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Immunological studies of human placentae: complement components in pre-eclamptic chorionic villi

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SUMMARY

Forty human placentae were studied by immunofluorescence for the presence of complement (C) components C1q, C4, C3d, C6 and C9 with the use of characterized antisera. The tissues were grouped as control placentae from 20 normal pregnancies and 20 from cases of pre-eclampsia (PE): the PE samples were sub-grouped as being obtained from patients with mild or severe PE. All the C components studied were found in the same distribution for test and control samples, but statistical analysis of each pattern of distribution revealed that the deposition of C1q, C3d and C9 were increased in PE as compared to normal tissues. This impression was strengthened by the finding that the deposition of these C components was further increased when chorionic villus immunopathology was compared between mild and severe PE. These data indicate that immunological mechanisms are operating in PE chorionic villi, and they suggest that, among other mechanisms, immune processes may be operative in the pathophysiology of this clinical disease, and that more studies along these lines are in order to rule either in or out this possibility.

Keywords placenta trophoblast complement placental sink pre-eclampsia

INTRODUCTION

The placenta is of central importance in pre-eclampsia (PE), for this disorder only occurs when trophoblastic tissue is present; removal of which causes regression of the associated clinical and biochemical manifestations. Although the mechanisms involved in normal pregnancy are not fully understood, immune reactions have long been suspected in PE (Redman, 1980). Early reports described lymphocytic infiltration and vascular changes in placental bed biopsies which were similar to those found in rejected kidney grafts, these findings being more marked in PE than normal pregnancy (Robertson, Brosens & Dixon, 1967; Robertson, 1976; Faulk & Fox, 1982). More recent investigations have shown that human placentae in normal pregnancy manifest evidence for immunological interplay between mother and fetus (Faulk & Johnson, 1977; Johnson & Faulk, 1978; McIntyre & Faulk, 1979), and complement components have also been reported to be in characteristic locations in both mature and immature placentae (McCormick *et al.*, 1971; Johnson & Faulk, 1978; Faulk *et al.*, 1980). We now report the results of a study of complement components in pre-eclamptic and normal placentae. The findings show a significant increase in complement deposition in PE.

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MATERIALS AND METHODS

Patients. Placentae from 40 women (aged 17–38 years) were studied. These came from Aberdeen Maternity Hospital (10 patients and 10 controls) and the Medical University of South Carolina (10 patients and 10 controls) in Charleston. The patients from both centres were primigravidae with singleton pregnancy, none had a history of previous abortions, and all were normotensive during the first two trimesters. For both the Aberdeen and Charleston patients, the degree of PE was grouped according to Nelson (1955). Each patient was paired with an age and race matched control from her obstetrical centre. The patients from both centres were further subdivided as mild and severe PE according to the amount of proteinuria, mild PE patients had at least 0·25 g/l and severe PE patients had more than 2 g/l. In both centres a systolic blood pressure below 140 mmHg was regarded as normal, and a diastolic pressure of 90 mmHg was chosen as the lowest compatible with a diagnosis of PE, according to MacGillivray (1961). Four normal and six pre-eclamptic mothers were delivered by caesarean section, the remainder had a vaginal delivery.

Placental Tissues. Placentae were collected in the delivery room and placed in plastic bags on ice until tissue samples measuring 0·5 cm^2 could be obtained from the middle aspect of the central cotyledon. This was done for each placenta by first identifying and dissecting out the central cotyledon; all samples were obtained from this tissue midway between the maternal and fetal surfaces. These were then snap frozen in isopentane/liquid nitrogen and kept at $-20^{\circ}C$ until sectioned (4·5 μm) on a cryostat (Bright Instruments, Huntingdon, UK). The maximum time between delivery and snap freezing was 15 min. Some tissue samples were collected in EDTA according to Faulk *et al.* (1980) to impede complement activation, but this practice was discontinued as there was no detectable difference in the immunohistological pattern of complement distribution when tissue from the same placenta was studied both with and without collection in EDTA. Sections were prepared fresh each day, transferred to microscope slides, air dried and washed at 4°C in phosphate-buffered saline (PBS), pH 7·2, for 20 min to remove blood and other loosely adherent material. Additional controls for passively trapped complement components were done by either (a) maintaining the fresh tissue in culture according to Faulk & Temple, (1976) for 48 h before snap freezing or (b) by exposing cryostat sections to the chaotropic effects of 0·5 M potassium thiocyanate for 10 min at 4°C before addition of antisera (Galbraith, Galbraith & Faulk, 1979). These procedures showed that complement was not non-specifically deposited within chorionic villi (Faulk *et al.*, 1978a).

Immunofluorescence. Tissue sections were reacted with 20 μl of 1:50 to 1:400 dilutions of anti-complement sera for 15 min in a humidified chamber to determine the end point dilution for each antiserum. The working dilution for each antiserum was then selected as one dilution below its end point, because at this concentration non-specific reactions were minimal and specific fluorescence was maximal (Faulk & Johnson, 1977). The slides were then washed twice for 10 min in excess PBS, reacted for 20 min with 20 μl of a 1:60 dilution of sheep anti-rabbit immunoglobulin (Ig) conjugated with fluorescein isothiocyanate (FITC) followed by three, 10 min washes in an excess of PBS and mounted in PBS-buffered 50% glycerol. All preparations were examined by epi-illumination employing a Zeiss Universal Microscope.

Antisera

(a) First antibodies. Rabbit antisera to human C1q, C4 and C3d were obtained from the Central Laboratory of Netherlands Red Cross Blood Transfusion Service, and an anti-C9 serum was obtained from Behringwerke AG, Marburg, Germany. Goat anti-human C6 was obtained from Atlantic Antibodies, Westbrook, Maine, USA. FITC conjugates of sheep anti-rabbit Ig (F:P ratio, 2·7) and rabbit anti-goat Ig (F:P ratio, 2·2) were purchased from Wellcome Laboratories, Beckenham, UK.

(b) Specificity studies. (1) *Immunoprecipitation.* The specificity of all antisera was determined by immunoprecipitation in gels, haemagglutination and immunofluorescence. The antigens sources were normal human serum (NHS), functionally pure human complement components from Cordis Laboratories (Florida, USA) and partially purified C3d (Bokish, Dierich & Muller-Eberhard,

1975). Immunoelectrophoresis (IEP) and double radial immunodiffusion showed that each antiserum recognized its homologous antigen and did not precipitate other components. (2) *Haemagglutination.* Specificity studies employing haemagglutination were performed with the use of sheep erythrocytes (SRBC) sensitized by rabbit IgM anti-SRBC antibodies together with either C4 and C2 (EAC4, 2) or C4 and C3 (EAC4, 3) according to Williams & Chase (1977). The anti-C4 serum agglutinated both EAC preparations, but anti-C3d agglutinated only EAC4, 3 and none of the other antisera reacted with these sensitized cells. (3). *Immunofluorescence.* Specificity studies employing immunofluorescence according to Faulk & Hijmans (1972) revealed that the anti-C sera demonstrated specific immunohistological reactions on cryostat sections of human placentae and that these were abolished following absorption with homologous antigen but not by absorption with other components.

(c) Antibody conjugates. The FITC conjugates were studied by immunoprecipitation in gels before and after absorption with goat and rabbit IgG prepared by ion exchange chromatography according to Faulk & Pondman (1969). To minimize non-specific fluorescence, the FITC conjugates were passed through a solid phase immunoabsorbent column containing insolubilized NHS according to Galbraith *et al.* (1978). Finally, all antisera were ultracentrifuged weekly for 1 h at 100,000*g* to remove Ig aggregates which might bind to Fc receptors in cryostat sections of placental tissues (Johnson & Faulk, 1977).

Data collection and evaluation. Having minimized inter-patient sampling errors as much as possible by obtaining all sections from the same anatomical site, efforts were made to semi-quantitate the amount of trophoblast basement membrane (TBM) reactivity with anti-C sera in all 20 normal and 20 toxaemic placentae. For this, it was assumed that villus size between normal and toxaemic placentae was the same, and 200 consecutive villi were examined by immunofluorescence for TBM reactivity with anti- C3d and anti-C9, any part of TBM fluorescence constituting a positive villus. Two hundred consecutive villi in all test and control tissues were then studied for circumferential reactivity with anti-C3d, and in this case only those villi that reacted throughout the entirety of their TBM were counted as being positive.

Efforts were also made to measure the number of fibrinoid areas that reacted with anti-C sera in the villi of all normal and toxaemic tissues. To do this it was assumed that no more than one fibrinoid area could form in one villus. These areas are easily identified by their location and optical properties, being circular, subsyncytiotrophoblastic and supra-TBM, and by their distinctive property of refracting blue to silver light. The number of fibrinoid areas were counted in 400 consecutive villi, and the percentage of those which reacted with each of the anti-C sera was determined and statistically evaluated for significance.

As stated in the Patients section, 40 placentae were grouped as coming from 20 normal pregnancies and 20 PE pregnancies. The PE group was then further sub-divided as 10 mild and 10 severe cases. The positive areas with each antiserum were thus recorded as being reactive (a) anywhere on TBM, (b) circumferentially on TBM or (c) within fibrinoid areas. From these counts, mean values and standard errors (s.e.) were calculated. Student's *t*-test and *P* values were obtained with the use of a table of Lindley & Miller (1968) for statistical significance.

RESULTS

As no differences were found between the Charleston and Aberdeen groups, results obtained from immunohistological studies done with their placentae are thus presented as pooled data.

C1

A patchy and granular distribution of fluorescence was noted in the larger fetal stem vessels (FSV) of placentae (Fig. 1). The number of positive FSV and the degree of extension through the vessel wall were more marked than that reported by Faulk & Johnson (1977) for normal villi. More stromal cells were positive with anti-C1q in chorionic villi from PE patients than in control placentae from normal pregnancies (Fig. 1b). Quantitative data also showed that anti-C1q serum reacted with more fibrinoid areas in PE than in normal placentae (Table 1).

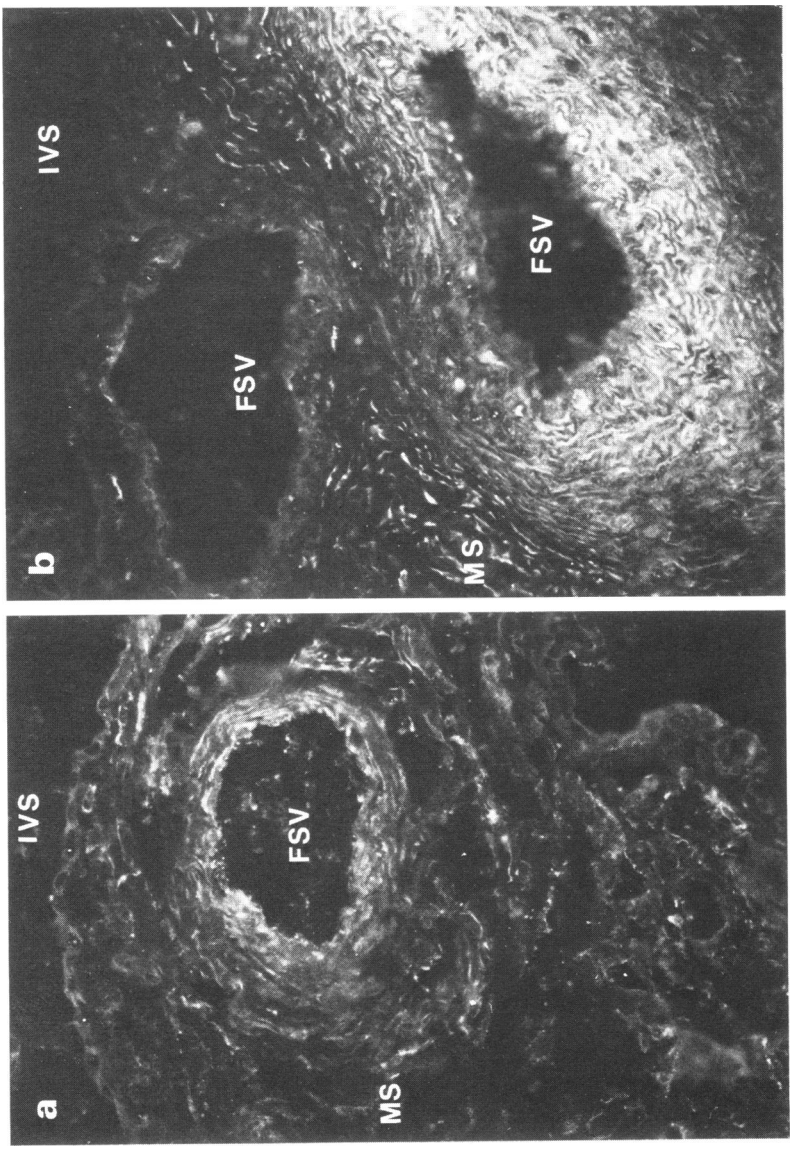

Fig. 1. C1 in walls of fetal stem vessels. Typical distribution of C1q within chorionic villi of (a) normal, term and (b) PE placentae. Note positive and negative vessels in PE villus. IVS intervillous space, FSV is fetal stem vessel and MS is mesenchymal stroma. Magnification × 360.

Table 1. Fibrinoid area C1q and C9 in normal and PE placentae

Antiserum reactivity in fibrinoid areas	Number of villi reactive*		Statistical analysis		Number of villi reactive*		Statistical analysis	
	normal† mean (s.e.)	PE† mean (s.e.)	*t*-value	significance at percentage level	mild PE‡ mean (s.e.)	severe PE‡ mean (s.e.)	*t*-value	significance at percentage level
C1q	10 (0·363)	16 (0·417)	10·86	0·1	14·9 (0·567)	17·1 (0·379)	3·23	1·0
C9	20 (0·603)	32 (0·562)	14·56	0·1	30·3 (0·559)	33·7 (0·616)	4·09	0·1

* 400 villi counted in each placenta.
† 20 placentae were studied.
‡ 10 placentae were studied.

C4

The distribution of C4 was mostly in inter- and peri-villus fibrin as described by Faulk *et al.* (1980) for normal placentae, and did not differ significantly in PE placentae. Immunohistological experiments on PE tissues with anti-C4 sera repeatedly failed to show the presence of C4 on TBM or FSV, or within mesenchymal stroma.

C3

In most normal villi the pattern of C3d reactivity was limited to segments of TBM (Fig. 2a), whereas in PE placentae the TBM reactivity was often circumferential (Fig. 2b). Anti-C3d reacted with more

Table 2. TBM, C3 and C9 in normal and PE placentae

Antiserum reactivity on TBM	Number of villi reactive*		Statistical analysis		Number of villi reactive*		Statistical analysis	
	normal† mean (s.e.)	PE† mean (s.e.)	*t* value	significance at percentage level	mild PE‡ mean (s.e.)	severe PE‡ mean (s.e.)	*t* value	significance at percentage level
reactive anywhere on TBM with anti-C3d circumferentially on TBM	128 (0·778)	160 (0·423)	19·73	0·1	156·1 (1·767)	163·9 (1·433)	3·43	1·0
with anti-C3d reactive anywhere on TBM	18 (0·363)	28 (0·562)	14·95	0·1	26·4 (0·452)	29·6 (0·748)	3·66	1·0
with anti-C9	56 (0·562)	80 (1·346)	16·46	0·1	75·4 (1·477)	84·6 (0·872)	5·36	0·1

*200 villi counted in each placenta.
† 20 placentae were studied.
‡ 10 placentae were studied.

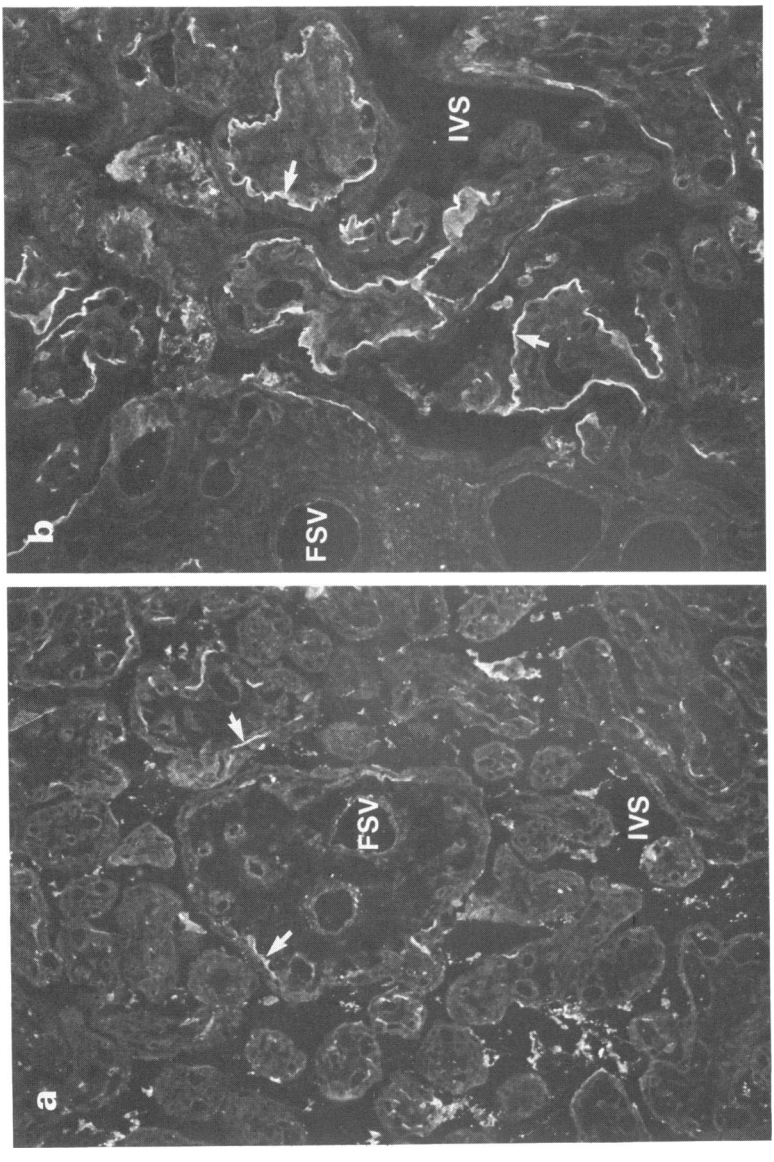

Fig. 2. C3 on trophoblastic basement membranes. Typical distribution (arrows) of C3d on TBM of (a) normal, term and (b) PE placentae. Note circumferential pattern of reactivity in some PE chorionic villi. Abbreviation as in Fig. 1. Magnification × 110.

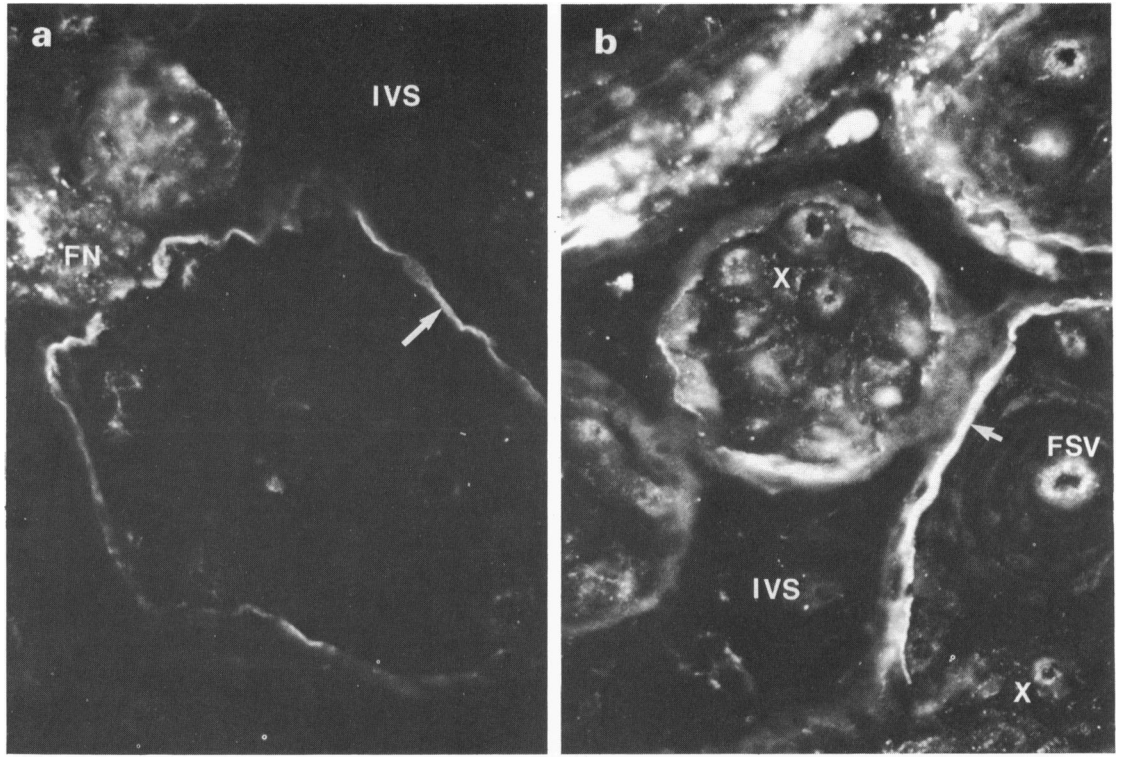

Fig. 3. C9 in chorionic villi. Distribution of C9 on TBM (arrows) of placentae from pregnancies complicated by (a) mild and (b) severe PE. Note number of positive cells in area (X) of mesenchymal stroma, and circumferention deposition of C9 on TBM in chorionic villus of mild PE (arrows) and increased thickening of C9 deposition on TBM in severe PE (arrows). FN is area of fibrinoid necrosis, containing a granular deposition of C9. Magnification × 360.

TBM segments in PE than in normal placentae (Table 2), and such TBM positivity was more marked in severe PE when compared with the group having mild PE (Table 2). In addition to a circumferential pattern of distribution, the TBM reactivity in severe PE often appeared to be coarse, laminated and occasionally extended into the underlying stroma. The circumferential distribution of TBM positivity in PE chorionic villi was yet again increased in severe PE when compared with the mild group (Table 2). There was also a higher percentage of C3d positive fibrinoid areas in PE placentae, and granular clusters of C3d were noticed within cells in the mesenchymal stroma, an uncommon observation in normal chorionic villi.

C6

The antiserum to C6 reacted in an intervillous fibrin (IVF) pattern similar to that obtained with anti-C4. Anti-C6 reactivity was however also present as small granular clusters within most endothelial cells of fetal stem vessels (Faulk *et al.*, 1978a), supporting the suggestion of Faulk *et al.* (1980) that this component is either synthesized or stored in vascular endothelium. More areas of IVF were reactive with anti-C6 in PE than in normal placentae (data not shown).

C9

The distribution of C9 appeared as a summation of C1 and C3 inasmuch as it included TBM, walls of FSV and occasional cells within mesenchymal stroma of chorionic villi (Fig. 3). Compared to normal placentae (Fig. 3a), the PE chorionic villi contained more C9 on TBM (Fig. 3b & Table 2) as well as more C9 within fibrinoid areas (Table 1). These reactivities were also more marked in tissues obtained from severe than from mild PE (Tables 1 & 2).

DISCUSSION

The results of this study show that certain components of the complement system can be reproducibly identified within characteristic locations in normal placentae, and reveal that their patterns of distribution are amplified in PE placentae. The validity of these observations is supported by control experiments with both the tissues and the antisera to minimize non-specific fluorescence and artefactual reactions. Inasmuch as C components are thought to be activated by underlying immune reactions, the findings in this report suggest a heightened level of immunological interplay between mother and fetus in PE as compared to normal pregnancy; however, neither the cause nor the effect of these reactions is known.

Considerations of the possible causes of immunopathology in PE must take into account the immunological phenomena which are known to occur in normal pregnancy, as one of the most consistent observations in this investigation was the increased deposition of C components in their normal patterns of distribution. For example, C1q was identified in more vessel walls and TBM, C3 and C9 extended from segmental to circumferential distributions. It is important to consider how C3 might have arrived on TBM, especially in light of the absence of C1q and C4. The identification of C3 not associated with earlier reacting components has been interpreted as evidence for alternative pathway activation (Faulk *et al.*, 1980), but it is also possible that C1 and C4 were insufficiently fixed to TBM and washed away during immunohistological processing. The presence of C3 receptors (Linder, 1981) on FSV endothelium or TBM is another possibility, we were however unable to demonstrate such receptors by using EAC 4, 3 rosettes in another study (Faulk *et al.*, 1980).

One of the most common immune reactions associated with normal pregnancy is the production of maternal antibodies to incompatible allotypes such as IgG heavy and light chain antigens (Nathenson, Schorr & Litwin, 1971; Faulk, van Loghem & Stickler, 1974) which are inherited by the fetus from its father, and immunological evidence has been put forward to support the concept that many of these antibodies are transported into placental villi where they bind their antigens and precipitate as immune complexes (Johnson *et al.*, 1977; Jeannet *et al.*, 1977). Chorionic villi with their myriad of Fc receptors thus serve as a physiological 'sink' for immune complexes (Faulk & Johnson, 1980) and as such they can provide a ready source of substrate upon which complement might be fixed. It is however unlikely that this accounts for abundant activation of placental

complement in PE, as contemporary information indicates that PE is more common in patients who manifest allotypic compatibility rather than incompatibility with their mates (Redman *et al.*, 1978; Scott, Jenkins & Need, 1978), at least as far as classical transplantation antigens are concerned (Faulk & McIntyre, 1981).

It would thus seem that neither the antigens nor the antibodies responsible for C activation within placentae are known, but the present state of knowledge is that a certain amount of C is normally fixed in chorionic villi and that the initiating reactions responsible for this seem to be amplified in PE. In this regard it may be pertinent that O'Sullivan *et al.* (1982) have shown trophoblast membrane antigens in maternal blood during pregnancy, and it has long been known that bits and pieces of trophoblast membranes are normally deported into the maternal lung (Ikle, 1964). Indeed, Jaameri, Koiruniemi & Carpen (1965) have claimed that this flux is increased in PE. If mothers normally respond to trophoblast membranes in an immune fashion which results in immune complex formation and complement activation within placentae, it would not be unreasonable to expect the immune reactions to increase as the antigenic flux increased. In support of this is the ever growing list of potential trophoblast antigens (Faulk *et al.*, 1978b; Faulk & Hsi, 1983), claims of maternal immunity to trophoblast antigens (Gaugas & Curzen, 1979; Faulk & McIntyre, 1983) and reports of circulating immune complexes in normal pregnancy which increase in PE (Masson, Delive & Cambiasco, 1977; Stirrat, Redman & Levinsky, 1978). When these findings are considered together with the present report of complement components in PE placentae, it seems fair to suggest that immunological mechanisms should probably not yet be deleted from the short list of candidates for the pathophysiological basis of PE.

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