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Immunological studies of human placentae: identification and distribution of proteins in mature chorionic villi

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SUMMARY

Cryostat sections of fifteen full-term, normal, human placentae have been studied by indirect immunofluorescence, using characterized antisera to a large number of proteins, in order to localize particular protein antigens to well-defined anatomical regions. Actin, plasminogen, and transferrin were identified in trophoblasts. Beta-2-microglobulin was uniformly absent from these cells. The complement component C3, the immunoglobulin IgG, and fibrinogen and collagen were identified in trophoblastic basement membranes. Of these, IgG was the most sparingly represented, and C3 was not found on all trophoblastic basement membranes. Many proteins were identified within the stroma of chorionic villi, collagen being the most abundant. Many of these persisted following prolonged washing of the tissues. All four IgG subclasses were present in stroma, IgG1 and IgG3 being most prominent. Stromal cells were positive for beta-2-microglobulin, suggesting a unique sequestration of this protein on stromal cells but not on trophoblasts. Placental stem vessels were surrounded by collagen, and the walls of these vessels stained brightly for myosin: the vascular endothelium contained beta-2-microglobulin and actin. Peri- and inter-villous fibrin were faintly positive for several proteins, but these areas stained intensely for fibrinogen, plasminogen, alpha-2-macroglobulin and C4. This approach has proven to be useful in establishing precise definitions of the molecular morphology of normal human placentae.

INTRODUCTION

Several lines of evidence indicate that immunological mechanisms are relevant in the materno–foetal relationship (Beer & Billingham, 1974; Faulk & Jeannet, 1975). These have generally focused on studies of the antigenicity, or lack thereof, of trophoblasts (Beer, Billingham & Yang, 1972; Loke, Joysey & Borland, 1971), foetal lymphocytes (Ceppelli *et al.*, 1971) and extracts of human placentae (Rocklin *et al.*, 1973). In addition, blocking factors for several *in vitro* assays of lymphocyte reactivity have been described in maternal sera (Kasakura, 1971), cord sera (Ayoub & Kasakura, 1971), and in immunoglobulins (Ig) eluted from human placentae (Bonneau *et al.*, 1973; Faulk *et al.*, 1974). Neither the biochemistry nor the specificity of these factors is presently clear.

Other lines of evidence concern the description within placentae of immunological phenomena that also occur in other tissues. For example, receptors for antibody-sensitized erythrocytes have been identified on trophoblasts (Matre, Tonder & Endresen, 1975; Jenkinson, Billington & Elson, 1976) and

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receptors for immune-complexes and aggregated-IgG have been reported on endothelial cells of placental stem vessels (Johnson, Trenchev & Faulk, 1975; Johnson, Faulk & Wang, 1976). In addition, glycoproteins binding phytohaemagglutinin (PHA) and concanavalin A (Con A) have been described on human trophoblastic basement membranes, (TBM) (Johnson & Faulk, 1976). On the other hand, IgG, complement and fibrinogen are normally found on TBM of human placentae (McCormick *et al.*, 1971), whereas in other (adult) tissues this would not be considered as being normal.

A more precise knowledge of antigens within placentae is required. In as much as many antigens are proteins, it appears appropriate to identify and describe the distribution of several relevant proteins in placentae. Although this approach has been found to be useful, we should like to stress at the outset that we have (a) studied a selected group of proteins, (b) examined only a very small piece of a very large organ, and (c) employed only full-term, normal human placentae. These observations do seem, however, to build a general pattern, and it is this that we wish to report.

MATERIALS AND METHODS

(a) *Tissues.* Fifteen full-term placentae were studied. The mothers were all healthy, and gave birth to healthy babies. Each placenta was judged to be normal by gross examination as well as by microscopical examination of histological preparations stained with haematoxylin-eosin, PAS, and Masson tri-chrome. Representative portions of tissue were cut from the middle aspect of the central cotyledon immediately following delivery. The tissues were then either processed for immunofluorescence or were washed for 12 hr with gentle shaking in 25 ml of complete growth medium at 5°C. This medium consisted of 10 ml of minimal essential medium, 5 ml of sodium bicarbonate, 85 ml of sterile distilled water, 10 ml of foetal calf serum, 1 u of glutamine and 200 u/ml of penicillin and 100 mg/ml of streptomycin (Faulk *et al.*, 1975). Use of the washed and unwashed tissues is described in the text. Tissues for histology were fixed in 10% buffered formalin and processed according to standard techniques. Tissues for immunofluorescence were snap-frozen in liquid nitrogen-cooled iso-pentane and sectioned at 4 μm on a cryostat. Sections were prepared fresh each day, and the tissues air-dried. These were exposed to 50 μl of a predetermined dilution of antisera for 20 min in a moist box. Slides were then washed in two complete changes of phosphate-buffered isotonic saline (PBS) for 20 min each, and each tissue was then exposed to 50 μl of a pre-determined optimal dilution of the appropriate fluorescein-conjugated antiserum for an additional 20 min in a moist box. Slides were then washed in three complete changes of PBS for 20 min each, and mounted in PBS-buffered 50% glycerol using methanol-washed cover-slips. The preparations were then ready for fluorescence microscopy.

(b) *Antisera and conjugates.* Antisera to intact heavy and light chains of normal adult human serum IgG were raised in sheep given an initial i.m. injection of antigen in Freund's complete adjuvant followed in one month by an i.m. injection of antigen in aluminium hydroxide. The antigens were prepared as previously detailed (Wang *et al.*, 1970). The anti-gamma chain sera did not precipitate light chains or alpha or mu chains in double radial diffusion analysis, and the anti-light chain sera failed to recognize any heavy-chain preparation. Both antisera precipitated their homologous antigens. The specificity of these sera has also been determined by haemagglutination using the chromic-chloride technique (Faulk & Houba, 1973). Both antisera haemagglutinated their homologous antigens at titres in excess of 1:10,000, and neither haemagglutinated any Ig preparations that they failed to recognize in double radial diffusion.

Antisera were raised in our laboratory to the subclasses of human IgG using Fc fragments from myeloma proteins representative of the four subclasses. The proteins used have been described previously (Johnson, Faulk & Wang, 1976). Antisera were raised in the following animals, as recommended by Giessen *et al.* (1974): rhesus monkey (IgG2 and IgG4), rabbit (IgG3) and guinea-pig (IgG1). Animals were injected i.m. with 1 mg antigen in Freund's complete adjuvant and after 4 weeks with a further 1 mg in Freund's incomplete adjuvant before being bled 2 weeks later. Appropriate absorption of the antisera was done using non-immune rhesus monkey serum and bovine gammaglobulin to remove any heteroantigenic specificities, as well as isolated intact human IgG1 and IgG3 myeloma proteins. The specificity of the resultant precipitating antisera was demonstrated by double radial immunodiffusion and immunoelectrophoresis (IEP) against their homologous antigens and other isolated human IgG myeloma preparations. Each antiserum was subclass-specific, except for a weak precipitin cross-reactivity of anti-IgG2 with IgG3 proteins. A rabbit antiserum specific for the hinge region (Fh fragment) of human IgG3 sublcass proteins (Michaelsen, Natvig & Sletten, 1974) was a gift of Dr T. E. Michaelsen, Oslo, Norway.

Human collagen was extracted from adult skin using 0·5 M glacial acetic acid and serially precipitated with sodium chloride according to Bornstein & Piez (1966). The amino-acid content and electrophoretic pattern of this collagen preparation was consistent with Type I human collagen (determined by Dr A. Bailey, ARC Meat Research Institute, Langford, Bristol). This antigen was not precipitated by sheep antisera to human serum proteins in either immunoelectrophoresis or double radial diffusion analysis, and rabbit antisera to the antigen did not precipitate normal human serum (NHS) in either of these systems. Tanned erythrocytes coated with human collagen (Conochie, Scott & Faulk, 1975) were agglutinated to titres in excess of 1:50,000 by the rabbit anti-human collagen serum, and tanned erythrocytes coated with rat collagen were not agglutinated.

Similarly, small amounts of native or denatured human (but not rat) collagen inhibited the haemagglutination reaction suggesting that the serum was specific for non-helical regions (Beard *et al.*, 1976). Actin and myosin were prepared from human uterus, as previously reported (Trenchev, Sneyd & Holborow, 1974). Antisera to these proteins were raised in rabbits and were found to be specific by double radial diffusion analysis as well as by the detection of characteristic immunofluorescence staining patterns using rat tissues as substrate (Trenchev *et al.*, 1974). In addition, neither actin nor myosin inhibited the collagen haemagglutination system, and antisera to these proteins did not agglutinate tanned collagen-coated erythrocytes.

Antisera to the human complement components B_1C/B_1A (C3), B_1A, B_1E, B_1F, and C1q were obtained from Dr K. W. Pondman, Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands. Antisera to B_1C/B_1A (C3) and B_1E were also purchased from Dakopatts A/S, Denmark. In addition, antisera to C1q were obtained from Professor Peter Lachmann, Royal Postgraduate Medical School, London, and from Professor Henri Isliker, University of Lausanne, Switzerland.

A large number of rabbit antisera to isolated human proteins were purchased from Dakopatts A/S, Denmark. These antisera are supplied as non-frozen, non-lyophilized, purified immunoglobulin fractions in 0·1 M sodium chloride containing 15 mM sodium azide. Antisera to the following human proteins were obtained from Dakopatts A/S: transferrin, beta-2-microglobulin, IgA, IgM, fibrinogen, plasminogen, alpha-1 foeto-protein (AFP), haptoglobin, albumin, caeruloplasmin, orosomucoid (alpha-1-acid-glycoprotein), alpha-1-antitrypsin, cholinesterase, muramidase (lysozyme), and C-reactive protein (CRP).

The specificity of all of these antisera were checked by immunoelectrophoresis and Ouchterlony analysis. Anti-Ig sera were studied for specificity as detailed above. The anti-fibrinogen serum precipitated fibrinogen from plasma but not from serum, and fibrinogen-anti-fibrinogen reactions in agar and on tissues were abolished when the anti-fibrinogen serum was passed over a column of Degalan V26 polymethymethylacrylate beads (Degussa Wolfgang, Frankfurt, Germany) coated with human fibrinogen (Kabi AB, Stockholm, Sweden). Similarly, plasminogen-anti-plasminogen, transferrin-anti-transferrin, and albumin-anti-albumin reactions in agar and on tissues were abolished by absorption of the antisera with plasminogen (Sigma, Kingston-upon-Thames, Surrey), transferrin (Sigma), or albumin (Sigma), respectively. The anti-CRP serum produced a well-defined precipitin band against sera from nine of ten rheumatoid arthritis patients in clinical exacerbation and were negative against ten normal human sera (NHS). Likewise, anti-AFP sera produced a characteristic precipitin line in IEP with foetal sera but not with NHS, and the anti-haptoglobin serum was positive against NHS in IEP but was either negative or faintly positive against cord-blood sera. Lines of complete identity in Ouchterlony testing against NHS were given by the Dakopatts anti-B_1A/B_1C (C3) and anti-B_1E and with the anti-B_1A/B_1C (C3) and anti-B_1E sera from the Netherlands Red Cross Blood Transfusion Service, and extensive specificity studies have been reported for these Dutch sera (Wolters, 1971; Molenaar, Velde & Pondman, 1973). A goat anti-CEA serum was obtained from Professor M. Neville, Chester Beatty Research Institute, Royal Cancer Hospital, London. This serum was specific at high dilution in radioimmunoassay for CEA (Tuberville, Darcy, Lawrence, Johns & Neville, 1973). A rabbit antiserum to human alpha-2-macroglobulin was obtained from Dr Keith James, University of Edinburgh, Scotland. This antiserum had been passed over an immunoabsorbent column containing human IgM, IgG, and foetal calf serum; it reacted only with alpha-2-macroglobulin in agar and haemagglutination systems. The anti-beta-2-microglobulin serum gave membrane staining of human leucocytes and patch-and-cap reactions at 37°C, but was negative on human erythrocytes. The antiserum also gave a precipitin reaction in agar with concentrated urine from an oligouric patient with secondary amyloid involvement of the kidneys, whereas concentrated normal urines were negative.

All of the above antisera were used in indirect immunofluorescence studies on cryostat sections of human placentae together with fluorescein-isothiocyanate (FITC) conjugates. All conjugates had a fluorescein:protein ratio of between one and two as determined by spectrophotometry (Faulk & Hijmans, 1972). The FITC-conjugates employed in this investigation were: rabbit anti-monkey Ig and rabbit anti-guinea-pig Ig (Nordic Pharmaceuticals, Tilburg, The Netherlands), rabbit anti-goat Ig (Behringwerke AG, Marburg, Germany), and sheep anti-rabbit Ig (Wellcome Reagents, Beckenham, Kent). The rabbit anti-monkey Ig conjugate was absorbed with NHS before use. The FITC-conjugates were used at dilutions that (a) did not stain placental sections when the middle layer was PBS, (b) did not stain placental sections when the middle layer was a 1:50 dilution of the species serum under study, and (c) did stain placental sections at one serial-dilution below end-point in a known positive system. Using these criteria, conjugates could usually be reliably used at dilutions in excess of those recommended by the manufacturer. Each unlabelled antiserum was then studied on placental sections using the predetermined dilution of conjugate. Antisera were arranged at dilutions ranging from 1:50 to 1:500, and the dilution chosen for use was one dilution below the end-point. These procedures maximized specificity and minimized non-specific staining. Positive reactions were controlled by blocking, absorption, and displacement techniques as described by Faulk & Hijmans (1972) and McCormick *et al.*, (1971).

(c) *Microscopy.* Two different sets of microscopic and photographic equipment were used in this investigation. Photomicrographs using diaphragm-fitted X90 and X40 objectives were taken using a Zeiss photomicroscope II equipped for epi-illumination with a Ploem epi-illuminator and an HBO 200 high-pressure mercury–vapour burner light source. Gaf 500 high speed 35-mm film was used. Photomicrographs using a X10 or a diaphragm-fitted X40 objective were taken using a Reichert Zetopan microscope fitted for transmitted light with a quartz-iodide light source, a Tiyoda wide-field dark-ground condenser incorporating a toric lens, and a FITC-3 Balzer interference filter and Wratten 12 barrier filter. Agfa–Gevaert CT18 daylight 35-mm film was used.

RESULTS

(a) *Antisera that predominantly stain trophoblasts*

The only antiserum used that stained all trophoblasts was anti-actin. This reaction involved the entire trophoblast (Fig. 1a). The antiserum also stained foetal stem-vessel endothelium as well as cytoplasmic elements, presumably microfilaments of stromal fibroblasts and macrophages. Anti-plasminogen also stained entire trophoblasts (Fig. 1b) but this reaction only involved occasional groups of cells. In this case, the positive trophoblasts were in areas near to deposits of intervillous fibrin. Anti-transferrin stained the apical aspects of trophoblasts (Fig. 1c). This reaction generally involved all trophoblasts, in areas presumed to be microvilli, and tended to circumnavigate each villous. Except for occasional weak staining of fibrinoid areas and rare staining of vessels, the apical staining of trophoblasts accounted for the majority of the staining given by anti-transferrin. The intensity and clarity of anti-transferrin staining were augmented by performing the entire reaction at 5°C.

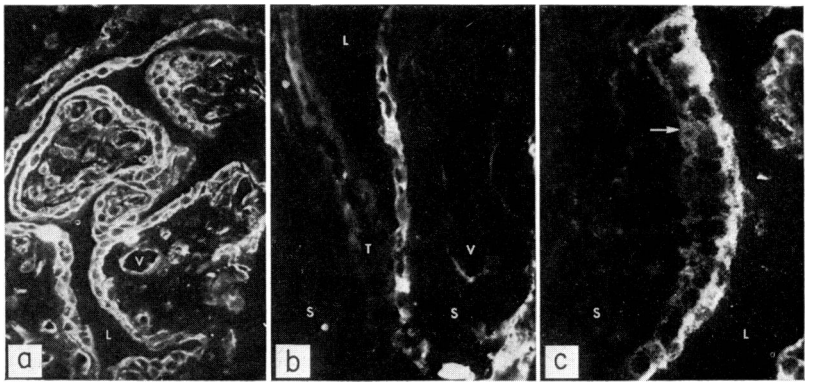

FIG. 1. Proteins identified in trophoblasts. (a) Actin. Note that all villous trophoblasts are stained, and that the entire cytoplasm of each cell is positive. (Magnification × 98.) (b) Plasminogen. Note that not all cells are stained, but positive cells involve entire cytoplasm. (Magnification × 176.) (c) Transferrin. Note that all cells are stained in their apical aspects. (Magnification × 280.) L, inter-villous space; S, villous stroma; T, trophoblast, and V, placental vessel. White arrow indicates trophoblastic basement membrane.

(b) *Antisera that predominantly stain TBM*

The most consistent and reproducible staining of TBM was obtained with antisera to either C3 or B_1A (Fig. 2a). Not all TBM were stained by these antisera, and often only a segment of a TBM within a villous was stained. Otherwise, these complement proteins were sometimes identified in fibrinoid areas, scattered throughout the stroma of occasional villi, and rarely in the vessel wall and endothelium of foetal stem vessels. The Dutch and Danish anti-C3 sera gave the same pattern of staining. None of the Ig classes studied except IgG could be identified on TBM, and this TBM staining was not as apparent as that for either C3 or B_1A. Also, the distribution of L-chains extended beyond TBM into stroma, as demonstrated by staining for IgG (Fig. 2b). IgG and L chains could not be demonstrated in trophoblasts in air-dried tissue sections, but were sometimes identified on TBM, and were usually found in great abundance in stroma, particularly in a peri-vascular distribution (not shown in Fig. 2b). All four IgG subclasses were present, and there was no significant difference in staining distribution using antisera against each of the subclasses. However, anti-IgG3 stained as intensely as anti-IgG1, and both gave considerably more marked staining than with anti-IgG2 or anti-IgG4. The strong reaction using an antiserum raised against the Fch fragment of human IgG3 was confirmed using an antiserum specific for the hinge-region (Fh fragment) of human IgG3. This suggests that the transfer of

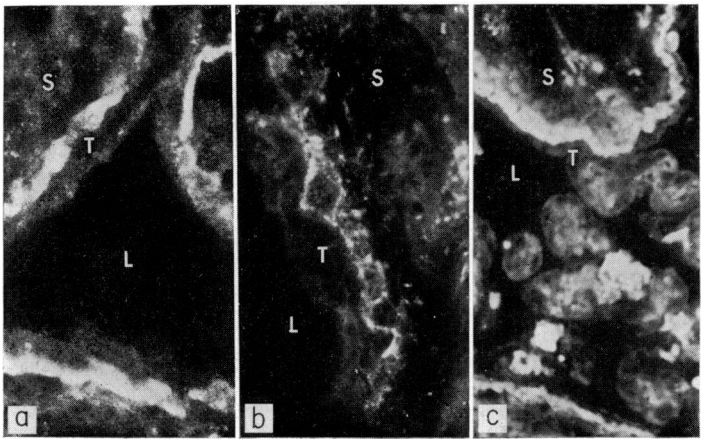

FIG. 2. Proteins identified on TBM. (a) C3. (Magnification × 240.) (b) IgG. (Magnification × 240.) (c) Fibrinogen. (Magnification × 840.) Note extension of fibrinogen into villous stroma and presence in intervillous fibrin. Code as in Fig. 1.

IgG3 may be retarded in placentae, since IgG3 values are not elevated in either maternal or cord blood (Hay, Hull & Torrigiani, 1971) although IgG3 is prominent in eluates prepared from human placentae (Faulk *et al.*, 1974).

Fibrinogen was commonly observed on TBM but, unlike C3 or B_1A, it was rarely identified solely on TBM, but was usually accompanied by staining of areas immediately below TBM and extending into the periphery of villous stroma (Fig. 2c). Collagen was commonly present in TBM.

(c) *Antisera that predominantly stain stromal components*

This is the most difficult section to describe because it ranges from the massive deposition of connective tissue elements to the discrete distribution of several enzymes. Collagen contributes much of the villous stroma whereas myosin was identified in the muscular coat of most of the larger vessels. Neither collagen nor myosin was identified in trophoblasts or in vascular endothelium. Occasional villi exhibit small clusters of staining for C3, B_1A, C1q, and alpha-2-macroglobulin. Fibrinogen is the most commonly identified plasma protein in human placentae, and its distribution is extremely variable. Within stroma, fibrinogen may be absent or it may be deposited in most of the stroma of isolated villi.

The distribution of several proteins within villous stroma can be altered by prolonged washing of the tissue. This is particularly exemplified by haptoglobin and albumin, both of which are represented as diffuse and patchy areas when studied without prior washing of the tissue, whereas they are only sparingly represented as discrete spots following a 12-hr wash in growth medium. Interestingly, the diffuse stromal distribution of IgG is also somewhat diminished by washing. Other proteins such as caeruloplasmin that are only sparingly represented in the first instance, are retained as a faint speckled distribution following washing. The distribution of beta-2-microglobulin was also not altered by washing. This protein is uniformly absent from trophoblasts and TBM, and is identified on stromal fibroblasts, pericytes, Hofbauer cells, and stem-vessel endothelium (Fig. 3a). Several proteins, of which orosomucoid is representative (Fig. 3b), are shown by immunofluorescence as a series of discretely clustered spots. These include alpha-1-antitrypsin, chymotrypsin, muramidase, and cholinesterase, listed in descending order of apparent staining intensities. These speckled patterns are found in three general areas; (a) below TBM; (b) in stroma, and (c) oriented about stromal vessels. The nature of these positively stained spots can not be resolved by immunofluorescence.

(d) *Antisera that predominantly stain stromal vessels*

Collagen constitutes the bulk of the peri-vascular tissue, myosin the bulk of the vessel wall, and actin and beta-2-microglobulin are identified in most endothelial cells. Fibrinogen can be identified in a peri-

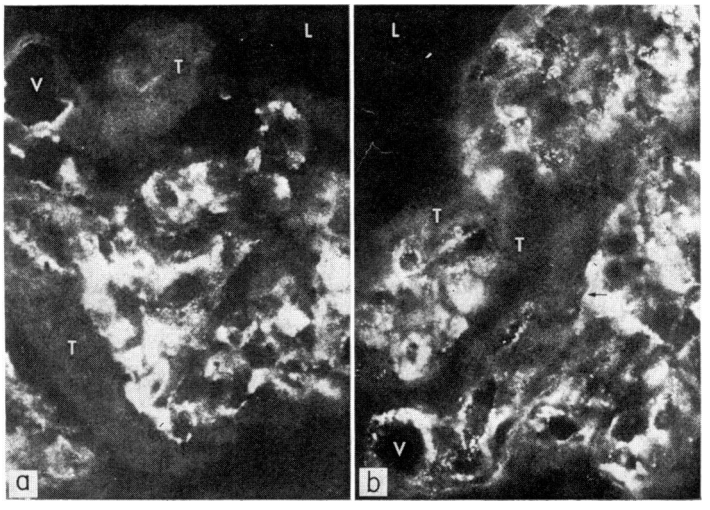

FIG. 3. Proteins identified in stroma. (a) beta-2-microglobulin. Note absence of trophoblast staining and positive vascular endothelial cells. Stromal staining is due to pericytes, fibroblasts, Hofbauer cells and endothelium on the stromal side of TBM. (Magnification × 240.) (b) Orosomucoid. Note negative trophoblasts, granular staining about vessels and dense accumulation beneath TBM (small black arrow). (Magnification × 240.) Code as in Fig. 1.

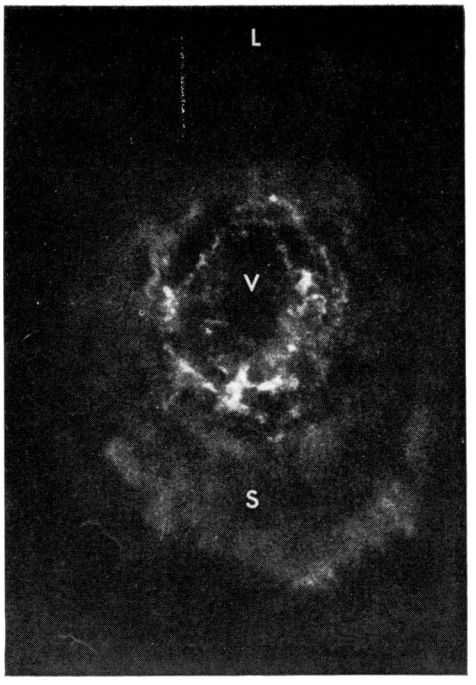

FIG. 4. (a) C1q in vessel (V) wall of placental stroma (S) bordered by inter-villous space (L). (Magnification × 240.)

vascular location, as can be IgG, B_1A, B_1E and alpha-2-macroglobulin, and C1q is occasionally found in a granular distribution in the walls of large vessels (Fig. 4).

Several proteins are regularly identified within endothelium. These produce homogenous staining reactions, and they include AFP, CEA, and albumin. The staining intensity of AFP and albumin is diminished by prior washing of the tissues, but that for CEA is not. A few proteins were identified as being expressed in the apical aspects of endothelium. These include IgG, IgM, and alpha-2-macroglobulin. Several of the staining reactions were performed using ultracentrifuged sera, suggesting that a positive reaction was not due to aggregates in the sera binding with Fc-receptors on placental stem vessel endothelium (Johnson, Trenchev & Faulk, 1975; Johnson, Faulk & Wang, 1976).

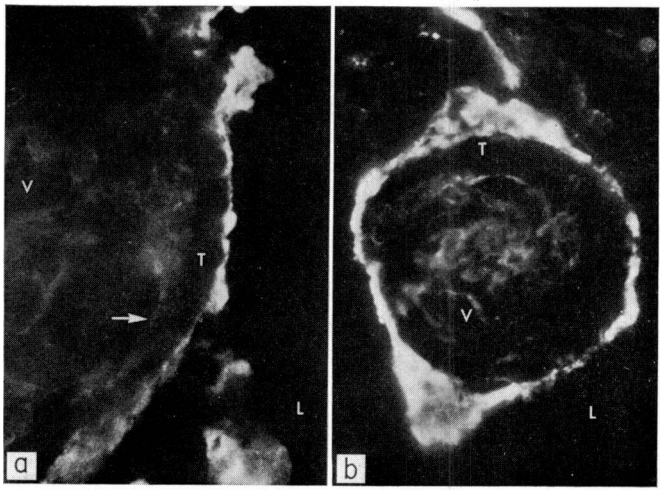

FIG. 5. Peri- and intervillous proteins. (a) C4. Note contact with trophoblasts as well as staining of intervillous fibrin (lower right). (Magnification × 240.) (b) Alpha-2-macroglobulin. These areas also contain fibrinogen and C4. (Magnification × 84.) Code as in Fig. 1.

(e) *Antisera that predominantly stain peri- and intervillous fibrin*

Many proteins are faintly positive in fibrin areas. On the other hand, beta-2-microglobulin, AFP and C-reactive protein were not identified; orosomucoid, chymotrypsin and muramidase were also usually absent. The only proteins that consistently gave intense reactions in fibrin areas were fibrinogen, plasminogen, alpha-2-macroglobulin and B_1E.

Fibrin areas on the surface of chorionic villi have a matrix of small circular profiles and appear as canalized structures. These areas stain intensely with anti-fibrinogen. In addition to containing fibrinogen, we have shown that these areas bind concanavalin A, and that the binding is inhibited by 10^{-1} M α-methyl-D-mannoside (Johnson & Faulk, 1976), suggesting the presence of glycoproteins. Alpha-2-macroglobulin and B_1E are identified primarily in peri- and inter-villous areas, (Fig. 5a, b), but their distribution is neither as impressive nor as wide-spread as that of fibrinogen. IgA and IgM were often, though faintly, identified in the inter-villous fibrin, and collagen was rarely recognized in these areas.

DISCUSSION

Within the three major constraints put forward in the Introduction, it would seem that the identification, localization, and distribution of many proteins in human placentae can be determined by immunohistological techniques. Notwithstanding traditional objections to immunofluorescence, several predictable observations were in fact confirmed. For example, proteins such as AFP and CEA that are

almost exclusively synthesized by the foetus were identified only in foetal stem vessels; proteins such as IgA that are almost exclusively synthesized by the mother and are not transported across placentae (Brambell, 1970), were identified in peri- and inter-villous fibrin areas; and, proteins such as C-reactive protein that are normally found in neither mother nor foetus were consistently negative in all placentae.

Actin is a common constituent of cells but it appears to be particularly abundant in trophoblasts (Faulk *et al.*, 1974, 1975). This may reflect the transport function of these cells, since microfilaments are important in the control of endo- and exo-cytosis (Allison, 1973) and actin is a fundamental component of microfilaments. Actin is also particularly abundant in certain malignant cells (Gabbiani, Trenchev & Holborow, 1975), with which trophoblasts have some analogies (Fauve *et al.*, 1974).

Fibrin and fibrinogen are identified very early in the development of human placentae (Hamilton & Boyd, 1960), and there is a general increase in fibrin deposition throughout gestation (Wilkin, 1958). Accentuation of fibrinolytic activity followed by clinical defibrination occurs in several complications of pregnancy such as premature detachment of placenta, *in utero* retention of dead foetus, amniotic fluid embolism, and infected abortion (Kawai, 1973). The pathogenesis of these lesions is not clear, but they may be associated with the capacity of trophoblasts to synthesize fibrinolysins such as plasminogen.

The identification of transferrin in the apical aspect of trophoblasts raises the question as to whether or not this is the foetal-type transferrin of Parker, Hagstom & Bearn (1963). The iron-binding property of trophoblastic transferrin may also serve a non-specific defence function against infection, analogous to the role of plasma iron in certain bacterial infections (Editorial, 1974). In addition, placental transferrin can bind iron from maternal blood and thereby contribute to iron-deficiency anaemia during pregnancy. Although feasible, this speculation has to be reckoned with the fact that syncytio-trophoblasts are released into the mother's venous circulation (Ikle, 1964), and some of the iron would presumably be returned to her through this process. In this regard it is pertinent to note that plasma transferrin levels rise during pregnancy (Tsung, Rosenthal & Milewski, 1975). Finally, the pattern of anti-transferrin staining is similar to that observed with colloidal iron by Bradbury *et al.* (1970) and attributed to a sulphated mucoprotein that functions to protect trophoblasts (Billington, 1975). The role of transferrin in these reactions seems to merit consideration.

The identification of C3 and B_1A on TBM, in the absence of B_1E, is suggestive of a complement by-pass system operating at this site. We rarely were able to demonstrate B_1E on TBM although it was commonly observed in peri- and intervillous fibrin. On the other hand, C3 and B_1A were often observed in fibrin areas in the presence of B_1E, suggesting a classical pathway of complement activation in these areas. Also, fibrinogen was commonly identified on TBM. It is often assumed that these proteins in placentae represent the consequence of immunological reactions. Alternative assumptions might however include complement activation by placental enzymes or glycoproteins on TBM, or activation by non-immune protein complexes such as those formed by collagen-glycosaminoglycan interactions (Conochie *et al.*, 1975). These possibilities seem to merit further study in terms of understanding the ageing and immunopathology of TBM.

The identification, biochemistry, and biological properties of IgG on TBM has previously been reported (McCormick *et al.*, 1971; Faulk *et al.*, 1974; Faulk & Jeannet, 1975). This IgG can inhibit mixed-lymphocyte culture reactions, and the inhibition seems to be independent of anti-HL-A activity. The precise specificity of this IgG remains to be established. It is interesting that C3 on TBM may be activated through a by-pass mechanism, since it has hitherto been difficult to reconcile the presence of a 'blocking' antibody on TBM in association with classical complement activation at the same site.

The identification of beta-2-microglobulin in villous stroma is of importance since beta-2-microglobulin is normally associated on cell membranes with HL-A antigens (Jones *et al.*, 1975). There have been many attempts, and one reported success (Loke, Joysey & Borland, 1971), to identify HL-A on trophoblasts. It is presently not clear whether HL-A antigens are expressed on trophoblasts *in vivo*. Cells that lack HL-A also lack beta-2-microglobulin, as for instance demonstrated by DAUDI cells (Goodfellow *et al.*, 1975). The absence of beta-2-microglobulin on trophoblasts and TBM (Fig. 3a) tends to speak against the often speculated presence of HL-A on these cells, and suggests that the immunobiology of the placental homograft may differ from that of other grafts. This speculation is

further supported by the observation that maternal beta-2-microglobulin levels do not rise significantly throughout gestation (Kithier *et al.*, 1974) as well as by reports by Faulk & Temple (1976), Faulk, Sanderson & Temple (1976) and Goodfellow *et al.* (1975) suggesting that human trophoblasts do not manifest antigens of the major histocompatibility complex. The identification of orosomucoid in sub-TBM and perivascular areas is of possible interest because this protein can interact with progesterone (Kawai, 1973). In this capacity it could act as a modulator of placental progesterone, although we have no data pertinent to this speculation.

The identification of C1q within walls of some foetal stem vessels could suggest the presence of immune complexes. It is assumed that the C1q in this circumstance is of foetal origin because newly synthesized C1q has been detected in culture fluids of human foetal tissues (Adinolfi, 1972). One possibility that might explain the presence of immune complexes in the vicinity of foetal stem vessels is that of materno-foetal allotypic incompatibility, such as has been reported for Gm and Inv antigens (Fudenberg & Fudenberg, 1964; Faulk, Loghem & Stickler, 1974). The most likely place for maternal (IgG) anti-allotype antibodies to encounter foetal antigens is at the level of placental stem vessels. In this event it would seem that such complexes could be prevented from entering the foetal circulation by precipitation into a macrocomplex within vessel walls by foetal C1q. Should this mechanism fail to impede immune complexes within placentae, we have previously described the presence of Fc-receptors on placental stem vessel endothelium (Johnson, Trenchev & Faulk, 1975; Johnson, Faulk & Wang, 1976) that may further retard the entrance of immune complexes into the foetal circulation.

Finally, the identification of a large number of proteins in peri- and inter-villous fibrin is suggestive of non-specific trapping. However, many proteins were absent. We were particularly impressed by the absence of collagen, as these areas are generally thought to contain collagen (Boyd & Hamilton, 1970). We do not wish to become embroiled in the embryological debate of whether these are 'fibrinoid' or 'fibrin' areas, or their apparent significance. However, the striking appearance of fibrinogen, plasminogen, alpha-2-macroglobulin, and B_1E has impressed us and we do feel that these proteins suggest fruitful approaches for future immunological studies of human placentae.

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