera are not yet available to test this. Indeed, allotypic syncytiotrophoblast antigens have been recently described by McIntyre & Faulk (1982) and by McIntyre *et al.* (1983).

One possible explanation for the HLA expression on metatrophoblast is that it may result from incorporation of cytoplasmic or nuclear material of maternal uterine cells. Fusion between trophoblast and endometrial cells has been reported in several mammals (Schlafke & Enders, 1975), and the results of autoradiographic studies have also suggested that trophoblast in mice and rats can incorporate maternal nuclear material (Galassi, 1967; Kaye, 1977). In addition, ingestion of maternal endometrial nuclei was noted in one of the youngest known human blastocyst studied by Hertig, Rock & Adams (1956). Uptake of maternal nuclear material by the cytotrophoblast might signal the secretion into plasma membranes of an altered class I MHC antigen capable of being recognized by anti-HLA (W6/32). This could occur through a mechanism similar to the HLA expression by Daudi cells following their hybridization with class I MHC antigen-positive cells (Kennett, Bengtsson & Bodmer, 1975; Ber *et al.*, 1978). Whatever the reason for the expression of class I MHC antigens and the suppresion of TA1 by metatrophoblast, it is no longer possible to construct generalities about the MHC status of extra-embryonic tissues, and this should stimulate a good deal of rethinking about the role of MHC antigens in the materno-trophoblastic relationship in human pregnancy.

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