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Immunological studies of human placentae: subclass and fragment specificity of binding of aggregated IgG by placental endothelial cells

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Summary. Fluorescein-conjugated heat-aggregated human IgG binds to endothelial cells of foetal stem vessels in cryostat sections of normal, full-term human placentae. No binding was observed using native human IgG or heat-aggregated human albumin, IgM or IgA2. No inhibition of binding of heat-aggregated human IgG was observed by pre-treatment of placental tissue sections with native IgG or non-aggregated Fc fragments. The binding was blocked using heat-aggregated Fc fragments prepared from IgG1, IgG2, IgG3 and IgG4 myeloma proteins, but not with heat-aggregated human light chains, Fab and $F(ab')_2$ fragments of human IgG, or with heat-aggregated human IgM and IgA2. It is suggested that the placental endothelial cell receptor for aggregated IgG may function to keep immune complexes from entering the foetal circulation.

INTRODUCTION

Cell membrane receptors for aggregated IgG and immune complexes have been characterized for several cells of the immune system, such as lymphocytes and monocytes (Frøland, Natvig & Michaelsen, 1974a; Frøland, Michaelsen, Wisløff & Natvig, 1974b; Lawrence, Weigle & Spiegelberg, 1975). Various immunological functions have been ascribed to them (Wisløff, Frøland & Michaelsen, 1974; Sachs & Dickler, 1975). However, several cells that are not generally thought of as directly involved in the immune system have also been shown to bind complexed or aggregated immunoglobulins. For example, heat-aggregated IgG binds to mouse fibroblasts (Papamichail, Faulk, Gutierrez & Temple, unpublished observations) and to human platelets (Henson & Spiegelberg, 1973), and antibody-sensitized erythrocytes bind to reticulum cells in normal liver and to certain neoplastic tissues (Tønder, Morse & Humphrey, 1974). Normal human serum IgG also binds through an Fc-dependent reaction to skeletal muscle and central nervous tissue (Aarli & Tønder, 1974; Aarli, Aparicio, Lumsden & Tønder, 1975). Similarly, a considerable interaction between heat-aggregated human IgG and liposomes has been reported (Weissmann, Brand & Franklin, 1974).

We have previously observed binding activity for aggregated immunoglobulin and soluble immune complexes on endothelial cells of foetal stem vessels in human placentae (Johnson, Trenchev & Faulk, 1975a). This report concerns studies on the immunoglobulin class specificity of this receptor on human placental endothelium, and furthermore its characterization by inhibition of the binding of fluorescein-conjugated heat-aggregated IgG in terms of specificity

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for subclasses and fragments of human IgG. It is suggested that this receptor may provide an immunobiological mechanism for the protection of the foetus from immune complexes such as those that may result from placental transfer of maternal antibodies to allotypically-incompatible foetal antigens (Fudenberg & Fudenberg, 1964; Faulk, van Loghem & Stickler, 1974).

MATERIALS AND METHODS

Tissues

Fifteen normal full-term human placentae were obtained immediately upon delivery and perfused with chilled Hanks's solution. 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' OCT Compound (Ames Company, Division of Miles Laboratories, Indiana) as embedding medium.

Immunoglobulin preparations and fragments

We prepared the following myeloma proteins and fragments for use in this study: intact IgG1(κ), Gm(1) protein, Gee; intact IgA2(λ), A_2m(2) protein, For; intact IgM(κ) protein, Til; Fc fragment from IgG1 protein, Fer; Fc fragment from IgG2 protein, Til; Fc fragment from IgG4 protein, Arp; Fab fragment from IgG1(κ) protein, Fer; Fab fragment from IgG2(κ) protein, Til; λ-type L-chain from IgG1 protein, Whi; κ-type L-chain from IgG3 protein, Hus.

Native IgG, IgM and IgA myeloma proteins were isolated from sera by standard procedures including sodium sulphate precipitation, ion-exchange chromatography, starch block electrophoresis and gel filtration on Sephadex columns (Pink, McNally, Wang & Fudenberg, 1972). Light chains were prepared from reduced and alkylated purified myeloma IgG by gel filtration on a Sephadex G-100 column equilibrated with 1 M acetic acid, as described previously (Wang, Faulk, Stuckey & Fudenberg, 1970).

Fab and Fc fragments of IgG myeloma proteins were prepared by digestion with twice recrystallized papain (Worthington Biochemicals Corporation, lot 35H 718) using a procedure similar to that of Porter (1959). IgG1 and IgG3 purified myeloma proteins were dissolved in 0·075 M phosphate buffer (pH 7·0, containing 0·15 M NaCl and 0·002 M EDTA) at a protein concentration of 10 mg/ml, and papain digestion carried out in the presence of 0·01 M cysteine at 37° for 2 h using 1 mg papain per 100 mg protein (Gergely, Medgyesi & Stanworth, 1967). IgG2 and IgG4 purified myeloma proteins were digested similarly except that 0·01 M 2-mercaptoethanol was used instead of 0·01 M cysteine (Wang, Gergely & Fudenberg, 1973). Fc and Fab fragments were separated by DEAE–cellulose ion-exchange chromatography using a column equilibrated with 0·005 M phosphate buffer, pH 8·0. Fab fragments were eluted directly under these conditions. The column was then washed with 0·015 M phosphate buffer, pH 8·0, before elution of Fc fragments with 0·5 M phosphate buffer, pH 8·0. Separation of Fab fragments and undigested IgG molecules was accomplished by gel filtration using a Sephadex G-200 column (90 × 2·5 cm) equilibrated with 0·1 M Tris–HCl buffer with 0·2 M NaCl, pH 8·0.

The specificity of each preparation was determined by immunoelectrophoresis using monospecific antisera and by molecular weight estimation according to the elution profile from a calibrated Sephadex G-100 column (90 × 2·5 cm) equilibrated with 0·1 M Tris–HCl buffer with 0·2 M NaCl, pH 8·0.

Intact IgG3, Gm(21), myeloma protein, Her, and the Fch fragment of this protein were kindly provided by Dr T. E. Michaelsen (Michaelsen & Natvig, 1973). The $F(ab')_2$ fragment of pooled human serum IgG (Kabi AB, Sweden) was a gift from Dr M. W. Turner: this fragment preparation has been used previously in cell-binding experiments (Johnson, Papamichail, Gutierrez & Holborow, 1975b). Polyclonal serum IgG was isolated by DEAE–ion exchange chromatography from normal human serum as described previously (Johnson, Watkins, Scopes & Tracey, 1974).

Heat aggregation of protein preparations

Heat aggregation of each of the various immunoglobulin preparations at a concentration of 10 mg/ml was carried out for 5–15 min at 60–66°, after which a slight turbidity in the protein solution was apparent (Spiegelberg, 1974). Fab fragments and light chains needed more extreme conditions than did Fc fragments or intact IgG. Human serum albumin (Nordic Pharmaceuticals, Tilburg, Netherlands) was heat-aggregated at 70° for 20 min. After heat-aggregation, each protein preparation was

centrifuged at 1,000 *g* for 15 min and the supernatant taken for use.

Preparation of heat-aggregated human IgG-fluorescein isothiocyanate

Fluorescein conjugation was always performed prior to heat-aggregation of human IgG (Kabi AB, Sweden). Conjugation with fluorescein isothiocyanate, FITC (BDH, Poole, Dorset), was performed by the method of Johnson & Holborow (1973), and unreacted FITC removed by gel filtration on a Sephadex G-25 column and extensive dialysis against phosphate-buffered isotonic saline (PBS), pH 8·0. Heat-aggregation of a 2% solution of human IgG-FITC was carried out at 63° for 15 min, followed by ultracentrifugation at 143,000 *g* for 90 min and reconstitution of the pellet in PBS, pH 7·2 (Johnson *et al.*, 1975a). Final fluorescein to protein ratios of between 0·8 and 1·5, determined by spectrophotometry, were accepted for heat-aggregated human IgG-FITC.

Fluorescein-conjugated antisera

FITC-conjugated sheep antiserum to human IgG, IgA and IgM (Wellcome Reagents, Beckenham, Kent) and FITC-conjugated rabbit antiserum to human serum albumin (Nordic Pharmaceuticals, Tilburg, Netherlands) were used. These were found to be potent, specific antisera suitable for use at high dilution with human placentae as substrates.

Immunofluorescence techniques

Sections prepared freshly each day were cut at 4–6 μm in a cryostat and air-dried without fixation. Sections were exposed for 20 min to the FITC-conjugate in a moist chamber, and subsequently washed in three changes of PBS, pH 7·2, for 20 min each before mounting in 80% glycerol buffered at pH 8·5.

Indirect immunofluorescence was performed according to McCormick, Faulk, Fox & Fudenberg (1971) by exposure of the sections to the first layer for 20 min, followed by washing in three changes of PBS, pH 7·2, for 20 min each before exposure to the appropriate FITC-conjugated antiserum. To ensure removal of any immunoglobulin aggregates from these FITC-conjugated antisera, it was necessary to ultracentrifuge the conjugates, diluted 1:12 in PBS, pH 7·2, at 143,000 *g* for 90 min before use at a final dilution (1:100 or greater) that gave no positive endothelial cell staining on placental tissue sections when used in direct immunofluorescence.

Blocking experiments were performed according to Faulk & Hijmans (1972) by exposure of the sections to the blocking agent for 30 min, then washing in three changes of PBS, pH 7·2, for 20 min each before exposure to FITC-conjugated heat-aggregated

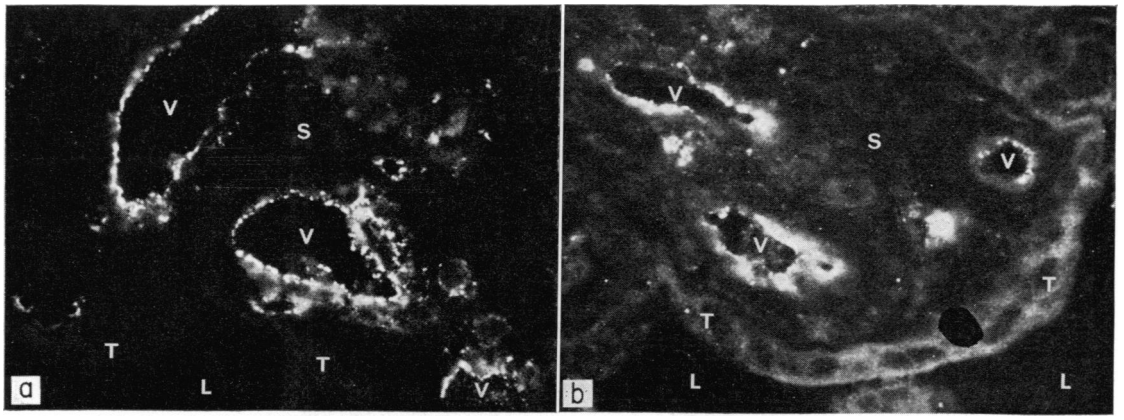

Figure 1. Cryostat sections of two separate human placentae, (a) and (b), stained with heat-aggregated human IgG-FITC at a concentration of 0·2 mg/ml. (a) Shows the common speckled appearance of positive staining in the apical aspect of foetal stem vessels, and the complete absence of any trophoblast staining. (b) Shows the same pattern, except that white light has been transmitted through the section in order to amplify morphological aspects while retaining the immunofluorescence image emitted by epi-illumination excitation. (V = foetal stem vessels; T = trophoblasts; S = villous stroma; L = maternal lake.) (Magnification ×270.)

IgG. Each blocking experiment was repeated at least twice.

RESULTS

Heat-aggregated human IgG-FITC (0·05–0·5 mg/ml) stains cryostat sections of human placentae in the apical aspect of endothelial cells of foetal stem vessels (Fig. 1). Bright fluorescent staining of stromal endothelial cells was given by most, but not all, vessels irrespective of size, and was commonly speckled in appearance. No staining could be observed using native human IgG-FITC at concentrations between 0·05 and 5 mg/ml. Similarly, no endothelial cell staining was observed after exposure to heat-aggregated human albumin, IgM or IgA2 (1 mg/ml) followed by FITC-conjugated rabbit antiserum to human albumin or FITC-conjugated sheep antiserum to human IgM or IgA. Indirect immunofluorescence experiments using heat-aggregated human IgG gave a positive staining pattern identical to that shown in Fig. 1. In all cases, there was clearly no staining of trophoblasts or trophoblastic basement membranes.

Table 1. Inhibition by non-aggregated human immunoglobulin preparations (10 mg/ml) of subsequent immunofluorescent staining of placental endothelium by heat-aggregated human IgG-FITC (0·2 mg/ml)

Blocking agent	Immunofluorescence intensity (scale from − to +++)
Normal serum IgG (polyclonal)	+++
Intact IgG1 (κ) protein	++
Intact IgG3 (λ) protein	++
Fc fragment (IgG1)	++
Fc fragment (IgG2)	+++
Fch fragment (IgG3)	++
Fc fragment (IgG4)	++
$F(ab')_2$ fragment (polyclonal IgG)	+++
Fab fragment IgG1(κ)	+++
Fab fragment IgG2(λ)	+++
λ-type L-chain	+++
κ-type L-chain	+++
Intact IgA2(λ) protein	+++
Intact IgM(κ) protein	+++
PBS	+++
Aggregated normal serum IgG* (polyclonal)	−

* Included as positive inhibitory control.

Pre-treatment of human placental tissue sections with native human immunoglobulin preparations or non-heat-aggregated IgG fragments had no inhibitory effect on the subsequent staining of placental endothelium by heat-aggregated human IgG-FITC (Table 1). Native immunoglobulin preparations at a concentration of 10 mg/ml were used in these blocking experiments; immediately before use they had been passed through a sterile 0·45 μm filter (Millipore Ltd, London) in order to remove any aggregates formed on storage. All blocking experiments included a positive inhibitory control using unlabelled heat-aggregated human serum IgG (5 mg/ml). In tissue sections where no inhibition of staining was observed, the staining pattern was identical to that in Fig. 1.

The inhibitory effect of aggregated human immunoglobulin preparations on the staining of placental endothelium by heat-aggregated IgG-FITC was next studied. Heat-aggregated immunoglobulin preparations at a concentration of 5 mg/ml were used in these blocking experiments: immediately before use they had been centrifuged at 1000 *g* for 15 min to remove any insoluble material. Positive inhibition of staining was obtained following pre-treatment of placental sections with heat-aggregated IgG1 and IgG3 myeloma proteins, as well as with heat-

Table 2. Inhibition by heat-aggregated human immunoglobulin preparations (5 mg/ml) of subsequent immunofluorescent staining of placental endothelium by heat-aggregated human IgG-FITC (0·2 mg/ml)

Blocking agent	Immunofluorescence intensity (scale − to +++)
Normal serum IgG (polyclonal)	−
Intact IgG1(κ) protein	−
Intact IgG3(λ) protein	+
Fc fragment (IgG1)	−
Fc fragment (IgG2)	−
Fch fragment (IgG3)	±
Fc fragment (IgG4)	−
$F(ab')_2$ fragment (polyclonal IgG)	++(+)
Fab fragment IgG1(κ)	+++
Fab fragment IgG2(κ)	+++
λ-type L-chain	+++
κ-type L-chain	+++
Intact IgA2 (λ) protein	++
Intact IgM (κ) protein	++
PBS	+++

aggregated Fc fragments prepared from myeloma proteins of each of the four human IgG subclasses (Table 2). No inhibition of staining was obtained following pre-treatment of placental sections with heat-aggregated Fab or $F(ab')_2$ fragments of human IgG, human IgM or IgA2, or with isolated κ or λ L chains.

DISCUSSION

Heat-aggregated human IgG-FITC binds to endothelial cells of foetal stem vessels in cryostat sections of normal, full-term human placentae (Fig. 1). Pre-treatment of sections with excess native human IgG or non-aggregated IgG fragments failed to block the uptake of heat-aggregated human IgG-FITC (Table 1), and also no direct binding of native human IgG-FITC to human placental endothelium could be demonstrated. Although these experiments cannot absolutely preclude the possibility of some low-affinity binding of native IgG, a similar lack of visualisation of any binding of unaggregated IgG compared with heat-aggregated IgG has also been reported using an analogous staining technique for human B-lymphocytes (Dickler & Kunkel, 1972).

Inhibition of the reaction between heat-aggregated human IgG-FITC and placental endothelial cells has shown that the binding can be mediated by the Fc fragment of all four subclasses of human IgG, and not by the other classes of immunoglobulins or IgG fragments that we have tested (Table 2). The intact IgG3 myeloma protein and its Fc fragment did not inhibit as efficiently as the other subclasses of IgG, although definite inhibition was clearly demonstrable. This may be related to our observation by immunofluorescence that relatively more of the IgG3 subclass is found in full-term human placental tissue (Faulk & Johnson, 1976). The observation that aggregated Fc fragments representative of the four subclasses of human IgG inhibit the binding reaction (Table 2) is consistent with the binding experiments of Henson and Spiegelberg (1973) using human platelets. On the other hand, human lymphocytes and neutrophils have been reported to express a higher affinity for IgG1 and IgG3 subclass proteins (Frøland *et al.*, 1974b; Lawrence *et al.*, 1975), and heat-aggregated IgG1 subclass proteins appear to predominate in the interaction of IgG with liposomes (Weissmann *et al.*, 1974). It is not clear why some subclasses are predominant in different systems.

Further evidence for Fc receptor activity in human placental tissue sections has recently been presented by Matre, Tønder & Endresen (1975), using a haemadsorption technique with antibody-sensitized erythrocytes. These authors, however, concluded from a study of hydatiform moles that the reaction occurred with trophoblastic tissue: furthermore, since the reaction could be inhibited by monomeric IgG, it was suggested that such a receptor might function in the selective transfer of maternal IgG across the placenta (Matre *et al.*, 1975). In the present study, we have not been able to detect any binding of native or heat-aggregated human IgG-FITC to trophoblasts or trophoblastic basement membrane (TBM). Similarly, immunofluorescence studies of the distribution of IgG in human placentae have shown considerable staining of villous stroma and vessels, including apical aspects of endothelium, but limited staining of TBM and no positive staining of trophoblasts (McCormick *et al.*, 1971; Faulk & Johnson, 1976).

Our data indicate that Fc receptors on human placental endothelium may function in protection of the foetus from immune complexes rather than in active transport of native IgG. In the human, all four subclasses of maternal IgG are transported into foetal circulation (Wang *et al.*, 1970; Hay, Hull & Torrigiani, 1971), and maternal antibody to incompatible foetal allotypes are probably represented in each subclass. These antibodies first meet their antigens in the placenta at the level of foetal stem vessels, where immune complexes would be expected to be formed. If immune complexes are to be kept from foetal circulation, a mechanism is required to remove them. Such a mechanism must be (a) in the placenta, (b) at the level of foetal stem vessels, (c) specific for the Fc fragment of all four subclasses of human IgG, and (d) express a high affinity for complexed IgG and little or no affinity for native IgG. The receptor described in this report seems to satisfy these criteria.

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