Ovine and Human Placental IgG inhibit Human Natural Killer Cell Cytotoxicity *in vitro*.

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SUMMARY

To determine the effect of ovine and human placental IgG on human Natural Killer (NK) cell cytotoxicity *in vitro* placental IgG was eluted at acidic pH and purified by ion exchange and subsequently by affinity chromatography on protein G and protein A sepharose columns. These antibodies were analysed for presense of IgG by immuno-electrophoresis and relative purity determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE). The effect of these antibodies on human NK cell cytotoxicity was investigated by ^{51}Chromium Release Assay using human K562 cells as targets and human peripheral blood lymphocytes as effector cells. Both ovine and human placental IgG inhibited human NK cell cytotoxicity in a dose dependent manner. Placental IgG may down-regulate the cytotoxic effects of NK cells *in vivo* by competitively excluding the binding of NK cells to their respective targets on the trophoblast. Alternatively, these antibodies may themselves be toxic to NK cells. Either way, the presence of these antibodies on the placental trophoblast may prevent the binding of NK cells and subsequent immunological rejection of the fetal allograft. Also, ovine placental IgG may be functionally similar to its human counterpart and may therefore be suitable as a model for the study of maternal fetal interaction during pregnancy in humans.

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Introduction

Maternal T cells have been associated with the cytotoxic events that lead to foetal losses in mammals and there is evidence to suggest that immunoglobulins (mainly IgG) purified from umbilical sera are cytotoxic towards maternal T lymphocytes [1]. These antibodies may downregulate maternal immune responses to allow successful gestation (2). Elsewhere, it has been shown that *in vivo* depletion of Natural Killer (NK) cells by anti-asialo GMI antisera reduces the incidence of embryo losses, suggesting that NK cells are essential for the initiation of embryo resorption in mice [3,4]. Also, ***in vivo*** activation of NK cytotoxic function or the adoptive transfer of ***in vitro*** activated NK or lymphokine-activated killer (LAK) cells to abortion resistant gravid mice induces foetal losses in normal matings [2,4]. Furthermore, it has been shown that exogenous Tumour necrosis factor-alpha (TNF-α) can induce resorption and that the treatment of gravid CBA females with inhibitors of expression and functions can prevent embryonic losses [3,5]. Given that both macrophages and NK cells can produce TNF-α [5] and that NK cells can produce

interferon gamma or macrophage activation factor which is important for priming macrophages for TNF-α production [6], the involvement of NK cells in failed pregnancies cannot be ruled out. Also, the observation that trophoblast cells express TNF receptors and that exogenous TNF causes both a direct placental and embryonic cytopathic effects [7-10], suggest that NK cell cytotoxicity may be linked with the direct lysis of trophoblast cells accompanying the cytotoxic events such as haemorrhage, thrombosis, and necrosis that are usually associated with TNF-α in resorbing CBA female X DBA male embryos.

Although several studies have been carried out to address embryonic losses in mammals, the role of NK cells and their potent *in vivo* inhibitors is not well studied. Thus, identification of potent *in vivo* inhibitors of NK cell cytotoxicity would provide a positive step towards the development of immunotherapy against recurrent spontaneous abortions in humans and other mammals. We have investigated *in vitro* the effects of ovine and human placental IgG on human Natural Killer cell cytotoxicity.

Materials and Methods

Ovine placentae

Ovine placentae were obtained at term from a group of sheep kept in open grazing yards at the University of Newcastle, Australia where their environment was not altered in any way.

Human placentae

Human placentae were obtained from the delivery suite of the John Hunter Hospital, Newcastle, Australia.

Elution of placental antibodies

Placental microvesicles (ovine and human), were prepared from the trophoblast containing layer of placental cotyledons as previously described for the humans [11] and immunoglobulins were eluted with 0.5M glycine buffer pH 2.5. These immunoglobulins were concentrated with 25% polyethylene glycol (PEG) and purified by ion exchange and subsequently by affinity chromatography on Protein G and protein A-Sepharose columns (Pharmacia) respectively for the ovine and human placental IgG.

Purification of ovine placental IgG

Eluate immunoglobulins were partially purified by ion exchange chromatography on Q-Sepharose High Performance column, using the FPLC system. These antibodies were further purified by affinity chromatography on a Protein G-Sepharose column. Protein G-Sepharose was suspended in chilled 0.15 M phosphate buffered saline pH 7.2 (PBS) and the slurry was packed in a 10ml polystyrene column. The column was connected to a peristaltic pump (Gilson Minipuls 2, Gilson Medical Electronics, France) and washed with at least ten volumes of PBS at 4°C. Approximately 1ml of eluate immunoglobulin or ram serum (control) was loaded on the column and washed with excess amount of chilled PBS to remove the unbound proteins. IgG bound to protein G was eluted with 10mM glycine buffer pH 2.5 at the rate of 12mls per hour and 3ml fractions were collected into tubes containing 400μl of 10mM Tris HC1 buffer pH 8.0 to neutralise pH of the eluate. The presence of IgG in the eluate was determined by Enzyme Linked Immunosorbent Assay (ELISA) using rabbit anti sheep IgG (specific for Fc and Fab fragments). Human placental IgG was similarly purified using protein A Sepharose column.

Analysis of eluate immunoglobulin by immuno-electrophoresis

The presense of immunoglobulin G (IgG) in placental eluates was confirmed by immuno-electrophoresis. Samples (5μl) were applied in wells cut out of 2% agarose gels on 2.5 x 7.5 cm glass plates. Samples were electrophoresed at 8.0 volts/sq.cm of the gel using 50mM Tris-Tricine buffer, pH8.0. Following electrophoresis, 15-20μl of rabbit antisera to sheep whole serum was dispensed into troughs made between the sample wells and incubated for 48 hours in a moist chamber. Plates were washed with PBS (0.15M, pH 7.2) for 72 hours and thereafter for 1 hour with distilled water. Plates were overlaid with moist filter paper to avoid gel cracking and dried in a stream of air in a fume hood. Gels were stained with 0.02% w/v Coomassie brilliant blue R (Sigma Chemicals) in 50% v/v methanol and 5%v/v glacial acetic acid. Slides were destained with 50%v/v methanol, 5% v/v glacial acetic acid until the background was clear enough to allow visual assessment of the precipitin arcs.

Analysis of Eluate Immunoglobulin by Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS PAGE)

The relative purity of eluate immunoglobulin was determined by electrophoresis under reducing conditions on a 10-20% gradient SDS polyacrylamide gel on a vertical electrophoresis unit using 50mM Tris-Tricine buffer (TTS) pH 7.5. Samples (4.0mg/ml) were diluted 1:1 with Laemmli [13] sample buffer containing 2%w/v SDS, 6%v/v 2-mercaptoethanol, 40%w/v sucrose, 0.02%w/v bromophenol blue in 0.125M Tris-HCl buffer pH 6.8 and were boiled for 5 minutes before applying 10µl (40µg) of protein per well. Electrophoresis was carried out at 50 volts for the first 30 minutes to stack the peptides. The voltage was increased to 75 and electrophoresis continued until the marker dye (bromophenol blue) reached the bottom of the gel. Proteins were fixed for 10 minutes in 10% trichloroacetic acid (TCA), and stained overnight in 0.2w/v Coomassie brilliant blue R (Sigma Chemicals) in 50%v/v methanol, 5%v/v glacial acetic acid. Gels were destained in 3 to 4 changes of excess 40%v/v methanol, 10%v/v glacial acetic acid to obtain a clear background.

Preparation of effector cells

Peripheral blood was collected from adult humans aged between 25-32 and lymphocytes prepared by the Ficoll Paque technique. These cells were resuspended at a concentration of 1×10^7 cells/ml in PBS pH 7.2 containing 20µg of either placental IgG or ovine serum IgG (control) and incubated for 2 hours at 37°C in a 5% CO_2/ 95% air incubator.

Labelling of target cells.

Target cells (K562, a human erythroleukemic cell line) at a concentration of 2×10^6 cells/ml of Dulbeco's Modified Eagles Medium (DMEM), was incubated for 1 hour at 37°C in a 5% CO_2/95% air incubator in presence of ^{51}Chromium (1mCi/ml). Cells were washed three times with DMEM by centrifugation at 100g for 4 minutes and re-suspended in DMEM supplemented with 10% heat inactivated foetal calf serum to a final concentration of 2×10^5 cells/ml.

Cytotoxicity assay

Natural Killer cell activity was determined in a ^{51}Cr release microcytotoxicity assay [14]. The effector and target cells were diluted to obtain an effector: target ratio of 40:1 as determined in preliminary assays. 100µl each of the effector and target cells (K562) at the desired concentration were dispensed into 5ml tubes and incubated for 4 hours at 37°C, 5%CO_2, 95% air incubator. In order to determine total (maximum) and background (spontaneous) release of ^{51}Cr, 100µl each of 0.4N HCl and DMEM respectively were used in place of effector cells. After incubation, 100µl of the incubation mixture was pipetted from each tube and the amount of ^{51}Cr released determined in a gamma counter. The inhibition due to Protein A purified human placental IgG was carried out as described above for the ovine placental IgG. All experiments were performed in triplicate.

Percent specific lysis was calculated using the formula below.

% Specific lysis=

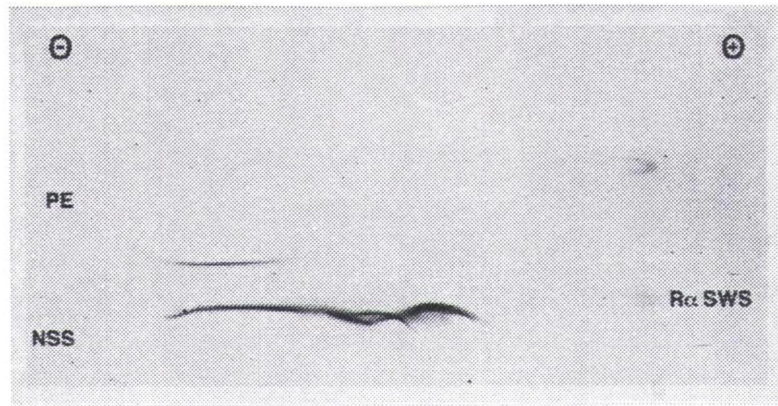
$$\frac{\text{Mean Exptl release - Spontaneous release}}{\text{Mean total release - Spontaneous release}} X100$$

% Inhibition was calculated as (100 - % specific lysis).

Results

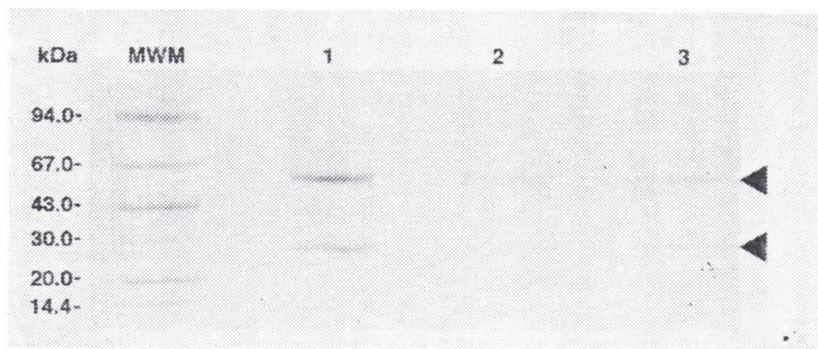
Ovine placental IgG was detected in the eluates by immuno-electrophoresis (Figure 1). Analysis of eluates by SDS PAGE showed the heavy and light chains of placental IgG at 57 and 27 kDa respectively giving a total molecular weight of 158kDa (Figure 2). Using the standard ^{51}Chromium release assay with human K562 cells as target cells and human peripheral blood leucocytes as effector cells, both ovine and human placental IgG inhibited human NK cell cytotoxicity in a dose dependent manner (Figures 3 and 4 and 5).

Figure1: Analysis of ovine placental IgG by immuno-electrophoresis.



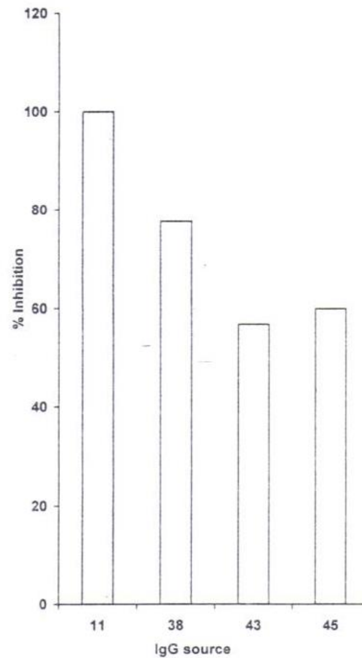
5μl samples were electrophoresed at 8.0 volts/sq.cm of the gel using 50mM Tris-Tricine buffer, pH 8.0. and reacted with Rabbit antisera to sheep whole serum. Gels were stained with 0.02%w/v Coomassie brilliant blue R (Sigma Chemicals) in 50%v/v methanol and 5%v/v glacial acetic acid and destained with 50%v/v methanol, 5%v/v glacial acetic acid. PE: Placental eluate IgG; NSS: Normal sheep serum R x SWS: Rabbit anti sheep whole serum

Figure 2: Analysis of ovine placental IgG by SDS-PAGE.



Eluate immunoglobulin was separated by SDS-PAGE on 10-20% gradient gels under reducing conditions. **KDa**: kiloDalton; **MWM**: Molecular weight markers. **Lanes 1, 2, and 3:** Eluate immunoglobulin from sheep placentae 1, 2, and 3 respectively.

Figure 3: Inhibition of human Natural Killer cell cytotoxicity by Protein G purified ovine placental IgG.

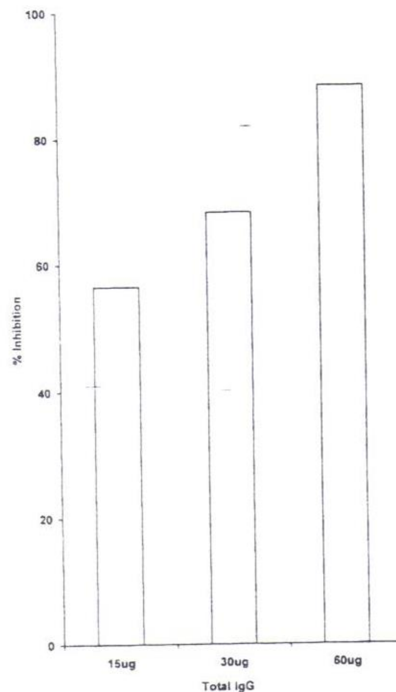


IgG was obtained from four different placentae (numbers 11, 38, 43 and 45) and used at a concentration of 30μg per assay. The cytotoxicity assay was performed using the standard ^{51}Chromium release assay using human peripheral blood lymphocytes as effector cells and K562 cells as the targets. Inhibition due to protein G purified ram serum IgG was subtracted from all experimental values. Each test was done in triplicate.

Discussion

We have reported for the first time that ovine placental IgG inhibits human NK cell cytotoxicity *in vitro* and that similar effects are shown by human placental IgG. Based on this observation, we propose that the binding of placental IgG to antigens expressed on the trophoblast may competitively exclude the binding of NK cells to the same target molecules on the trophoblast. Alternatively, these antibodies may be themselves toxic to NK cells. Either way, the presence of these antibodies on the trophoblast may downregulate the cytotoxic effects of NK cells *in vivo* and may prevent immunological rejection of the foetal allograft by an immunologically competent mother. This observation also suggests that placental IgG, their trophoblast specific antigens, and NK targets may be conserved in several mammalian species for reproductive purposes. This thesis may be re-enforced by the observation elsewhere that a monoclonal antibody, BA 11 raised to a non polymorphic epitope of an 80kDa human trophoblast specific antigen R80K, recognises a mouse analogue of R80K antigen, inhibits both mouse and human NK cell activity and reduces the incidence of foetal resorption in mouse abortion models [15]. In addition, there is evidence to show that NK targets may be conserved across several animal species. Thus, a monoclonal antibody 5C6 raised against catfish non specific cytotoxic (NCC) cells recognises a vimentine-like molecule and blocks the cytotoxic effects of catfish nonspecific cytotoxic (NCC) cells and the human NK cell killing of the K562 cells [16,18].

Figure 4: **Dose dependent inhibition of human Natural Killer cell cytotoxicity by protein G purified ovine placental IgG.**



IgG was obtained from one ovine placenta (number 45) and used in three different concentrations (15μg, 30μg and 60μg) per assay respectively. The cytotoxicity assay was performed using the standard ^{51}Chromium release assay with human peripheral blood lymphocytes as effector cells and K562 cells as the targets. Inhibition due to protein G purified ram serum IgG was subtracted from all experimental values. Each test was done in triplicate.

Conclusion

We propose that mammalian placental IgG may block rejection of foetal allograft by competitively excluding or sterically hindering the binding of NK cells to the trophoblast or by way of being toxic to NK cells. In addition, we suggest that ovine placental IgG may be functionally similar to its human counterpart and that it may be useful as a model for the study of maternal foetal interactions in human pregnancy.

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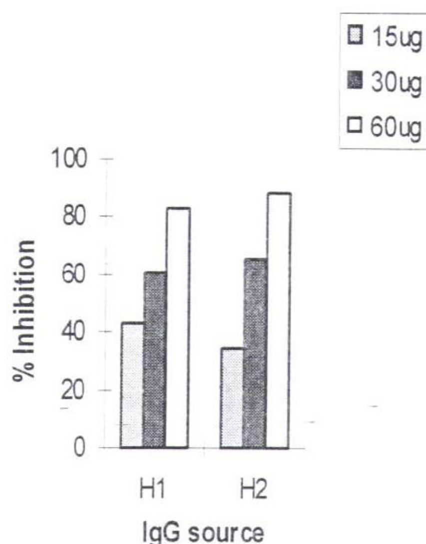
Figure 5: Inhibition of human Natural Killer cell cytotoxicity by human placental IgG.



30µg of IgG was used in each assay. The cytotoxicity assay was performed using the standard [51]Chromium release assay with human peripheral blood lymphocytes as effector cells and K562 cell lines as the targets. Inhibition due to protein A purified human serum IgG was subtracted from the experimental value. Each test was done in triplicate. H1 and H2:, Human placentae 1 and 2

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