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IMMUNOGLOBULIN G BOUND TO OVINE PLACENTA IS ELUTED BY SURGICAL CANNULATION AND ACID PERFUSION *IN SITU*
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## ABSTRACT

***Objective:*** **To elute placental bound immunoglobulin G (IgG) *in situ*.**
***Design:*** **Laboratory based experimentation.**
***Setting:*** **Biological Sciences Department, The University of Newcastle Australia and the Department of Biochemistry, University of Nairobi, Kenya.**
***Subjects:*** **Twelve pregnant ewes 10 to 15 days before the onset of natural parturition.**
***Results:*** **Placental eluates were rich in $IgG_1$ and $IgG_2$. The relative molecular weight of placental IgG was estimated at 158kDa by gel filtration chromatography. Analysis of eluate by SDS PAGE revealed the heavy and light chains of IgG at 57 and 27kDa respectively together giving a relative molecular weight of 168kDa.**
***Conclusion:*** **Placental bound IgG may be crucial in immunology of pregnancy and together with the cognate antigen thereof may be useful as models for the study of maternal-fetal interaction in human pregnancy and in the development of experimental immunotherapy to immunologically compromised pregnancies in humans and livestock.**

## INTRODUCTION

Placental bound immunoglobulins have been reported in mice, humans and sheep and have been shown to comprise mainly of IgG although small amounts of IgM and IGA have been detected (1-3). The IgG rich fractions derived from full term placentae are able to neutralise catalytic activities of reverse transcriptases from several retroviruses(2) and have been characterised as blocking antibodies capable of inhibiting blastogenic reactions of maternal Iymphocytes directed against MHC determinants in humans (4). These immunoglobulins have also been shown to inhibit spontaneous IgE synthesis by Iymphocytes from healthy and atopic subjects *in vitro* (5) suggesting that they may be involved in the down-regulation of maternal immune responses to feto-placental antigens. That these antibodies may be protective to feto-placental antigens is supported by the evidence that immunisation with IgG raised to paternal strain antigens reduces the incidence of fetal resorption in mouse abortion models (6). We have described an *in situ* procedure for elution of immunoglobulins bound to ovine placenta, and partially characterised eluate immunoglobulins for class and IgG sub-class composition.

## MATERIALS AND METHODS

*Elution of antibodies in situ:* 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. Ewes were put down at 130 days of gestation (10-15 days before the onset of natural parturition) with 20ml of 300mg/ml pentobarbitone sodium injected into the jugular vein. Uterine artery and vein were cannulated and a ligature tied around the cervix proximal to the cannulated site to prevent flow of eluates into systemic circulation. The tubing from the cannulated artery was connected to a peristaltic pump (Minipuls 2, Gilson Electronics, France) to aid in washing the uterine vascular system with buffers. At first, 0.15M PBS pH 7.2 with 10U/ml heparin was perfused through the uterus until the eluate became clear and no protein could be detected by measuring optical density at $\lambda$ 280nm. Immunoglobulins bound to placenta were thereafter eluted by perfusing the uterus with 0.5M glycine-HCI buffer (pH 2.5) until the pH of eluate stabilized at 2.55 for 5 minutes and no further proteins could be detected in the eluate 50 ml fractions were collected and their pH determined using a portable pH meter. Each fraction was titrated to pH 7.2 with 3M Tris-HCI buffer (pH 10) and centrifuged at 1000g for 10 minutes to clarify the eluate. Fractions were concentrated by vacuum dialysis, tested for IgG by radial immunodiffusion (7) and two dimensional immunodiffusion and thereafter for presence of $IgG_1$ and $IgG_2$

by ELISA (8) using mouse monoclonal antibodies to sheep IgG. All fractions were pooled, concentrated with 50% saturated ammonium sulphate and purified by gel filtration chromatography using Sephacryl S-300 and eluates tested for presence of $IgG_1$ and $IgG_2$ by ELISA. The IgG containing fractions were pooled, concentrated with 25% polyethylene glycol, PEG (Ajax Chemicals, Australia), dialysed in five changes of chilled PBS and analysed for polypeptides by polyacrylamide gel electrophoresis.

*Detection of IgG in the eluates by immunodiffusion:* The presence and relative concentrations of immunogloblin G in the crude eluates was tested by single radial immunodiffussion using donkey anti-sheep gamma globulin (Silenus Laboratories, Australia). Presence of IgG in the eluates was further confirmed by two dimensional immunodiffusion and immuno-electrophoresis. Subsequently, all fractions were pooled and concentrated using ammonium sulphate precipitation method.

*Concentration of immunoglobulins with ammonium sulphate:* Placental eluates were pooled and precipitated in 50% saturated ammonium sulphate (v/v) and pelletted by centrifugation at 1,500g for 30 minutes. The pellet was washed in 45% saturated ammonium sulphate solution (v/v), pelleted by centrifugation at 1,500g for 30 minutes, re-suspended in minimal amount of PBS, then dialysed for 20 hours at 4°C with five changes of excess PBS and purified by gel filtration chromatography using Sephacryl S-300 matrix.

*Purification of eluates on Sephacryl S-300 column:* A glass column of internal diameter 2.1cm and 100cm height was used. Sephacryl S-300 (Biorad Laboratories, Sydney Australia) was supplied in 20% ethanol. Ethanol was decanted and the gel re-suspended in excess chilled PBS, washed several times with gentle stirring then re-suspended in PBS to make a slurry. The slurry was gently poured into the column (half filled with PBS) and allowed to pack under gravity until the desired column height was achieved. The column was washed with at least ten times its volume of chilled PBS at a flow rate of 12 mls per hour with the help of a peristaltic pump (Minipuls 2, Gilson Electronics, France).

The void volume was determined by loading 300 µls of 0.5% solution of Blue Dextran 2,000 (Pharmacia) and 3 ml fractions collected at the rate of 12 mls per hour as soon as the coloured solution disappeared into the gel column. Elution volume at the inflection point of the leading edge of Blue Dextran was taken to represent the void volume. The column was calibrated by running molecular weight marker protein standards (Pharmacia), four times and a standard curve constructed. In order to make the calibration independent of the individual experimental conditions and thus more applicable, the concept of the volume fraction available for a solute in the gel was introduced: Thus

$$Kav = \frac{Ve-Vo}{Vt-Vo}$$

Where Ve is the elution volume of the species in question, Vt, the total volume of the column and Vo, the void volume of the gel bed (equivalent to elution volume of Blue Dextran in this case). Between 600 to 800 µls of eluate immunoglobulin was loaded on the column and elution carried out at 4°C using chilled PBS and 3 ml fractions collected at the rate of 12 mls per hour. Eluates were scanned for presence of proteins by measuring optical densities at $\lambda$280 nm using Ultrospec III spectrophotometer (Pharmacia) and individual fractions tested for presence of $IgG_1$ and $IgG_2$ by ELISA using mouse monoclonal antibodies to sheep $IgG_1$ and $IgG_2$. The IgG containing fractions were pooled, concentrated with 25% polyethylene Glycol, PEG (Ajax Chemicals Australia), dialysed for 20 hours against five changes of excess chilled PBS. The resulting eluates were analysed for presence of IgG by polyacrylamide gel electrophoresis.

*Detection of $IgG_I$ and $IgG_2$ in Sephacryl S-300 eluates:* Presence of $IgG_I$ and $IgG_2$ in Sephacryl S-300 eluates was detected by ELISA (8). Ninety six well flat bottom plates (Linbro; Flow Laboratoreis, McLean, VA, USA) were coated with 100µl per well of 1/500 dilution of mouse monoclonal antibodies to sheep $IgG_I$ and $IgG_2$ in PBS and incubated at 37°C for one hour. Plates were washed in running water and blocked for one hour with 5% (w/v) fat-free skim milk at room temperature. After washing, 100 µl of Sephacryl S-300 eluates (experimentals), positive control (ram serum of equivalent protein concentration) and negative control (PBS containing Tween 20 to a final concentration of 0.05% (v/v), PBST) respectively were added to experimental and control wells and incubated for one hour at 37°C. Plates were washed and 100µl of alkaline-phosphatase-conjugated donkey-anti-sheep immunoglobulin diluted 1/2,000 in PBST was added to all wells and incubated for one hour at room temperature. After washing, 100µl of substrate (lmg/ml of 2,4-dinitrophenyl phosphate in 50mMol diethanolamine buffer pH 9.8), was added to all wells and kept in the dark for 30 to 60 minutes at room temperature until colour was adequately developed. Further reaction was stopped by adding 50 µl of 3M NaOH to all wells and optical densities read at $\lambda$405nm using Biorad's Model 450 microplate reader. Absorbance due to positive control was subtracted from experimental values.

*Analysis of eluates by SDS PAGE under non reducing conditions:* IgG rich fractions of the Sephacryl S-300 eluates were pooled, concentrated with 25% polyethylene glycol, PEG (Ajax Chemicals Australia), and analysed for polypeptides by gel electrophoresis on a 6-20% gradient polyacrylamide gels. Samples (2 to 4 mg/ml) were mixed 1:1 (v/v) with sample buffer containing 2% (w/v) SDS, 40% (w/w) sucrose, 0.02% (w/v) bromophenol blue in 0.125M glycine buffer pH 8.2. Approximately 20 to 40µg of sample was applied in each sample well and electrophoresis carried out at 50 Volts for the first 30 minutes to stack the polypeptides. The voltage was increased to 100V and electrophoresis continued until the marker dye (bromophenol blue) reached the bottom of the gel. Proteins were fixed for 10 minutes in 10% (w/v) Trichloroacetic acid (TCA), and stained overnight in 0.02% (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. For purposes of comparison, *in vitro* eluted IgG (methods not described here) was electrophoresed on the same gel.

*Analysis of eluate immunoglobulins by SDS PAGE under reducing conditions:* IgG rich fractions of the Sephacryl S-300 eluates were pooled, concentrated with 25% polyethylene glycol, dialysed against five changes of excess chilled PBS then analysed by SDS PAGE under reducing conditions. Electrophoresis on 10-20% gradient polyacrylamide gels was carried out under reducing conditions on a vertical electrophoresis unit using 50 mMol Tris-Tricine buffer (TTS) pH 7.5. Samples were diluted 1:1 with Laemmli sample buffer (9) containing 2% (w/v) SDS, 6% (v/v) 2-Mercapto-Ethanol, 40% (w/v) sucrose, 0.02% (w/v) bromophenol blue in 0.125M Tris-HCL (pH 6.8) and boiled for 5 minutes before applying

40μg of protein per well. Electrophoresis was carried out at 50Volts for the first 30 minutes to stack the proteins and thereafter at 75Volts 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, 75% (v/v) glacial acetic acid. Gels were destained in 4 to 5 changes of excess 40% (v/v) methanol, 10% (v/v) glacial acetic acid.

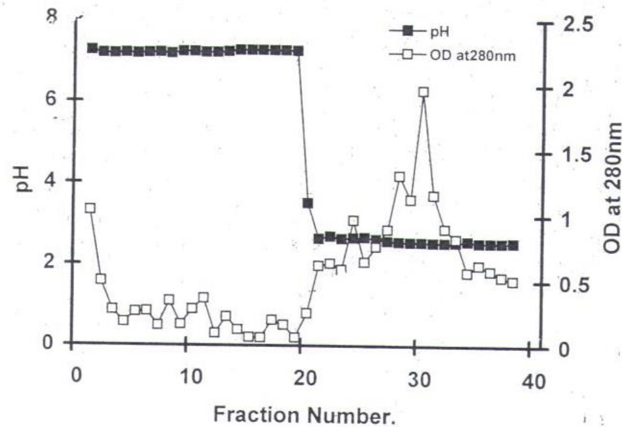
## RESULTS

This study revealed that IgG bound to ovine placenta is easily dissociated by perfusion with 0.5M glycine buffer pH 2.5 (Figure 1) and its presence readily demonstrated in crude eluates by single radial immunodiffusion following vacuum dialysis (Figure 2). Following filtration of eluates on Sephacryl S-300. presence of $IgG_1$ and $IgG_2$ was demonstrated by ELISA using mouse monoclonal antibodies to sheep $IgG_I$ and $IgG_2$.

These antibodies were recovered at the same elution volume as Aldolase (a 158 kDa molecular weight marker protein), suggesting that ovine placental IgG has a relative molecular weight of approximately 158kDa (Figure 3). Electrophoresis of these eluates under non reducing conditions gave a relatively poor resolution of polypeptides.(Figure 4).

**Figure 1**

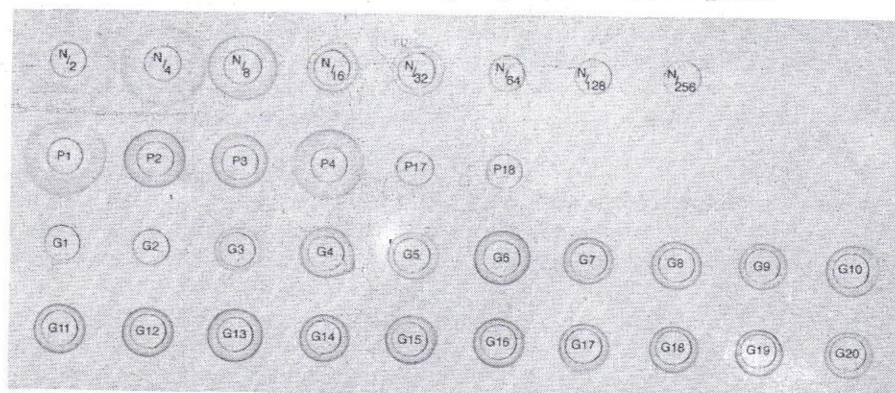
*Elution profile of proteins bound to ovine placenta*




This figure presents elution profile of proteins bound to ovine placenta number 11. The maternal side of the uterus was perfused with PBS until no more protein was detected in the eluates as determined by measuring optical densities at λ 280nm and eighteen 50 ml fractions (1-18) collected. Thereafter, placental bound immunoglobulins were eluted by perfusing the maternal side of the uterus with 0.5 M glycine butter pH 2.5 and eluate (fractions 19-38) collected

**Figure 2**

*Detection of IgG in placental elutes by single radial immunodiffusion*




Each fraction represents 1ml obtained by concentrating each of the 50 ml *in situ* fractions by vacuum dialysis

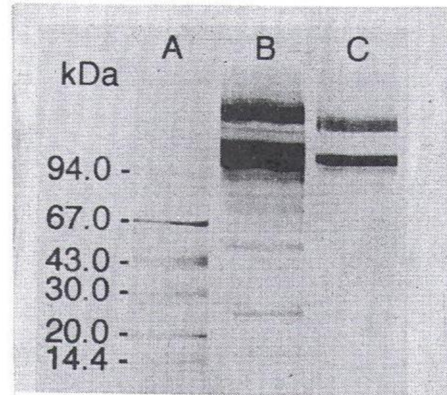
N/2 - $\frac{N}{256}$: $\frac{1}{2}$ to $\frac{1}{256}$ dilution of ram serum

P1 - P18: PBS eluates (fractions 1-18)

G1 - G20: Glycine eluates (fractions 1 to 20)

**Figure 3**

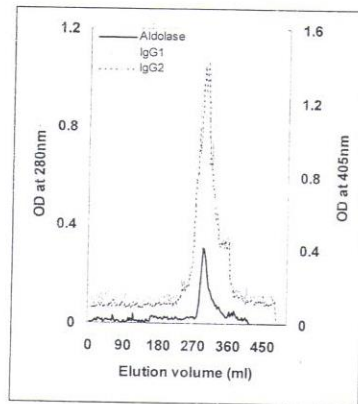
*Analysis of placental eluate immunoglobulin by SDS PAGE under non reducing conditions*




Electrophoreis was carried out on a 6 - 20% gradient polyacrylamide gel under non reducing conditions
Lane A: Molecular weight marker
B: Crude *in situ* eluate immunoglobulin following concentration of 50 ml fraction by vacuum dialysis
C: *In vitro* eluate immuglobulin following concentration with 25% polyethylene glycol (PEG)

**Figure 4**

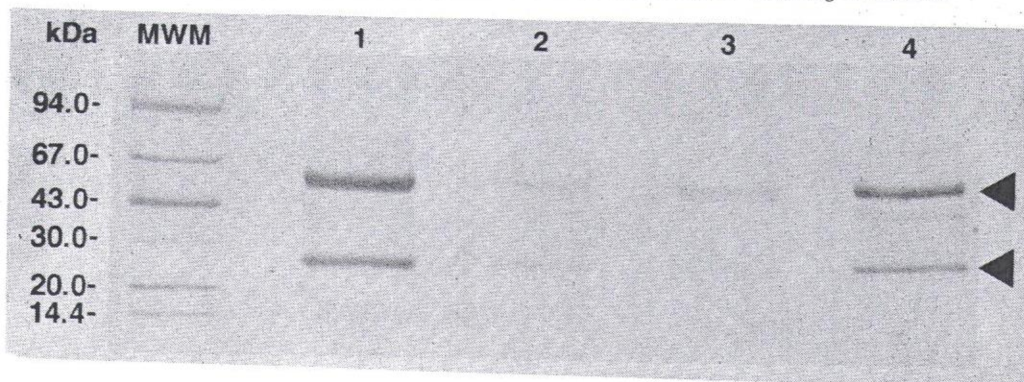
*Elution profile of placental $IgG_1$ and $IgG_2$ on Sephaayl S-300 column*




A glass column of internal diameter 2.1 and column height 100 cm was packed with Sephacryl S-300 to a bed height of 96.6cm. Elution profile of Aldolase was determined by measuring optical densities at $\lambda$280nm while the profiles for $IgG_1$ and $IgG_2$ in the eluates were determined by ELISA using mouse anti sheep $IgG_1$ and $IgG_2$

**Figure 5**

*Analysis of elute immunoglobulin by SDS PAGE under reducing conditions*



However, electrophoresis of the same under reducing conditions showed two distinct polypeptides of approximate molecular weight 57 and 27kDa representing the heavy and light chains of IgG respectively suggesting that ovine placental IgG is approximately 168kDa in molecular weight (Figure 5). This apparent discrepancy of 10kDa in relative molecular weight presents a shift of approximately 6% which falls well within the normal experimental and measurement error margins.

## DISCUSSION

Results of this study have demonstrated that placental bound IgG is readily eluted *in situ* by surgical cannulation and perfusion with 0.5M glycine buffer pH 2.5. The finding in this study that an intact ovine placental IgG is approximately 158kDa (by gel filtration chromatography) and 168kDa (by gel electrophoresis) presents a minor shift of approximately 6% which falls well within the normal experimental and measurement error margins. However, this shift may well have resulted partly from retarded mobility of molecular weight marker proteins relative to placental IgG due to the steep gradient (10-20%) of gel used during electrophoresis. The relative molecular weight of ovine placental IgG as observed in this study suggests some degree of structural similarities with the human placental IgG reported elsewhere (10) to be approximately 160kDa in molecular weight. These estimates are also at variance with previous reports where sheep serum IgG was estimated to have a relative molecular weight of 190kDa by gel filtration on Sephadex G-100 and 192.6kDa by density gradient sedimentation (11). This variation (approximately 20 to 30kDa) suggests a substantial deviation in the overal molecular structures of the ovine placental IgG from the systemic ones and may be a reflection of the differences in their respective functions.

Elsewhere, it has been suggested that $IgG_1$ is the antibody that is predominantly produced in reponse to fetal antigens in pregnant mice (12) and that it may impede the binding of cytotoxic maternal antibodies by competitive exclusion. Whether or not this is the case in ovines is not clear but the demonstration of both of $IgG_1$ and $IgG_2$ sub-classes in these eluates suggest that these two antibody subclasses may be crucial in immunology of pregnancy. We propose that placental IgG may down-regulate maternal immune responses to feto-placental antigens in order to facilitate successful gestation.

In conclusion immunolobulin G may be crucial in immunology of pregnancy and would be a suitable candidate for experimental immunotherapy in the management immunologically compromised pregnancies in humans and livestock.

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