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Immunological studies of human placentae

LECTIN BINDING TO VILLOUS STROMA AND TO TROPHOBLASTIC BASEMENT MEMBRANE

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SUMMARY

It has been demonstrated immunohistologically using cryostat sections that the lectins PHA and Con A bind to human placentae. Both lectins stain trophoblastic basement membrane (TBM), perivascular tissue and stroma. No staining of trophoblasts was observed. It has been shown by blocking experiments that the lectin binding sites on placental TBM are glycoproteins, whereas experiments involving pretreatment of placental sections with anti-collagen antisera or highly purified bacterial collagenase have indicated that lectin binding to stromal and perivascular structures is collagen-associated. The possible relation of TBM lectin-binding sites to immune response gene products is briefly discussed.

INTRODUCTION

Immunological mechanisms are thought to be responsible for the successful maintenance of a placental graft (Medawar, 1953; McCormick *et al.*, 1971; Faulk *et al.*, 1974). The placenta is a foetal structure endowed with paternal as well as maternal gene products (Beer & Billingham, 1971), and is the principal site of contact and exchange between mother and foetus (Fox, 1971). Antigens in the placenta have not been well characterized. HL-A antigens have been claimed to be present on trophoblasts *in vitro* (Loke, Joysey & Borland, 1971) and there are heteroantigens associated with trophoblast membranes (Curzen, 1970; Beer & Billingham, 1971), although it has been suggested that these may be masked by sialomucins *in vivo* (Bradbury *et al.*, 1969). Immunohistological studies (McCormick *et al.*, 1971) have shown the presence of immunoproteins on human trophoblastic basement membrane (TBM), suggesting that TBM may be immunologically important in the materno-foetal relationship. This point is strengthened by the observation that IgG eluted from human placentae contains a maternal blocking antibody to uncharacterized TBM antigens that may protect the placenta from maternal cell-mediated immune rejection mechanisms (Faulk *et al.*, 1974). There is evidence that antigens may be common to both trophoblasts and lymphocytes (Beer, Billingham & Yang, 1972), although such antigens may not be HL-A in character (Faulk *et al.*, 1974).

The purpose of this study was to investigate the tissue distribution and character of human placental antigens by immunofluorescence using the plant lectins, concanavalin A (Con A) and phytohaemagglutinin (PHA). Con A has two active sites, both of which react preferentially with carbohydrate structures containing α-D-mannopyranosyl or α-D-glucopyranosyl residues (Goldstein, Hollerman & Smith, 1965): the specificity of PHA for carbohydrate structures is less clear, although the major affinity is for *N*-acetylgalactosamine derivatives (Sharon & Lis, 1972). Plant lectins are useful molecular probes for saccharide-containing structures on the surface of a variety of cells and they have provided considerable data on the topography and relative mobility of membranes of several different cells (Inbar & Sachs, 1969; Nicholson, 1974). We report here the specificity of these lectins for components of human placentae.

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MATERIALS AND METHODS

Reagents. Jack bean concanavalin A (Sigma Chemicals, St Louis, U.S.A.; Grade IV) and purified phytohaemagglutinin (from *Phaseolus vulgaris*; Wellcome Reagents, Beckenham, Kent) were prepared fresh in phosphate-buffered isotonic saline, PBS, pH 7·2, before use. α-Methyl-D-mannoside (α-MM) was obtained from Sigma Chemicals, St Louis. Enzymes employed in this investigation included highly purified bacterial collagenase (fraction A of type III from *C. histolyticum*; Sigma Chemicals, St Louis), trypsin (2·5% in normal saline; Flow Laboratories, Irvine, Scotland), neuraminidase (from *Vibrio cholerae*, 500 u/ml; Behringwerke, Marburg, Germany) and hyaluronidase B.P. (from ovine testicular extract, 1500 i.u./ml; Fisons, Loughborough, England).

Antisera. Two sheep were intramuscularly immunized twice with 7·5 mg of either PHA or Con A: the first injection was given in Freund's complete adjuvant and the second in aluminium hydroxide, according to the Medical Research Council's schedule (1966). Precipitating antisera were shown to be monospecific by double radial immunodiffusion analysis in agar gel. Specificity of the sheep antisera for PHA and Con A was also demonstrated on cell membranes according to Faulk & Temple (1975): this was done by showing that the immunofluorescence reaction of fluorescein-conjugated antiserum to PHA with fibroblast membrane-bound PHA was inhibited by PHA but not Con A, and that the analogous Con A– anti-Con A reaction was inhibited by Con A but not PHA. Rabbit anti-human acid-soluble skin collagen was prepared according to Bornstein & Piez (1966). The amino-acid composition of this antigen, obtained by Dr A. J. Bailey, ARC Meat Research Institute, Langford, Bristol, was compatible with human skin collagen. Anti-collagen sera were raised in six rabbits by multiple injections of collagen according to Faulk *et al.* (1975a). The specificity of these sera were determined by haemagglutination, immunofluorescence, and immunoelectron microscopy according to Faulk *et al.* (1975a). In addition, one serum was found to agglutinate sheep erythrocytes tanned with native collagen, but not erythrocytes tanned with denatured collagen; the other sera agglutinated sheep erythrocytes tanned with either native or denatured collagen. The former serum will be referred to as anti-collagen helical conformational site (ACC), and the latter sera as anti-collagen telopeptide (ACT). Both ACC and ACT had haemagglutination titres in excess of 1:50,000. Both anti-collagen antisera diffusely stained stromal collagen in human placentae by immunofluorescence, but neither stained TBM nor trophoblasts (Faulk *et al.*, 1975b).

An antiserum was prepared to a human placental extracellular glycoprotein by immunizing rabbits with an antigen prepared by isoelectric-point precipitation at pH 4·0 of placental eluates prepared according to Faulk *et al.* (1974). The antigen contained large amounts of mannose and very little sialic acid (Faulk & Clamp, unpublished observations). The resultant antiserum contained an anti-fibrinogen antibody that was removed by passage over an immunoadsorbent column of human fibrinogen (Kabi AB, Sweden) coated on Degalan V26 polymethylmethacrylate beads (Degussa Wolfgang, Frankfurt). Immunoelectrophoretic and double radial immunodiffusion analysis of the immunoadsorbent-prepared antiserum revealed no precipitin reaction to human or animal serum or plasma, but the antiserum gave intense staining of human placental TBM by immunofluorescence when used at a dilution of 1:500. This antiserum will be referred to as anti-precipitate (Appt).

Fluorescein conjugation of antisera. Samples of gamma globulin-enriched antisera were prepared by 33% saturated ammonium sulphate precipitation, reconstitution and extensive dialysis against physiological saline. These preparations were then labelled with isomer I fluorescein isothiocyanate (FITC) (BBL, Division of BioQuest, Cockeysville, Maryland, U.S.A.) by the dialysis technique recommended by Faulk & Hijmans (1972). Fluorescein conjugates were freed of excess FITC by extensive dialysis, and fluorescein:protein (F:P) ratios determined by spectrophotometry according to Johnson & Holborow (1973). F:P ratios of between 1·0 and 1·5 were accepted, and all FITC conjugates were used at dilutions of between 1:50 and 1:100. PHA and Con A were also labelled with FITC by the dialysis technique, with the modification that Con A was conjugated at pH 8·5 rather than pH 9·0 due to its conformational instability at the higher pH (Pflumm, Wang & Edelman, 1971). When indirect immunofluorescence was employed, FITC-conjugated sheep antiserum to rabbit Ig or FITC-conjugated rabbit antiserum to sheep Ig were used (Wellcome Reagents, Beckenham, Kent, England). These are potent, specific antisera that can be satisfactorily used at high dilution using human placentae as substrate.

Tissue sections. Ten normal full-term human placentae were used. Blocks of tissue (0·5 × 0·5 cm) from the mid-portion of the central cotyledon were snap-frozen in isopentane cooled in liquid nitrogen using 'Tissue-Tek' O.C.T. compound (Ames Company, Division Miles Laboratories, Indiana, U.S.A.). Sections were cut at 6 μm in a cryostat and air-dried without fixation. Sections were prepared fresh each day.

Immunofluorescence techniques. Immunofluorescent staining and examination of tissue sections was performed as described previously (Johnson, Trenchev & Faulk, 1975). For indirect immunofluorescence, sections were exposed for 20 min to unlabelled PHA or Con A (1 mg/ml) in a moist chamber and subsequently washed in three changes of PBS, pH 7·2, for 20 min each before exposure to the appropriate FITC-conjugated antiserum for 20 min. Sections were then washed in three changes of PBS, pH 7·2, for 20 min each before mounting in 40% glycerol buffered at pH 8·5. FITC-conjugated antisera to PHA or Con A were always used at a dilution (1:80) that did not stain tissue sections that had not been exposed to PHA or Con A. Blocking experiments were performed by exposure of the sections to neat unlabelled antiserum, then washing in three changes of PBS, pH 7·2, before treatment with lectins. Enzyme digestion of tissue sections was performed similarly for 30 min with solutions of enzyme activity as stated in text.

Photomicrographs were taken using a Zeiss photomicroscope II equipped for epi-illumination with a Ploem illuminator and a HBO 200 high-pressure mercury-vapour burner light source. Gaf 500 high-speed 35 mm film was used.

RESULTS

Bright immunofluorescent staining of cryostat sections of human placentae was given by both PHA and Con A (Fig. 1). The pattern of staining achieved for each lectin was independent of whether direct or indirect immunofluorescence was used, and was consistent for all placentae examined. The most striking feature of the fluorescence was the strong reaction of the lectins with placental TBM: this reaction was more consistent for PHA staining where sometimes strong positive staining of the total TBM of placental villi was achieved, whereas with Con A often only segments of thickened TBM were positively stained. Both lectins gave perivascular staining and a generalized staining of stromal connective tissue. The staining of villous stroma was more diffuse for Con A compared with PHA. Both PHA and Con A also stained areas of fibrinoid necrosis, notably on the periphery of such areas. Neither lectin gave any staining of trophoblasts.

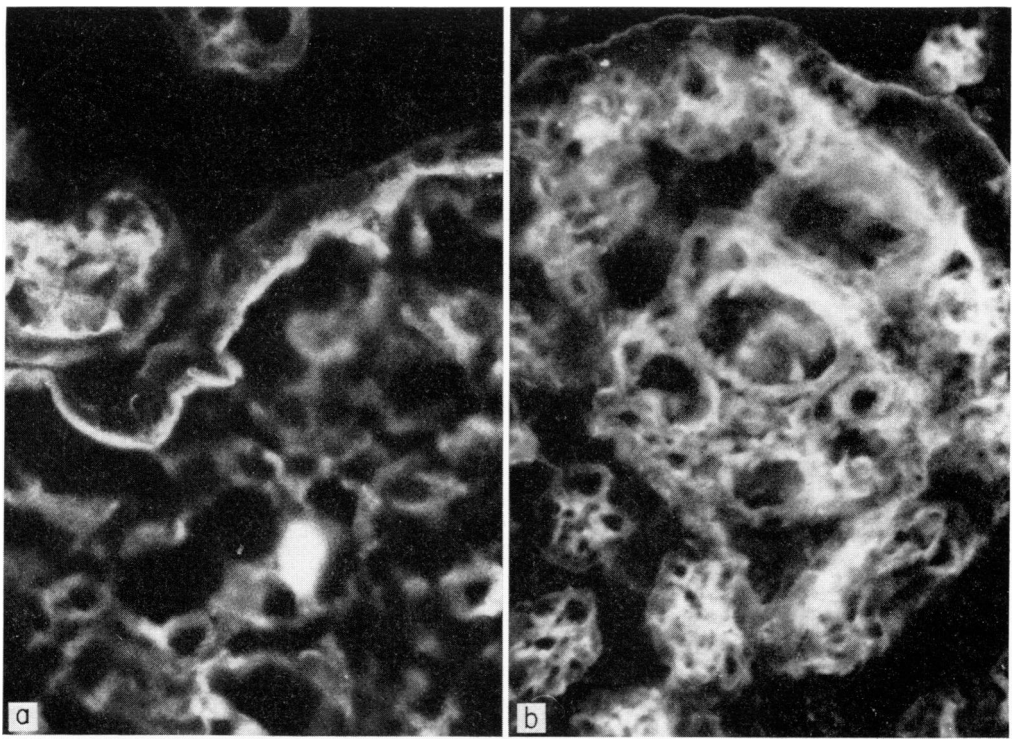

FIG. 1. Cryostat sections of human placentae after indirect immunofluorescent staining with (a) PHA (1 mg/ml) followed by FITC-conjugated sheep anti-PHA antiserum, and (b) Con A (1 mg/ml) followed by FITC-conjugated sheep anti-Con A antiserum. (Magnification × 540.)

Immunofluorescent staining experiments in the continuous presence of α-MM demonstrated that the binding of Con A to placental tissue was completely inhibited using 10^{-1} M α-MM, and that using 10^{-2} M α-MM binding to TBM was reduced whereas binding to perivascular and stromal structures was completely inhibited. The continuous presence of 10^{-1} M α-MM had no effect on the immunofluorescent staining pattern given by PHA.

The effect of enzyme treatment on subsequent lectin staining of placentae is summarized in Table 1. All enzymes were used at activities that were found to keep the morphology intact. Collagenase digestion at low concentration reduced stromal and perivascular staining by lectins, but had no effect on TBM staining. At higher concentrations, TBM staining was also reduced by collagenase. Collagenase activity is Ca^{2+}-dependent: if no $CaCl_2$ was added to collagenase solutions the effect on subsequent lectin staining was similar to that of a broad specificity protease, i.e. a slight reduction in perivascular and stromal staining only. The removal of TBM staining and marked reduction of stromal and perivascular

TABLE 1. Effect of various enzymes on components of subsequent immunofluorescent staining by PHA and Con A on human placental tissue sections

Enzyme	Enzyme activity per tissue section	Placental tissue component giving positive fluorescence (scale − to +++)					
		TBM		Perivascular		Stroma	
		PHA	Con A	PHA	Con A	PHA	Con A
Collagenase	10 u in PBS+1 mM $CaCl_2$	+++	+++	++	++	++	++
Collagenase	40 u in PBS+1 mM $CaCl_2$	+++	+++	+	+	+	+
Collagenase	100 u in PBS+1 mM $CaCl_2$	+	+	±	±	−	−
Collagenase	100 u in PBS (No Ca^{2+})	+++	+++	++	++	++	++
Trypsin	0·1 ml of a 0·1% solution in PBS	+++	+++	+	++	++	++
Neuraminadase	1·5 u in PBS	+++	++	++	+	++	+
Hyaluronidase	75 u in PBS	+++	+++	+++	+++	+++	+++
PBS		+++	+++	+++	+++	+++	+++

staining by lectins following digestion of placental tissue sections with Ca^{2+}-activated collagenase therefore reflects specific enzyme activity. Trypsin caused a slight reduction in perivascular and stromal staining by lectins, whereas hyaluronidase had no effect whatsoever (Table 1). Neuraminidase was found to have a similar effect to trypsin, except that there was a more marked general reduction of staining intensity for Con A.

The effects of anti-collagen and anti-placental glycoprotein antisera on subsequent lectin staining of placentae are summarized in Table 2. Pre-treatment of placental tissue with non-immune rabbit serum was used as a control. Both ACC and ACT had no effect on lectin staining of TBM. However, pre-treatment with ACT, unlike ACC, significantly reduced perivascular and stromal staining by both lectins. Conversely, pre-treatment of placental sections with Appt caused a striking reduction in TBM staining but had little effect on perivascular or stromal staining by both lectins. The loss of TBM staining by PHA following pretreatment of placental sections with Appt is shown in Fig. 2.

DISCUSSION

The results of pretreatment of human placental tissue sections with anti-collagen antisera or collagenase indicate that components giving perivascular and stromal immunofluorescent staining with PHA or Con A are collagen-associated. Lectin binding to these structures is removed by mild collagenase digestion. The ability of ACT rather than ACC to block this lectin staining suggests that telopeptide may be involved in the collagen–lectin interaction. Some non-specific proteolytic cleavage of collagen telopeptide

TABLE 2. Effect of anti-collagen antisera and antiserum to extractable placental glycoprotein on components of subsequent immunofluorescent staining by PHA and Con A on human placental tissue sections

Blocking antibody	Placental tissue component giving positive fluorescence (scale − to +++)					
	TBM		Perivascular		Stroma	
	PHA	Con A	PHA	Con A	PHA	Con A
ACC	+++	+++	+++	+++	+++	+++
ACT	+++	+++	+	+	+	+
Appt	±	+	++	++	+++	+++
Normal rabbit serum	+++	+++	+++	+++	+++	+++

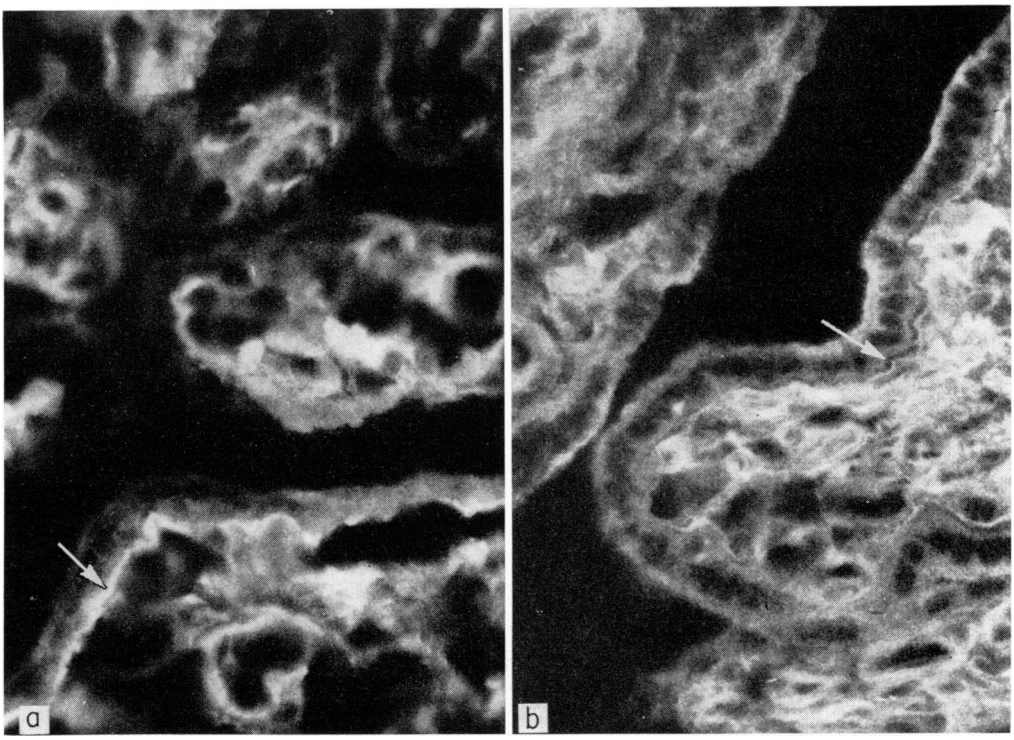

FIG. 2. Cryostat sections of human placentae after indirect immunofluorescent staining with PHA (1 mg/ml) followed by FITC-conjugated sheep anti-PHA antiserum. Sections had been pre-incubated with (a) non-immune rabbit serum (arrow points to positively-stained trophoblastic basement membrane), and (b) rabbit anti-placental glycoprotein antiserum, Appt (arrow points to unstained trophoblastic basement membrane). (Magnification × 540.)

may also account for the slight reduction in perivascular and stromal staining by lectins following treatment with other enzymes (Table 1) as telopeptide, unlike helix, is susceptible to many proteases.

Neither ACC not ACT blocked placental TBM staining by lectins (Table 2). However, digestion with high concentrations of collagenase did reduce TBM staining. In this circumstance, positively-stained aggregates formed on the surface of the section indicated that collagenase digestion was only partially releasing the lectin-binding component and that the lectin binding sites on TBM are not collagen. The ability of Appt to block TBM staining suggests that the lectin-binding sites are glycoproteins since the placental glycoprotein used as the antigen to produce Appt contains substantial amounts of monosaccharides that interact with Con A and PHA, i.e. mannose and *N*-acetylgalactosamine (Faulk & Clamp, unpublished observations).

It is interesting to speculate on the nature of the lectin-binding glycoproteins in human placental TBM. A cross-reactivity of trophoblast and lymphocyte antigens has previously been described (Beer *et al.*, 1972). This cross-reactivity could be due to alloantigens defined by immune response genes. A group of lymphocyte membrane alloantigens (Ia antigens) determined by the I region of the H-2 histocompatibility complex in mice has been described (Shreffler & David, 1975). These antigens are also found on non-lymphoid cells such as macrophages, epidermis and spermatozoa (Hammerling *et al.*, 1975). Such antigens appear to be glycoproteins that can bind lectins (Sachs *et al.*, 1975), and probably are not associated with β_2-microglobulin (Delovitch & McDevitt, 1975). Lymphocyte membrane alloantigens with similar characteristics have been reported in man (Arbeit *et al.*, 1975) and are presumably not normally associated with β_2-microglobulin (Jones *et al.*, 1975), unlike HL-A antigens (Poulik *et al.*, 1974). We have studied several human placentae by indirect immunofluorescence using rabbit antiserum to human β_2-microglobulin (Dakopatts A/S, Denmark) and have been unable to identify β_2-microglobulin on trophoblasts or TBM. If immune response gene products are present on placental

TBM, they might become detached and could explain the observation of glycoproteins in pregnancy sera that are capable of binding Con A and may suppress lymphocyte transformation (Gaugas, 1974). Maternal sensitization to these antigens could result in antibodies to non-HL-A alloantigens expressed on human lymphocytes, and such antibodies have been described in pregnancy sera (Winchester *et al.*, 1975). Also, elution studies of IgG on placental TBM have revealed the presence of maternal antibodies to non-HL-A antigens that are capable of inhibiting T-cell responses in mixed lymphocyte culture reactions (Faulk *et al.*, 1974). These observations prompt us to put forward the concept that antigens on placental TBM may be important in the maternofoetal relationship and its possible genetic implications, and that lectin probes such as PHA and Con A could prove useful in the isolation and characterization of these antigens.

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