Ovine placental eluate immunoglobulins recognise isologous and third party acid-treated trophoblast microvesicle antigens *in vitro*

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ABSTRACT

Placental microvesicles were prepared from ovine placentae and immunoglobulins eluted with 0.5 M glycine buffer pH 2.5. The ability of eluate immunoglobulins to re-associate with isologous (self) and third party acidified microvesicles was tested by ELISA. Ovine placental immunoglobulins re-associated with isologous and third party acidified microvesicles suggesting that at least 2 types of antigenic epitopes I and II may be expressed on the ovine placentae. Type I antigens may be present on placentae of all ovines while type II epitopes may be paternally derived, hence unique to each pregnancy. Analysis by SDS PAGE revealed the heavy and light chains of IgG at 57 and 27 kDa, respectively, together giving a relative molecular weight of 158 kDa. Results suggest that immunoglobulins produced to placental microvesicle antigens may be directed to some but not all antigenic epitopes expressed on the trophoblast, possibly defining a mechanism by which the foetus evades maternal immunological rejection.

Key words: immunoglobulins, microvesicles, ovine placenta, pregnancy.

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INTRODUCTION

There is evidence that administration of IgG raised to paternal strain antigens reduces the incidence of foetal resorption in mouse abortion models[1], suggesting a crucial role of paternally derived antigens in the immunology of pregnancy. Human placental eluate immunoglobulins are capable of re-associating with isologous and third party acidified placental microvesicles following acid elution of antibodies[5]. The authors observed that acidified microvesicles re-associated more strongly with isologous than third party eluate immunoglobulins, suggesting that placental antigens are polymorphic and that the paternally derived antigens may provoke stronger antibody responses to feto-placental unit during pregnancy. We investigated re-association of ovine placental eluate immunoglobulins with acidified placental microvesicles in order to determine whether or not placental-bound immunoglobulins and their cognate antigens thereto may be conserved for reproductive purposes in mammals.

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MATERIALS AND METHODS

Experimental animals

Eleven pregnant ewes (Corriedale) were purchased from commercial farms in the Hunter Region of New South Wales, Australia and kept in open grazing yards at the University of Newcastle, Australia. The animals were handled according to provisions of The Animal Handling and Ethics Committee of the University of Newcastle, Australia.

Recovery of placentae and preparation of microvesicles

Ewes were put down at 130 days of gestation (10–15 days before the onset of natural parturition) with 20 mℓ of 300 mg/mℓ pentobarbitone sodium injected into the jugular vein. The abdomen was surgically opened and placenta excised and placed in chilled 0.15 M phosphate buffered saline pH 7.2 (PBS). Approximately 10 g trophoblast containing layer of placental cotyledons was dissected and suspended in 500 mℓ of chilled PBS, homogenised in a kitchen blender, stirred for 30 minutes at 4 °C and centrifuged at 1500 *g* to remove cell debris. The supernatant was centrifuged at 25 000 *g* for 1 hour to pellet microvesicles and the latter re-suspended and washed twice in PBS at 4 °C by centrifugation at 25 000 *g*.

Elution of antibodies from ovine placental microvesicles

Immunoglobulins were eluted from microvesicles as previously described by Jalali *et al.* 1989[5] for human placental antibodies but with minor alterations. Microvesicles were re-suspended in 0.5 M glycine buffer pH 2.5, allowed to stand for 1 hour at 4 °C with occasional stirring and centrifuged at 25 000 *g* for 1 hour. The supernatant was decanted to recover eluate immunoglobulins and titrated immediately to pH 7.2 with 10 mM Tris HCl buffer pH 10. Acidified microvesicles were re-suspended in PBS and titrated to pH 7.2 with 10 mM Tris-HCl buffer pH 10 and kept at –20 °C and subsequently used as the antigens in re-association experiments. Eluate immunoglobulin was dialysed for 20 hours in 5 changes of PBS at 4 °C, concentrated with 25 % polyethylene glycol, PEG (Ajax Chemicals Australia), redialysed against chilled PBS, kept at –20 °C and later used as antibodies in re-association studies.

Concentration of eluate immunoglobulin with ammonium sulphate

Eluate immunoglobulins were precipitated in 50 % saturated ammonium sulphate (v/v). The resulting pellet was washed in 45 % saturated ammonium sulphate solution (v/v), pelleted and subsequently suspended in minimal amount of PBS, dialysed for 20 hours at 4 °C with 5 changes of PBS and kept at –20 °C until use.

Purification of eluate IgG on protein G-Sepharose column

Protein G-Sepharose (Pharmacia) was suspended in chilled PBS and the slurry packed into a 10 mℓ polystyrene column. The column was connected to a peristaltic

pump (Gilson Minipuls 2, Gilson Medical Electronics, France) and washed with 10 volumes of PBS at 4 °C. Approximately 1 mℓ of eluate immunoglobulin was loaded on the column and washed with chilled PBS until all unbound protein was removed from the column as determined by measuring optical densities at λ 280 nm. Protein G-bound IgG was eluted with 0.1 mol/ℓ glycine buffer (pH 2.5) at 12 mℓ per hour and 3 mℓ fractions collected into tubes containing 400 µℓ of 10 mMol/ℓ Tris-HCl buffer, pH 8.0 to neutralise pH of eluates. Eluates were scanned for presence of protein by measuring optical densities at λ 280 nm and for presence of IgG_1 by ELISA. IgG-containing fractions were pooled, concentrated with 25 % polyethylene glycol, dialysed against 5 changes of chilled PBS and analysed for purity using SDS Polyacrylamide gel electrophoresis.

Detection of IgG_1 in protein G-Sepharose eluates

Presence of IgG_1 in protein G-Sepharose eluates was detected by ELISA[2]. Ninety-six-well flat-bottom plates (Linbro; Flow Laboratores, McLean, VA, USA) were coated with 100 µℓ per well of 1/500 dilution of mouse monoclonal antibodies to sheep IgG_1 (donated by Ken Beh, CSIRO, Sydney, Australia) in PBS and incubated at 37 °C for 1 hour. Plates were washed in running water and blocked for 1 hour with 5 % (w/v) fat-free skimmed milk at room temperature. After washing, 100 µℓ of protein G-Sepharose eluates, and PBS containing Tween-20 to a final concentration of 0.05 %(v/v), PBST (blank) were added to respective wells and incubated for 1 hour at 37 °C. Plates were washed and 100 µℓ of alkaline-phosphatase-conjugated donkey-anti-sheep immunoglobulin (Silenus Laboratories, Australia) diluted 1/2000 in PBST added to all wells and incubated for 1 hour at room temperature. After washing, 100 µℓ of substrate (1 mg/mℓ of 2,4-dinitrophenyl phosphate (PIERCE Laboratories, Rockford, Illinois, USA) in 50 m mol/ℓ diethanolamine buffer pH 9.8) was added to all wells and kept in the dark for 30–60 minutes at room temperature until colour was adequately developed. Reaction was stopped by adding 50 µℓ of 3 mol/ℓ NaOH to all wells and optical densities read at λ 405 nm using a Biorad Model 450 microplate reader.

Analysis of eluate immunoglobulins by SDS PAGE

Electrophoresis on 10–20 % gradient SDS polyacrylamide gels was carried out under reducing conditions on a vertical electrophoresis unit using 50 mol/ℓ Tris-Tricine buffer (TTS) pH 7.5. Samples were diluted 1:1 with Laemmli sample buffer[6] containing 2 % (w/v) SDS, 6 % (v/v) 2-Mercapto-Ethanol, 40 % (w/v) sucrose, 0.02 % (w/v) bromophenol blue in 0.125 mol/ℓ Tris-HCl (pH 6.8) and boiled for 5 minutes before applying 40 µg of protein per well. Electrophoresis was carried out at 50 Volts for the 1st 30 minutes to stack the proteins and thereafter at 75 Volts until the marker dye (bromophenol blue) reached the bottom of the gel. Proteins were fixed for 10 minutes in 10 % (w/v) TCA and stained overnight in 0.2 % (w/v) Coomassie Brilliant blue R (Sigma Chemicals) in 50 % (v/v) methanol, 5 % (v/v) glacial acetic acid. Gels were destained in 4 to 5 changes of 40 % (v/v) methanol, 10 % (v/v) glacial acetic acid.

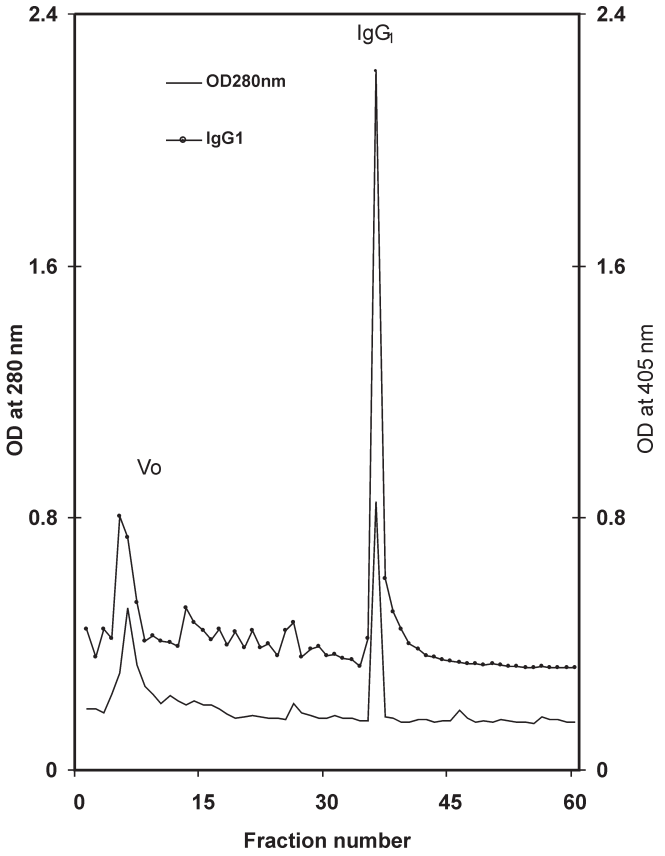

Fig. 1: **Elution profile of ovine placental IgG on protein G-Sepharose. IgG-containing fractions were detected by ELISA using mouse monoclonal antibodies to sheep IgG_1.**

Re-association of ovine placental immunoglobulins with acidified ovine placental microvesicles

Ninety-six-well ELISA plates (Linbro) were coated with 100 µℓ/well of 0.35 mg/ml acidified ovine placental microvesicles (oVa) and incubated for 1 hour at 37 °C.

Plates were washed in running water and blocked with 5 % (w/v) fat free skimmed milk for 1 hour at room temperature, washed and blocked a 2nd time with 3 % (v/v) human serum in PBS. After washing 100 µℓ of 0.21 mg/mℓ eluate immunoglobulin, oVe (experimental), ram serum (positive control), isologous Immunoglobulin (reference) and PBS (blank) were dispensed into respective wells. Plates were incubated for 1 hour at 37 °C, washed, then 100 µℓ of 1/1000 alkaline phosphatase conjugated donkey anti-sheep immunoglobulin (Silenus Laboratories, Australia) dispensed into all wells and incubated for 1 hour at room temperature. After washing, colour was developed by adding 100 µℓ of 1 mg/mℓ 2,4 dinitrophenyl phosphate (Pierce Laboratories, Rockford, Illinois, USA) in 50 mM diethanol amine buffer pH 9.8 into all wells. Plates were kept in the dark until colour was adequately developed and optical densities read at 405 nm in a Model 450 microplate reader (Biorad). The absorbance due to positive control was subtracted from experimental and reference values.

RESULTS

Purification of eluate IgG on protein-G sepharose gave a relatively clean eluate containing IgG_1 (Fig. 1). Analysis of purified IgG by SDS PAGE showed the heavy and light chains of IgG at 57 and 27 kDa, respectively (Fig. 2), giving a relative molecular weight of 158 kDa.

Three patterns of re-association were observed. I: acidified microvesicles oVa32 oVa33 re-associated strongly with both isologous and 9 of 10 (90 %) third party

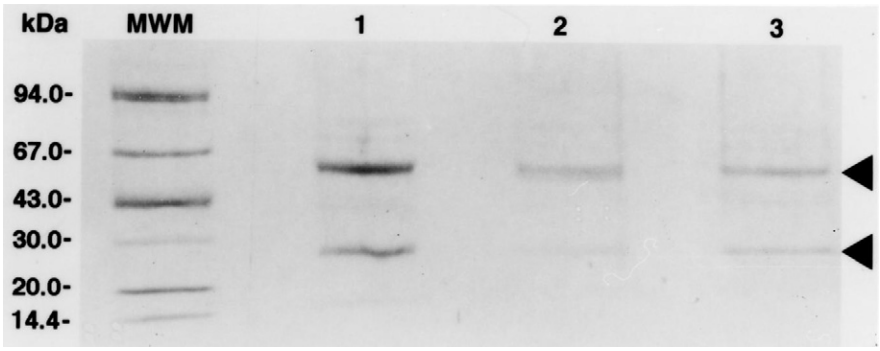

Fig. 2: **Analysis of protein-G purified IgG by SDS PAGE. 40 µg of IgG was loaded in each well and electrophoresis carried out at 50 V for the 1st 30 minutes and thereafter at 75 V until the marker dye (bromophenol blue) reached the bottom of the gel. Proteins were fixed for 10 minutes in 10 % (w/v) TCA and stained overnight in 0.2 % (w/v) Coomassie Brilliant blue R (Sigma Chemicals) in 50 % (v/v) methanol, 5 % (v/v) glacial acetic acid then destained in 4–5 changes of 40 % (v/v) methanol, 10 % (v/v) glacial acetic acid.** MWM**: molecular weight marker.** Lane 1: **placental IgG no. 19.** Lane 2: **placental IgG no. 24.** Lane 3: **placental IgG no. 30.**

immunoglobulins tested (type I re-association); II: acidified microvesicles oVa26 re-associated strongly with isologous but weakly with 9 of 10 (90 %) of the third party eluate immunoglobulins tested (type II re-association). The rest of the microvesicles (oVa19, oVa21, oVa22, oVa24, oVa29) re-associated strongly with some but weakly with other third party immunoglobulins (type III re-association).

Interestingly, while acidified microvesicles oVa32 and oVa33 re-associated strongly with 9 of 10 (90 %) third party eluate immunoglobulins tested, suggesting that these microvesicles were antigenically polymorphic, isologous immunoglobulins oVe32 and oVe33 re-associated weakly with 10 of 10 (100 %) third party acidified microvesicles tested showing immunoglobulins eluted from the same microvesicles had a restricted antigenic specificity. In addition, while oVa26 re-associated weakly with 9 of 10 (90 %) third party immunoglobulins tested, suggesting that these microvesicles were antigenically non polymorphic, isologous eluate immunoglobulin, oVe26 re-associated strongly with 9 of 10 (90 %) of the third party acidified microvesicles tested, showing that immunoglobulins eluted from the same microvesicles had a wider antigenic specificity. These data suggest that antibodies produced to antigenic epitopes expressed on the ovine placenta do not bind to all antigenic epitopes equally but rather to a selection of these epitopes.

DISCUSSION

The data presented in this study suggest that ovine placental IgG has a relative molecular weight of 158 kDa and that at least 2 types of antigenic epitopes may be expressed on the ovine placentae. Type I antigen (examples oVa32, and oVa33) re-associate strongly with isologous and third party eluate immunoglobulins suggesting that they may be expressed on all ovine placentae. Type II antigen (example oVa26) re-associate strongly with isologous but weakly with third party eluate immunoglobulins suggesting that they are unique to each pregnancy and may be paternally derived. Given that some acidified microvesicles re-associated strongly with isologous and third party eluate immunoglobulins, whereas respective isologous immunoglobulins showed restricted re-association with third party acidified microvesicles, we suggest that ovine placental immunoglobulins may be synthesised and directed to a selection but not all epitopes expressed on the placental trophoblast. Alternatively, if all epitopes expressed on the placenta evoked antibody production, then it is likely that only a selection of these antibodies may bind to their respective epitopes on the placenta. This apparent discrepancy in antibody production and interaction with antigen may define a mechanism that protects the foetus from excessive maternal immunological attack. Our observation agrees to some extent with earlier reports that pregnant mothers produce antibodies to some and not to all the mismatched HLA antigens of the foetus as early as 8 weeks of pregnancy[5] and may be further strengthened by observations of Malan-Borel *et al.* 1991[7] that 2 types of IgG are bound to human placenta, symmetrical and asymmetrical. The authors reported that symmetrical IgG molecules are divalent while asymmetrical IgG are glycosylated on 1 of the 2 Fab arms hence behave like monovalent rather than divalent, cannot precipitate antigens or participate in effector functions and may largely function as blocking antibodies.

Although it had been reported elsewhere that placental microvesicle antigens preferentially re-associate with self (isologous) immunoglobulin[5,4], suggesting that paternally derived antigens may provoke stronger antibody response by the mother during pregnancy, our results did not show such restricted re-association pattern. Instead, some acidified microvesicles re-associated more strongly with third party eluate immunoglobulins than with isologous immunoglobulins (Tables 1, 2). This discrepancy between our results and those of Jalali's group[5,4] may reflect a higher divergence in HLA haplotypes in their sample population compared to those used in our study. Cross-reactivity between eluate immunoglobulins and third party acidified

Table 1: **Re-association of ovine placental eluate immunoglobulins** (oVe) **with acidified ovine placental microvesicles** (oVa)**.**

	oVe19	oVe21	oVe22	oVe23	oVe24	oVe26	oVe29	oVe30	oVe31	oVe32	oVe33
oVa19	**1.83**	1.81	1.62	1.68	2.08	1.79	1.47	1.48	2.49	1.88	1.47
oVa21	1.90	**2.08**	1.48	1.81	1.91	1.89	0.81	1.58	2.66	1.81	1.40
oVa22	1.86	1.98	**1.67**	1.89	1.88	1.97	1.47	1.51	2.58	1.73	1.46
oVa23	2.34	1.97	1.81	**1.80**	2.15	2.10	1.04	2.10	2.95	2.04	1.65
oVa24	2.16	1.69	1.75	1.57	**1.76**	1.65	1.52	1.65	2.28	1.60	1.32
oVa26	2.91	1.65	0.45	1.54	0.47	**1.60**	0.45	0.15	2.30	1.66	1.29
oVa29	1.78	1.65	1.54	2.86	1.74	1.83	**1.72**	1.44	2.55	1.73	1.52
oVa30	1.97	1.70	1.61	1.41	1.83	1.46	1.57	**1.6**	2.08	1.4	1.13
oVa31	1.95	2.22	0.53	1.99	1.55	2.06	0.78	1.71	**2.88**	1.88	1.47
oVa32	2.24	2.43	1.87	1.95	2.25	2.29	0.82	1.93	3.09	**2.14**	1.61
oVa33	2.07	2.23	1.69	1.74	2.06	2.06	0.86	1.79	2.79	1.92	**2.77**

Results are presented as optical density readings at λ 405 nm.

Table 2: **Patterns of re-association between ovine placental eluate immunoglobulins** (oVe) **with acidified ovine placental microvesicles** (oVa).

	oVe19	oVe21	oVe22	oVe23	oVe24	oVe26	oVe29	oVe30	oVe31	oVe32	oVe33
oVa19	**100**	–1	+1	–1	+1	+1	–1	–1	–1	–1	–2
oVa21	+1	**100**	–1	+1	+1	+1	–3	+1	–1	–1	–2
oVa22	+1	–1	**100**	+1	+1	+1	–1	–1	–1	–1	–2
oVa23	+1	–1	+1	**100**	+1	+1	–2	+1	+1	–1	–2
oVa24	+1	–1	+1	–1	**100**	+1	–1	+1	–1	–2	–3
oVa26	+2	–1	–3	–1	–3	**100**	–3	–3	–1	–1	–3
oVa29	+1	–1	–1	+2	+1	+1	**100**	–1	–1	–1	–2
oVa30	+1	–1	+1	–1	+1	–1	–1	**100**	–2	–2	–3
oVa31	+1	+1	–3	+1	–1	+1	–3	+1	**100**	–1	–2
oVa32	+1	+1	+1	+1	+1	+1	–3	+1	+1	**100**	–2
oVa33	+1	+1	+1	+1	+1	+1	–2	+1	+1	–1	**100**

Key:
–3: 0–49 % re-association; –2: 50–75 % re-association; –1: 76–95 % re-association.
+1: 96–150 % re-association; +2: 151–200 % re-association; +3: >200 % re-association.
Re-association between acidified microvesicles and isologous eluate immunoglobulins (reference) was taken as 100 % re-association (in bold).
and those due to experimental samples expressed as percentages of the reference.

microvesicles was 16.7 % in humans, 13.6 % in ovines and none between humans and equines[4].

We propose that immunological regulation of pregnancy may involve synthesis of immunoglobulins directed to some but not all antigenic epitopes expressed on the mammalian placental trophoblast and that some of these epitopes may be conserved for reproductive purposes in mammals. This hypothesis may be supported by previous reports that a mouse monoclonal antibody, BA 11 raised to a non polymorphic epitope of an 80 kDa human placental trophoblast antigen (R80K) recognises a similar epitope on the mouse analogue of the human R80K, is capable of inhibiting Natural Killer cell cytotoxicity in both humans and mice and reduces the incidence of foetal resorption in mouse abortion models[3]. Based on these observations by Jalali *et al.*[3], and the data presented in this study that some placental antigens are strongly recognised by both isologous and third party eluate immunoglobulins, it is likely that these antigens may be conserved in mammalian species for reproductive purposes.

CONCLUSION

Immunological regulation of pregnancy may occur at a primitive level involving synthesis of antibodies directed to some but not all epitopes expressed on the placenta. These target epitopes may be conserved in mammals for reproductive purposes and may together with their cognate antibodies be suitable as models for the study of maternal-foetal interactions in pregnancy, and for the development of immunotherapy to immunologically compromised pregnancies in livestock and humans.

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REFERENCES

1. Chaouat G, Menu E, Kinsky R, Brezin C 1990 Immunology mediated abortions: one or several pathways. *Research in Immunology* 141: 188–195
2. Engvall E, Perlmann P 1971 Enzyme-linked immunosorbent assay (ELISA) quantitative assay of immunoglobulin G. *Immunochemistry* 8: 871–879
3. Jalali G R, Arck P, Surridge S, Markert U, Chaouat G, Clark D A, Underwood J L, Mowbray J F 1996 Immunosuppressive properties of monoclonal antibodies and human polyclonal alloantibodies to the R80K protein of trophoblast. *American Journal of Reproductive Immunology* 36: 129–134
4. Jalali G R, Rezai A, Underwood J L, Mowbray J F, Surridge S H, Allen W R, Mathias S 1995 An 80 kDA syncytiotrophoblast alloantigen bound to maternal alloantibody in term placenta. *American Journal of Reproductive Immunology* 33: 213–220
5. Jalali G R, Underwood J L, Mowbray J F, 1989 IgG on normal human placenta is bound to both antigen and Fc recepters. *Transplantation Proceedings* 81: 572–574
6. Laemmli U K 1970 Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227: 680–685
7. Malan-Borel I, Gentile T, Angelucci J, Pividori J, Guala M C, Binaghi R A, Margni R A 1991 IgG asymmetric molecules with anti-paternal activity isolated from sera and placenta of pregnant human. *Journal of Reproductive Immunology* 20: 129–140
8. Reed E, Beer A E, Hutcherson H, King D W, Sociu-Foca N 1991 The alloantibody response of pregnant woman and its suppression by soluble HLA antigens and anti-idiotypic antibodies. *Journal of Reproductive Immunology* 20: 115–128