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Immunoglobulin isotype isolated from human placental extract does not interfere in complement-mediated bacterial opsonization within the wound milieu

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ABSTRACT

The wound healing potency of an aqueous extract of placenta can be evaluated through the presence of numerous regulatory components. The presence of glycans was detected by thin layer chromatography and fluorophore-assisted carbohydrate electrophoresis. Mass spectrometric analysis revealed the existence of multiple fragments of immunoglobulin G (IgG). IgG was present in the extract at a concentration of 25.2 ± 3.97 μg/ml. IgG possesses anti-complementary activity by diverting the complement activation from target surface. Thus, effect of placental IgG on complement–bacteria interaction was investigated through classical and alternative pathway and the preparation was ascertained to be safe with respect to their interference in the process of bacterial opsonization.

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1. Introduction

Immunoregulatory effects of human placental extract along with wound healing ability, hormonal regulation, prevention of recurring respiratory infections, asthmatic bronchitis, etc. are clinically well established [1–7]. Consistent maintenance of fetal-maternal nutrient exchange through placenta endures the organ to have remarkable therapeutic potential. This is exhibited through multiple independent mechanisms in order to exhibit the cumulative effect. An aqueous extract of human placenta, the preparation of which has been elaborated in the text, plays an imperative role in various stages of healing. Studies have demonstrated its role in modulation of cytokine induction during different phases of healing, effective stimulation of collagen synthesis during regeneration and epithelialisation and placental growth factor (PIGF) as an accelerator of granulation tissue maturation [8–10]. Characterization of this placental extract lead to the identification of numerous biological regulators such as growth factors, receptors, glycosaminoglycans and PDRNs [11–19]. These findings indicate that placental extract plays a definitive role in wound repair through multiple regulatory mechanisms.

The role played by complex carbohydrates in wound repair is an active topic of investigation since the carbohydrate moieties of glycoconjugates are often important as recognition determinants in cellular interactions, modulation of immunogenicity etc. [20,21]. A previous study showed that in a *Pax6*$^{+/-}$ mouse model of Aniridia-related keratopathy, mutations in glycoconjugate composition of the cell surface might lead to an abnormal cellular migration phenotype [22]. This could cause impaired re-epithelialisation which in turn could elevate risk of infection, inflammation and undermine normal stromal remodeling. Another study suggested that cell surface proteins which are N-glycosylated with terminal fucosylation mediated wound closure of airway epithelial monolayer by promoting cellular adhesion and migration [23]. These observations have important implications in understanding the critical role played by the glycans moieties in augmenting wound repair.

Injury to the skin is equivalent to an immunological crisis since the sub-epidermal exposed tissue is vulnerable to bacterial colonization [24–28]. The innate immune system, which is composed of phagocytes and the complement system, provides the primary line of defense against invading pathogens [29]. The complement system which is composed of more than 50 plasma and membrane

Abbreviations: IgG, immunoglobulin G; ATP, adenosine triphosphate; NAD$^+$, nicotinamide adenine dinucleotide; CNBr, cyanogens bromide; PNGase F, peptide N-glycosidase F; BHI, Brain–Heart Infusion; ANTS, 8-aminonaphthalene-1,3,6-trisulfonate; BCIP, 5-Bromo 4-Chloro 3′ indolylphosphate; NBT, nitro-blue tetrazolium chloride; G6PDH, glucose-6-phosphate dehydrogenase; BSA, bovine serum albumin; EDTA, ethylenediamine tetra acetic acid; EGTA, ethylene glycol tetra acetic acid

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associated proteins, is a key mediator of inflammatory and immune responses [30–32]. It generates a flux of active signaling molecules that includes C1q, C3, C4 and C5, which help to orchestrate the recruitment of phagocytes from blood. The complement activation proceeds primarily through two pathways: classical and alternative. Another pathway involved in the complement activation is the Mannan-Binding Lectin (MBL) pathway. MBL triggers complement similar to C1q. All these pathways are activated by different molecules but in the end, they all converge to generate the same set of effector molecules [33]. The inflammatory response promotes the recruitment of neutrophils which cause the debridement of necrotic and apoptotic cells and eliminate infectious agents from the wound bed. Role of complement in wound healing has been exemplified by the involvement of C3a and C5a in promoting liver regeneration [34]. C1q, another member of the complement cascade, possesses proangiogenic activity along with the increase in tissue strength, inflammation, fibroblast migration and collagen deposition post application of C3 on wounded surface [35–37]. In wound physiology, bacteria compete for the nutrients in the wound milieu and cause significant destruction to the wound matrix [38]. Wound debridement is an effective method to physically remove the dead, devitalized tissue and reduce the bacterial bioburden. On the other hand, the complement system, based on its ability to interact with bacteria, provides an element of natural immunity. In order to evaluate the effect of promoter/inhibitor molecules on the complement mediated opsonization of bacteria, *pseudomonas aeruginosa* was used as the chosen strain because of its common availability in infected wound matrix. It is an opportunistic gram negative pathogen which causes serious infections in patients with severe burns, wounds injuries [39,40].

This study was initiated to study the role of glycoconjugates present in the placental extract in wound healing. Multiple chromatographic and biophysical evidences indicated the presence of IgG as a major constituent of glycoprotein content in human placental extract. IgG is known to be a preferential acceptor of activated C3 and might interfere in the process of opsonization of bacteria inside the wound milieu [41,42]. Thus, effect of placental IgG on complement-bacteria interaction was investigated, through classical and alternate pathways, to elucidate its role in complement regulation.

2. Experimental procedures

2.1. Reagents

Fine chemicals were obtained as follows: Glucose, ammonium molybdate, sodium dihydrogen arsenate, sodium potassium tartrate, ATP, NAD^+, hexokinase, G6PDH, copper sulphate, diphenylamine, aniline, ovalbumin, Concanavalin A, CNBr activated Sepharose 4B, methyl-α-D-mannopyranoside, PNGase F from *Elizabethkingia miricola*, sodium cyanoborohydride, reference human IgG, subtilisin, protein-A conjugated alkaline phosphatase, protein-A agarose, BSA, protein A-alkaline phosphatase, p-nitrophenyl phosphate, EDTA, EGTA from Sigma, USA; *P. aeruginosa* (ATCC 51679), BHI, phosphoric acid, agar from Himedia; C_{18} zip-tip, silica gel TLC plates from Merck, Germany; periodic acid, acidic fuschin sulfate, sodium metabisulfite from Pierce, USA; ANTS from Invitrogen; nitrocellulose membrane, BCIP, NBT from Promega; veronal buffer saline from Lonza. Other reagents were of analytical grade and purchased locally.

2.2. Placental extract

The drug house M/s Albert David Ltd., Kolkata, India supplied aqueous extract of human placenta that is sold as a licensed drug under the trade name 'Placentrex'. Preparation of the extract holding the confidentiality of the manufacturer's proprietary terms has been described [11]. In short, the extract is prepared from fresh term pooled placentae using single hot (90 °C) and cold (6 °C) water extractions followed by sterilization under saturated steam pressure of 15 psi at 120 °C for 40 min. It is routinely tested for HIV antibody and Hepatitis B surface antigen. It contains 1.5% benzyl alcohol (v/v) as preservative which does not interfere with the experiments presented here. Collection and handling of the placenta and manufacturing of the drug were done under the export license of the drug controlling authority of India.

2.3. Analysis of free/bound carbohydrates

2.3.1. Nelson-Somogyi method for reducing sugars

To 0.5 ml of glucose solution (0.0125–0.150 mM) or test samples, 0.5 ml of Somogyi's alkaline copper tartrate reagent was added and kept in a boiling water bath for 10 min. The tubes were cooled to room temperature and 0.5 ml of Nelson's arsenomolybdic reagent was added. The tubes were incubated at 25 °C for 10 min and the $A_{620\ nm}$ was read. Somogyi's and Nelson's reagents were prepared after [43]. Colorimetric estimation of molybdenum blue at 620 nm generated a standard curve of glucose showing linear dependence between absorbance and concentration (R^2 = 0.996, where R^2 is the regression coefficient).

2.3.2. Coupled enzyme assay for glucose

The test sample was added to 0.1 M Tris–HCl, pH 7.5 containing 2.1 mM $MgCl_2$, 1 mM ATP and 1 unit/ml of hexokinase to a final volume of 1 ml and incubated at 25 °C for 30 min. Then 1.5 mM NAD^+ and 1 unit/ml of glucose-6-phosphate dehydrogenase was added and again incubated for 30 min followed by measuring NADH production at 340 nm [44].

2.3.3. Detection of glycoconjugates by TLC

Glucose and galactose (5 μl, 10 mg/ml) and placental extract (5 μl, 100×) were loaded on a silica gel TLC plate (E Merck, Germany). Chromatograms were developed with the solvent system chloroform: acetic acid: water (12:7.5:1.5). Diphenylamine-aniline-phosphoric acid spray reagent was used in the detection of glycoconjugates after heating at 105 °C for 2 h [45].

2.3.4. Fluorophore assisted carbohydrate electrophoresis (FACE)

The oligosaccharides present in placental extract were analyzed using FACE [46,47]. Firstly, the placental extract was deglycosylated using PNGase F (peptide-N-glycosidase F from *E. miricola*). Briefly, the extract was dissolved in 50 mM Na-phosphate, pH 7.5 containing 0.005 ml of denaturation solution (0.2% SDS with 0.1 M β-mercaptoethanol) and boiled at 100 °C for 10 min. The solution was cooled and 10 μl (50 units/ml) of PNGase F was added to the reaction mixture. The solution was incubated at 37 °C for 3 h. The released oligosaccharides were dried and labeled by adding 5 μl of 0.2 M ANTS in acetic acid/water (3:17, v/v) and 5 μl of 1 M Na-cyanoborohydride in DMSO. The mixture was incubated at 37 °C for 15 h. Oligosaccharides labeled with ANTS were subjected to 32% PAGE. The stacking gel was 8% acrylamide/0.6% bisacrylamide. The running buffer was 0.025 M Tris/ 0.192 M Glycine, pH 8.4 and the electrophoresis was run at a constant current of 15 mA for 2 h.

2.3.5. Quantification of glycoprotein

Glycoprotein content of the extract was estimated by Schiff-periodic acid staining procedure using ovalbumin as reference. Ovalbumin (10–200 μg) and placental extract (50 μl; 100× conc.) were electrophoresed in 15%/20% SDS–PAGE respectively and stained using GelCode® Glycoprotein Staining Kit (Pierce) [19]. ImageJ densitometric analysis was performed to quantify glycoprotein staining. It

generated a linear dependence correlating image area with concentration of ovalbumin (10–200 μg/ml; R^2 = 0.967).

2.4. Purification and identification of glycoproteins in placental extract

2.4.1. Concanavalin A-Sepharose affinity chromatography

Glycoproteins of placental extract were purified by Concanavalin A- Sepharose affinity chromatography [19]. Further, 0.05 M β-mercaptoethanol and 26 N sulfuric acid were added to each fraction and absorbance was measured at 413 nm to determine the presence of glycans.

2.4.2. Reverse phase HPLC

The released glycoproteins/peptides were separated using RP-Nova-Pak C_{18} HPLC column (Waters, 3.9 × 150 mm, 4 μm). The unbound components were washed with water while the bound fractions were eluted by a linear gradient of 0–80% acetonitrile for 60 min. Elution was followed at 220/280 nm.

2.4.3. MALDI ToF MS/MS

The fractions from RP-HPLC were deglycosylated using PNGase F as mentioned above. The reaction mixture was applied to a Concanavalin A Sepharose spin column at 1500 rpm for 5 min to remove the released oligosaccharides. To each sample were added 15 μl of 50 mM Na-bicarbonate and 1.5 μl of 100 mM DTT [48]. The samples were incubated at 60 °C for 1 h. Then 3 μl of 100 mM iodoacetamide was added and incubated in the dark for 30 min. Post alkylation, the peptides were digested using chymotrypsin (2 μg) for 16 h at 37 °C. Digested peptides were desalted using a C_{18} zip-tip (0.2 μl bed volume), eluted with 50% acetonitrile/0.1% trifluoroacetic acid and applied to the MALDI plate along with the matrix α-cyano-4-hydroxycinnamic acid (CHCA) at a ratio of 1:1 (v/v, 0.45 μl each). MS analysis were performed using a 4700 MALDI ToF (Applied Biosystems) operated in reflectron mode. The MS/MS of the most intense chymotryptic peptide mass ion were searched against Swissprot and NCBI database using Mascot (Matrix Science Ltd., London, U.K; http://www.matrixscience.com/) search program with fixed and variable modifications; carbamidomethyl and oxidation respectively.

2.5. Identification and Purification of IgG

2.5.1. Dot-Blot

Reference IgG, placental extract and subtilisin (negative control) were spotted on a nitrocellulose membrane and dried completely and incubated with PBS containing 0.1% Tween 20 and 1% BSA at 4 °C for 15 h. The strip was washed thrice with PBS containing 0.1% Tween 20 for 10 min each. The strip was then incubated with Protein A-conjugated alkaline phosphatase (1:500) for 2 h. Strip was again washed and presence of IgG was confirmed on the basis of color development using BCIP-NBT in 0.1 M Tris–HCl, pH 8.8. Subtilisin is a protein of bacterial origin thus, eliminating the possibility of cross reaction with proteins of mammalian origin.

2.5.2. Protein A-agarose affinity chromatography

A 2 ml Protein A-agarose affinity column was prepared and equilibrated with 0.05 mM Na phosphate, pH 8.0. Placental extract (10 ml; 10× conc.) was loaded in the column and 2 ml fractions

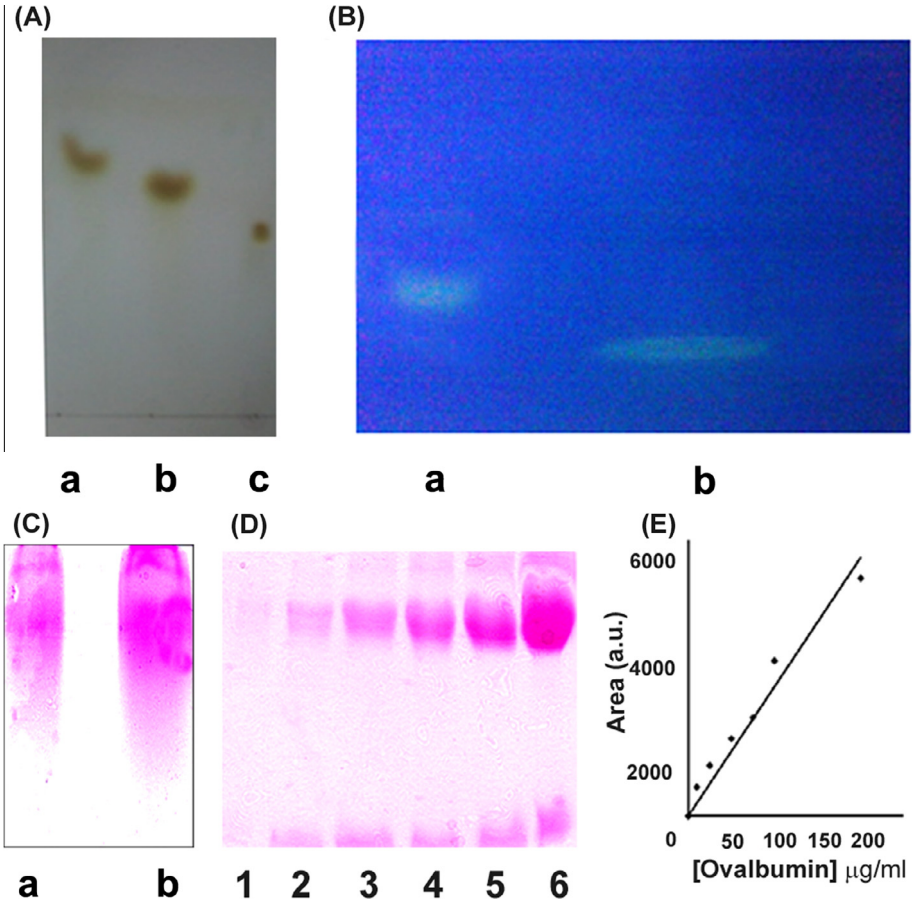


Fig. 1. (A) TLC profile of glycans. Lane a: glucose; Lane b: galactose and Lane c: placental extract. (B) FACE profile. Lane a: glucose; Lane b: placental extract. (C) Schiff-periodic acid stain after 20% SDS–PAGE. Lane a: 25 μl and Lane b: 50 μl of placental extract. (D) Schiff-periodic acid stain after 15% SDS–PAGE of ovalbumin. Lane 1, 10; 2, 25; 3, 50; 4, 75; 5, 100; 6, 200 μg of ovalbumin. (E) Densitometric scan of (D) by Image J software to quantify band intensities in terms of area. A linear correlation between area and amount of protein is demonstrated (R^2 = 0.967).

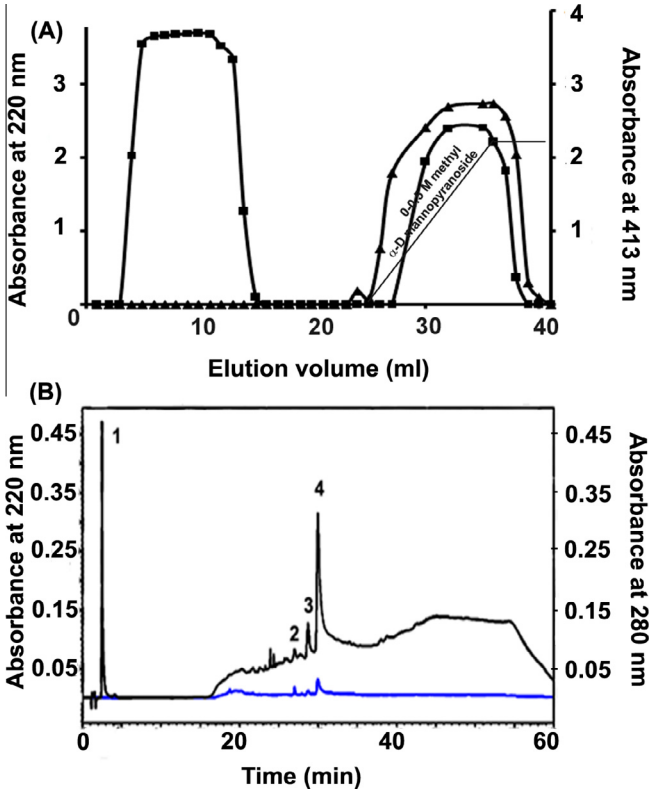


Fig. 2. (A) Separation of glycoproteins using Concanavalin A-Sepharose affinity column. The unbound fraction was eluted using the equilibration buffer followed by application of a linear gradient of 0–0.5 M methyl α-D-mannoside in the same buffer to elute glycoproteins. Elution profile at 220 nm (■) has been shown. Presence of glycans in the fractions was detected at 413 nm by the method of sulfuric acid-β mercaptoethanol (▲). (B) RP-HPLC profile of the pool of glycoproteins obtained from (A). Fractions 1, 2, 3 and 4 were collected for further analysis. Chromatograms were recorded at 220 nm (black) and 280 nm (blue).

were collected. The column was washed with 10 volumes of the same buffer. Bound fractions were eluted using 0.1 M Gly-HCl, pH 2.2. Eluted fractions were followed at 220 nm.

2.5.3. ELISA

Immunoglobulin G in placental extract was quantified using enzyme-linked immunosorbent assays (ELISA) using protein A-alkaline phosphatase conjugated. Briefly, both commercial IgG (0.005–0.1 mg/ml) and placental extract were incubated at 37 °C for 3 h in 96 well microplates. Non-specific binding sites were blocked using 1% BSA in PBS pH 7.5 for 2 h. The plates were incubated with 0.05% protein A alkaline phosphatase for 2 h and the reactions were revealed by incubation with the substrate p-nitrophenyl phosphate disodium salt. To obtain the A_{405} units, plates were read in an ELISA plate reader. Negative controls included uncoated, no IgG wells. For determination of IgG concentrations, absorbance values were plotted against the standard curve obtained for the dilutions of the standard IgG within a linear range wherein a linear curve was generated between absorbance and concentration ($R^2 = 0.960$).

2.6. Analysis of Complement uptake onto bacteria

2.6.1. Complement buffers

Isotonic veronal buffer (VBS) containing 0.5 mM $MgCl_2$, 0.15 mM $CaCl_2$ and 0.1% gelatin in presence (EDTA-GVBS) and absence ($GVBS^{2+}$) of 10 mM EDTA were prepared as described in [49]. Isotonic $GVBS^{2+}$ containing 8 mM EGTA and 5 mM $MgCl_2$ (EGTA-GVBS) for alternative pathway activation assays was prepared after [50].

2.6.2. Bacterial stains

P. aeruginosa (ATCC 51679; SS), a serum-sensitive mucoid strain was obtained from the sputum of a patient with cystic fibrosis, and its serum resistant mucoid derivative, SR, was isolated by passage of SS strain in the presence of increasing concentrations of human serum [51]. Both strains were maintained on BHI agar at 37 °C and transferred daily. Each strain was grown to mid-log phase (5–6 h) in BHI broth at 37 °C with agitation, harvested by centrifugation at 5000 rpm for 10 min at 4 °C, washed twice with VBS and resuspended in appropriate buffer.

2.6.3. Pooled normal human serum (PNHS)

Blood was obtained by venipuncture of 5 healthy volunteers and was allowed to clot at 25 °C for 30 min. After centrifugation at 1000*g* for 15 min at 4 °C, the serum was pooled, filter sterilized through a 0.22-μm-pore-size filter, and stored at −80 °C in small fractions until use. For some experiments, complement in PNHS was inactivated by heating at 56 °C for 30 min. In some assays, serum was pre-adsorbed with bacteria to be tested, to remove pre existing reactive antibodies (1 ml of serum adsorbed 4 times with 10^8 CFU of mid log phase grown bacteria, on ice for 30 min). The volunteers were non-smokers and were not under antibiotic medication. Collection of blood was approved by the institutional human ethics committee.

2.6.4. Complement deposition assay

Mid log phase bacteria were used in all assays. Cells were washed in assay buffer and resuspended to a final concentration of 1×10^8 cells/ml. Different concentrations of placental IgG were added to bacteria before incorporation of a source of complement (20% PNHS). Reference IgG was used as the control in each experiment.

Bacteria were incubated with shaking at 37 °C for 1 h. At the end, reaction volume was diluted with EDTA-GVBS and washed three times with the same buffer. Then, Rb pAb anti-human C3 (1:400) was added and incubated for 60 min at 25 °C. Cells were again washed thrice with EDTA-GVBS and then incubated in goat anti-rabbit IgG (1:2000) for 1 h in dark. The reactions were revealed by incubation with the substrate pNPP. Results were expressed as a percentage of maximal labeled Ab binding to complement components bound to the bacterial surface (human serum alone). Non-specific C3 binding was examined by incubating bacteria with buffer alone.

2.6.5. Erythrocytes as a model for evaluation of anti-complementary activity of placental IgG

Complement binding to sensitized erythrocytes is a well studied system to evaluate the anti-complementary activity of IgG. Human erythrocytes were sensitized with rat anti human red blood cell IgG. Then sensitized erythrocytes were incubated with various concentrations of placental IgG. Deposition of C3 onto erythrocytes was measured using Rb pAb anti-human C3 as described above.

3. Results and discussion

Elucidation of roles played by different components present in placental extract provides an understanding into its cumulative effect on tissue repair and healing. Assessment of the role played by carbohydrates was initiated to investigate the same. Quantification of the reducing sugar content present in human placental extract was followed using the Nelson–Somogyi method

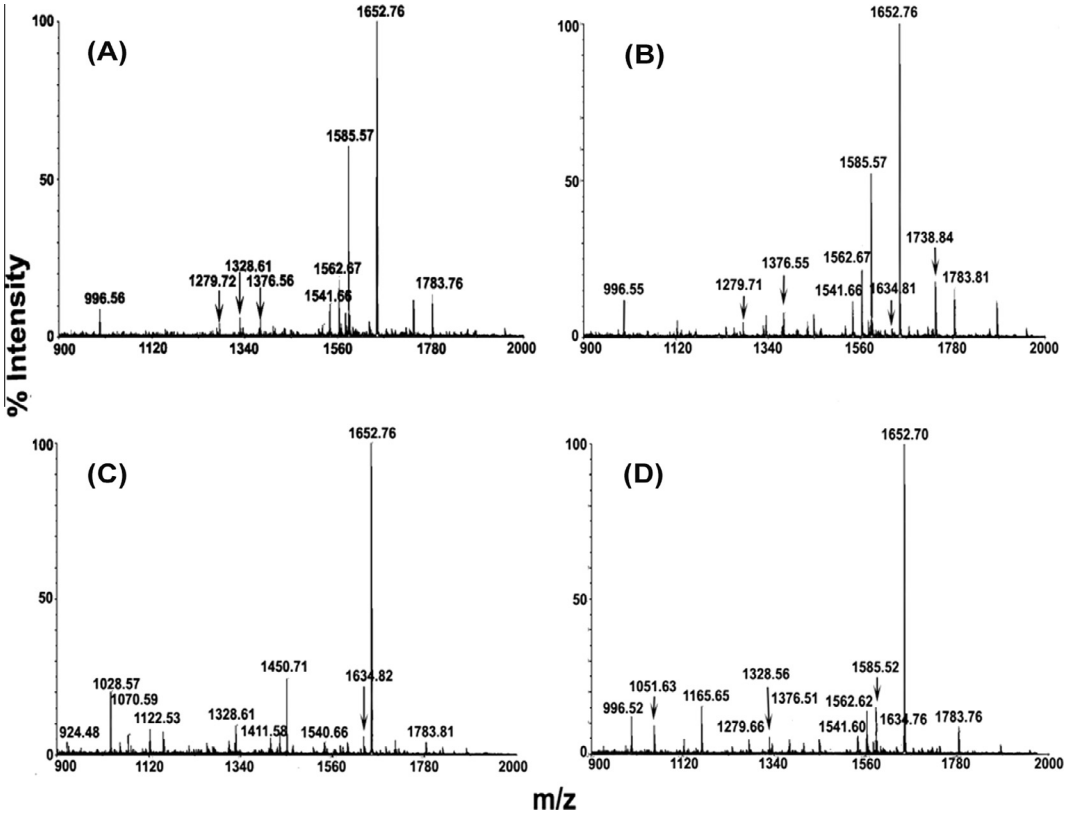


Fig. 3. (A–D) Mass spectrum of the fractions 1, 2, 3 and 4 of Fig. 2B respectively. Peaks of low intensity and close proximity have been marked by downward arrows.

[43]. The concentration of reducing sugar was estimated to be 9.84 ± 2.42 μg/ml (n = 4). Since glucose is one of the most prevalent monosaccharide present in human, a coupled enzyme assay was performed for specific determination of glucose in placental extract. The concentration of glucose was estimated to be 9.31 ± 2.82 μg/ml (n = 5). Thus, glucose was a major component of the reducing sugar content of human placental extract.

Carbohydrates are expected to exist in both free and bound state in placental extract. Thus, TLC was performed to detect the presence of glycoconjugates. Application of glucose, galactose and placental extract (100× conc.) resulted in R_f values 0.67, 0.61, 0.41 respectively (Fig. 1A). Apart from a faint band, no separation of components was observed in placental extract. In comparison to monosaccharides like glucose and galactose, no spot for TLC was obtained for placental extract at equivalent migration point. This indicated that probably free glycans are much less in amount as compared to conjugates. Subsequently, FACE was done to detect any separation of components.

After their enzymatic release from glycoproteins, glycans were reductively aminated and separated using FACE. Glucose was run as a reference marker of the electrophoretic mobility. In the FACE profile of placental extract, ANTS derivatives showed a single diffused band with higher electrophoretic mobility than labeled glucose. No separation of components was observed. Thus, TLC profile and FACE indicated presence of glycans in placental extract (Fig. 1B and C). Previous reports on Schiff-periodic acid staining confirmed the presence of glycoconjugates in placental extract [19]. However, in the absence of any specific method to quantitatively estimate the glycoprotein content of placental extract, ImageJ densitometric analysis was employed (Fig. 1D). Glycoprotein content of placental extract was estimated to be 59.77 ± 9.01 μg/ml (n = 5).

The glycopeptide content of the placental extract was isolated by eluting the bound fraction of Concanavalin A-Sepharose column using methyl α-D mannopyranoside as the eluent (Fig. 2A). The broadness of the unbound fraction was attributed to large volume of placental extract applied. Presence of glycans in the pooled bound fraction was determined by specified protocol (Fig. 2A). This bound fraction was further applied to Nova-Pak C_{18} RP-HPLC column (Fig. 2B). Chromatography profile revealed a pattern with multiple peaks eluted both in the aqueous as well as organic solvent mediums. Peak fractions with significant absorbance were separately collected and analyzed by MS analysis.

The fractions from RP-HPLC were deglycosylated using PNGase F, subjected to chymotrypsin digestion and the resulting fragments were analyzed by MALDI ToF/ToF combined with Mascot search (Fig. 3, Table 1). The components identified were lymphocyte cytosolic protein, trophinin and immunoglobulin G (IgG). Recurrent occurrence of IgG fragments in the list was, however, almost always with a low mascot score. This could be attributed to the impure form of the extract which was used for the analysis. Various fragments of IgG heavy chain variable region were found to be repeatedly matching in chromatographic fractions (Table 1). Reports indicate that the hypervariable (HV) regions within the variable domain are directly involved in antigen binding [52]. One such hypervariable fragment (HV 2, residues 48–62, Accession No. CAB37157) has been indicated on the data list (Table 1). This region is also referred as the complementarity determining region (CDR). These observations gave an indication of IgG being a major component of the glycoprotein content of placental extract.

Immunoglobulins are glycoproteins which play a critical role in immune response. However, presence of immunoglobulin in placental extract has to be attributed to fetal-maternal exchange which provides passive immunity to the fetus. Literature provides strong evidences to support the fact that IgG is exclusively transported across the placenta. Other isotypes are unable to cross the placental barrier as IgM exist as pentamer; IgA exists as dimer

Table 1
MALDI ToF/ToF analysis and Mascot search of chymotryptic fragments of components separated by RP-HPLC.

Fraction No	Protein	Accession No.	Score	Peptide ion m/z	Sequence	MS/MS derived sequence
1,2,4	Immunoglobulin heavy chain variable region (fragment) human	CAB37157	48	1279.6311	71–81	TISRDDSKNML
				1376.5643	98–109	CTVGTCISTACF
				1562.6437	98–110	CTVGTCISTACFW
				1634.8608	48–62	VGRIKNRADGGTIDY
				1783.912	67–81	KGRFTISRDDSKNML
1,2,4	Lymphocyte cytosolic protein 1 (L-plastin) (fragment) human	Q5TBN4_HUMAN	48	996.536	82–90	KSTDVAKTF
				1328.5753	64–74	DQDGRISFDEF
				1541.6901	58–71	MATGDLDQDGRISF
				1600.6652	1–14	MARGSVSDEEMMEL
1	Immunoglobulin superfamily member 8 protein (fragment) human	Q9BTG9_ HUMAN	37	1279.6866	59–69	ARTSTQKHTHL
				1376.6917	263–275	RLEAARPGDAGTY
				1541.6591	414–426	HCAPSAWVQHADY
				1634.8683	217–233	EMAPAGAPGPGRLVAQL
				1652.8213	213–229	SVGWEMAPAGAPGPGR L
3,4	Trophinin-human	I38488	45	996.4455	468–477	GGAPSTSLCF
				1028.4718	318–327	GGTLSTSVCF
				1121.5221	568–581	GGGPGTSTGFGGGL
				1154.5688	88–99	SNTASISFGGTL
				1165.5483	368–381	GGSPSTSAGFGGAL
				1652.7299	705–724	SGGPSTGAGFGGGPNTGAGF
				1652.7299	715–734	GGGPNTGAGFGGGPSTSAGF
				1783.8496	676–695	SSGPSSIVGFSGGPSTGVGF
3	Ig heavy chain V region (clone RIV) – human fragment	PH1660	46	924.4421	79–86	RAEDTAVY
				1070.5629	52–60	YVDSVKGRF
				1411.7177	61–72	TISRDNSKNTLY
				1540.6948	104–118	GMDVWGQGTTVTVSS
3	Ig kappa light chain, A28 V-segment protein (fragment) – (human)	CAA31203	42	1070.5001	57–67	SSSGSGTDFIL
				1464.7693	68–80	KISRVEAEDVGVY
				1627.8325	68–81	KISRVEAEDVGVYY
				1708.7959	4–19	LGEPSSISCRSGQSPF
3	Immunoglobulin heavy chain variable region precursor (fragment) human	CAD60375	42	924.4421	97–104	RAEDTAVY
				1411.7177	79–90	TISRDNSKNTLY
				1540.6948	111–125	GMDVWGQGTTVTVSS
				1687.8431	6–21	RGVQCEVQLVESGGGL
3	Immunoglobulin heavy chain VHDJ region (fragment) – human	BAC02383	41	936.4632	122–131	GQGTTVTVSS
				1154.6165	1–11	QVQLQQSGPGL
				1328.6005	87–98	NSVTPEDAAVYY
				1540.6948	117–131	GMDVWGQGTTVTVSS
				1627.6904	21–36	TCAISGDSVSSDTAAW
3	Ig heavy chain V-III region (But) – human	A2HUBU	40	924.4421	86–93	RAEDTAVY
				1065.4307	28–36	TVSBHSMSW
				1427.6761	68–79	TISRDDSRBTVY
				1539.7762	68–80	TISRDDSRBTVYL
				1540.7603	68–80	TISRDDSRBTVYL

The numbers 1–4 refer to the HPLC fractions described in Fig. 2B.

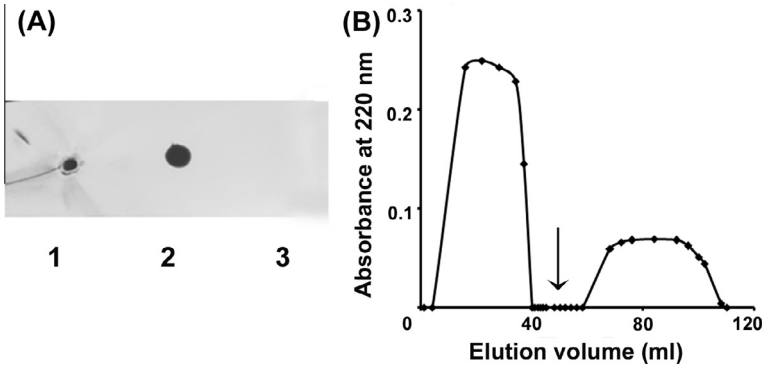


Fig. 4. (A) Dot blot for the detection of IgG using protein A-conjugated alkaline phosphatase. (1) Human placental extract, (2) reference human IgG as positive control and (3) subtilisin as negative control. Partial diffusion of the spot in l is a characteristic feature of concentrated placental extract due to floating of lipid particles. (B) Purification of IgG using protein A-Sepharose affinity column. Bound IgG fraction was eluted using 0.1 M Gly-HCl, pH 3.1 (marked by arrow).

and is localized to mucosal layers; IgD is bound to B cells and IgE normally exists in concentration below measurable limit [53–56]. This emphasized the possibility of presence of IgG as the sole immunoglobulin isotype in placental extract.

This prediction was confirmed by immunoblot analysis. Protein A is a bacterial protein with the ability to bind immunoglobulins. Thus reactivity between placental extract and protein-A conjugated alkaline phosphatase was tested using commercial IgG and subtilisin as positive and negative control respectively. It was observed that placental extract developed a deep blue spot along with commercial IgG. No spot was developed with subtilisin (Fig. 4A). Next, IgG pool was purified using protein A-Sepharose

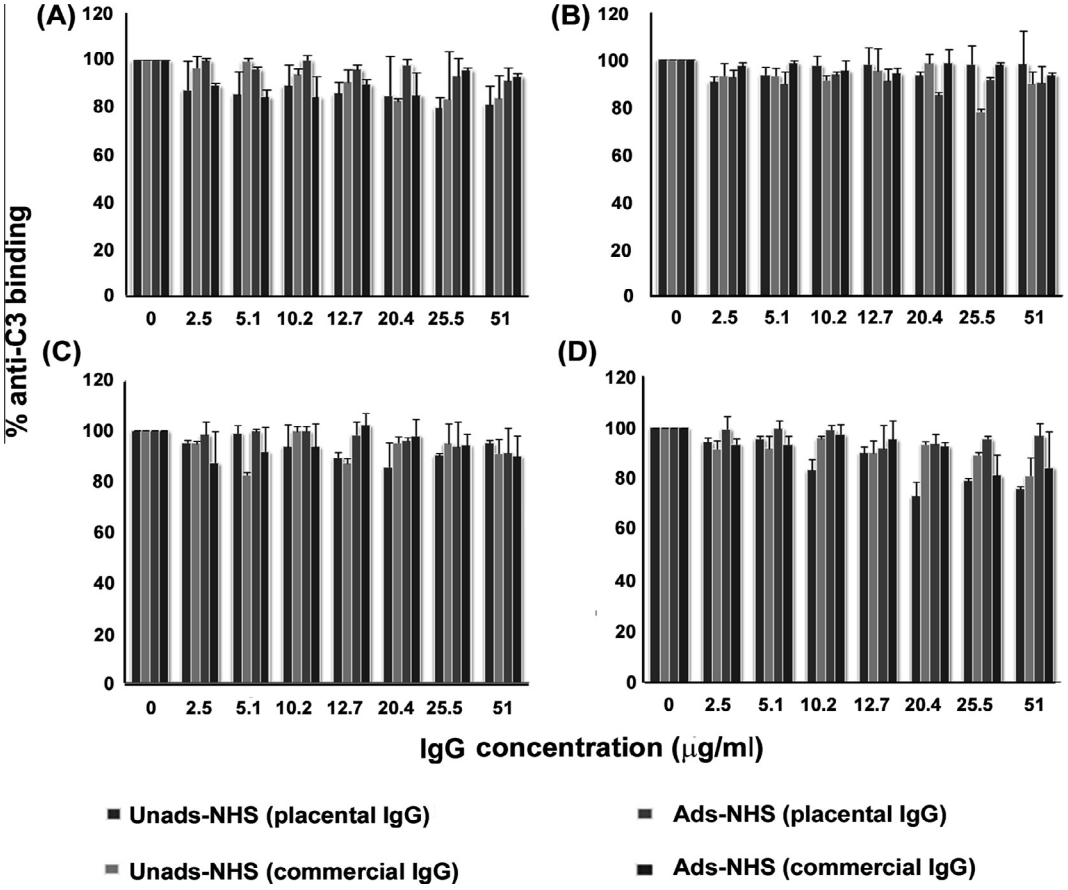


Fig. 5. Effect of placental IgG on C3 binding to *P. aeruginosa* serum sensitive (A and C) and the derived serum resistant strain (B and D); via the alternative pathway (A and B) and classical pathway (C and D) using unadsorbed IgG and unadsorbed human serum or extensively adsorbed IgG and human serum. Bacteria were incubated with 20% human serum in appropriate buffer with varying concentrations of placental IgG or reference human IgG. Bound C3 was detected with an anti C3 antibody. Controls included cells incubated with buffer alone which is indicative of maximal anti-C3 binding (100%, human serum alone).

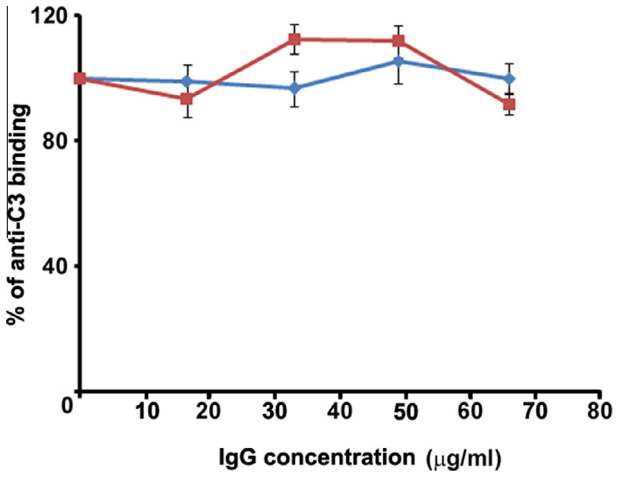


Fig. 6. Effect of IgG on C3 binding to sensitized human erythrocytes with rat anti-human red blood cell IgG. The cells were incubated with varying concentrations of placental IgG. Bound C3 was detected with an anti C3 antibody. Controls included cells incubated with buffer alone which is indicative of maximal anti-C3 binding (100%, human serum alone). Effect of placental IgG and reference IgG have been presented by (◆) and (■) respectively.

affinity chromatography. Bound fraction was eluted using Gly-HCl, pH 2.4. Purified IgG was estimated to be 25.2 ± 3.97 μg/ml using ELISA (Fig 4B).

Pooled IgG preparations are routinely administered to patients with autoimmune and systemic inflammatory diseases. However, reports have indicated the complement regulatory activity of IgG by acting as a preferential acceptor of activated C3, thus diverting complement activation from target bacterial surface [57]. In patients with dermatomyositis, intravenously administered IgG substantially inhibited activated C3 deposition on target tissue, thus supporting the anticomplementary potential of IgG [58]. Human placental extract shows significant potential as a wound healer as well as an immune-regulator. Removal of bacterial burden in the wound milieu prevents the progression of an acute wound into a chronic one. Presence of IgG in placental extract raised the possibility of its interference in the process of opsonization of bacteria. Thus, ability of purified placental IgG to modify complement bacteria interaction was investigated through both alternative as well as classical pathway. *P. aeruginosa* was the bacterial strain of choice due to its persistent occurrence in wound milieu. Effect of IgG on complement mediated lysis of both serum sensitive and serum resistant strains was examined to elucidate its mechanism of action in complement regulation. *P. aeruginosa* isolated from the sputa of CF patients are generally mucoid, nontypable, deficient in LPS O-side chains, and serum sensitive (SS) [59]. These were transformed into serum resistant (SR) strains by repeated passage of increasing concentrations of serum. Resistance to bactericidal activity of serum is reflected in the increase in lipopolysaccharide O-side chain composition [51]. Both the strains were tested for their susceptibility towards serum lysis before experimental studies.

Activation of different pathways for bacterial opsonization is initiated by different mechanisms. Alternative pathway activation was studied on *P. aeruginosa* (SS) and the derivative strain (SR) in

presence of 2.5–51 μg/ml of placental IgG. Within this concentration range, IgG did not inhibit C3 binding to *P. aeruginosa* (Fig. 5A and B). Although GVBS-EGTA was used as the appropriate buffer to study activation of alternative pathway, adsorption procedures were performed to eliminate the possibility of complement activation by antibacterial antibodies. Similar concentrations of commercial IgG produced comparable results (Fig. 5A and B). The concentration of commercial IgG used in the experiments was in accordance with placental IgG, since the potential of human placental extract towards its effect on innate immunity was being investigated. Classical pathway activation, in absence of antibodies, might occur through direct C1q binding. Thus, serum was heated to 55 °C in order to destroy alternative pathway activity. The serum was further adsorbed with the bacteria to study antibody independent classical pathway activity. No significant modification in complement binding to bacterial surface was observed in both serum sensitive and serum resistant strains (Fig. 5C and D). Sensitized human erythrocytes were used as a model to complement activation through classical pathway. It has been reported that C3 binding to sensitized targets might be observed at higher concentration of IgG [60]. Thus, it was necessary to ascertain whether this concentration range of IgG was high enough to cause any such effects. However, within the specified range of 2.5–51 μg/ml of placental IgG, no inhibition of C3 binding was observed (Fig. 6). These results clearly demonstrated the safety of the human placental extract as an immunoregulatory agent with respect to interference in the process of normal host defense.

The placental extract used in this study does not show detectable proteolytic activity against azoalbumin and azocasein but contains collagenolytic activity [18] and fibrinolytic activity (P. Bhattacharyya, unpublished data). This indicates that the extract contains proteases of narrow specificity. Considering the elevated temperature and pressure used in the manufacture of the extract, activation of proteases therein and denaturation of such large proteins like IgG (150 kDa), presence of fragmented IgG instead of the intact molecule is expected. The binding between IgG and C3 occurs through ester and amide bonds. If the IgG fragments are unable to form these bonds then, inadvertently they will not have any effect on the complement-bacteria interaction and would thus ensure the safety of human placental extract towards maintaining the natural innate immunity of the host.

4. Conclusion

The present study was initiated with the aim to decipher any specific role exhibited by glycans in the process of wound healing. Identification of immunoglobulins in placental extract was followed by evaluation of their regulatory role in complement pathway. Using bacterial strains of serum sensitive and resistant types, it was indicated that placental IgG, apart from its role as an immunostimulator, does not interfere in the bacterial opsonization in the wound milieu. Thus, the administration of placental extract to a wound patient would not tamper with his/her innate bacterial defense potential.

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