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Reactive Oxidant Species Augment Complement Function in Human Blood Independently of Classical and MBL-Pathways

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Abstract: Recent evidence suggests that complement plays an important role in the pathogenesis of ischaemia-reperfusion injury and that this may be mediated by reactive oxidant species (ROS). However, it is not clear how individual complement pathways could be affected by ROS. In this study purified C3 and C4 proteins were subjected to a 30 min treatment with ROS generators at 37°C: 1mM authentic peroxynitrite ($ONOO^-$), 10 μM/10 μM xanthine/xanthine oxidase mixtures to generate O_2^- and100 μM H_2O_2. We also studied the effect of ROS on complement activation in normal human serum and mannan binding lectin (MBL) deficient serums. In addition, normal human blood was taken from healthy volunteers treated with ROS during 30 min extracorporeal circulation. Sensitive ELISA assays were used to determine the effect of ROS on complement reactivity respectively and whether this related to haemolytic activities. Blood treated with the competitive ligand for MBL binding, N-acetyl-D-glucosamine (GlcNAc=100mM) and untreated blood were used as controls. There was a concentration-dependent modification of protein residues by ROS and that ROS treatment of normal and MBL deficient serums inhibited C4 deposition originating from both MBL and classical pathways and interestingly this was associated with an increased haemolytic activity. The results may suggest that ROS plays an important role in regulating complement activation independently of the classical and MBL pathways and that increased activities may be attributable to a direct effect on the alternate pathway.

Key words: Oxygen species, recirculation, proinflammatory cytokines, mannan-binding, lectin

INTRODUCTION

Reperfusion, although necessary for the preservation of reversibly injured tissue, is associated with additional injury mediated largely by blood leukocytes[1,2]. Throughout the ischaemic period, levels of intrinsic enzyme Xanthine Oxidase (XO) and hypoxanthine rise. Upon the introduction of molecular oxygen during reperfusion, the resulting reaction leads to the formation of superoxide (O_2^-)[3] . In addition, the restoration of blood flow to ischaemic tissues initiates a cascade of inflammatory-like events, including increased microvascular permeability, protein leakage, neutrophils sequestration and complement activation that contribute to post-ischaemic injury[4]. As a result of their interaction with chemotactic cytokines (IL-8)[5,6] neutrophils at the site of inflammation adhere to endothelial cells triggering an activation of endothelial Nicotine Adenine Dinucleotide Phosphate (NADPH) oxidase complex[2,7] that generates superoxide (O_2^-) by transferring electrons from NADPH, found in the cytoplasm to molecular O_2. The resultant O_2^- reacts with Nitric Oxide (NO) to form peroxynitrite ($ONOO^-$)[7,8]. Protonation of $ONOO^-$ creates its conjugate acid, peroxynitrous acid (ONOOH) that gives rise to $HO^?$ by haemolysis and cause tissue damage by acting on proteins, DNA and lipids, ultimately resulting in cell apoptosis[9]. In addition, TNF-á in association with other cytokines activate neutrophils that release oxygen radicals[6,10]. Although, the action of neutrophils and the production of oxygen free radicals (ROS) in ischaemia-reperfusion injury (IR) have been extensively studied, the role of complement still remains less understood.

The complement system is a major constituent of the humoral branch of the immune system. The soluble and cell bound proteins that constitute the complement system interact in highly regulated cascades to serve four important functions; cell lysis, opsonisation, removal of harmful immune complexes from the circulation and mediating the immune response (inflammation and release of immunoregulatory molecules) by interacting with other immune cells. The complement system is a complex cascade of proteins that are activated in 3 distinct ways, designated the classical, alternative and lectin pathways. Activation of classical pathway is initiated by C1q binding to antigen-antibody complexes, whereas activation of the alternative or lectin pathway is antibody independent[11]. The alternative pathway is activated by spontaneously cleaved C3 or by C3b generated by one of the other pathways[12]. The lectin pathway is activated by Mannose Binding Lectin (MBL) interacting with its ligand

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leading to activation of 2 MBL-associated serine proteases (MASPs)[13,14].

As well as its purpose in complement activation, MBL also functions to promote opsono-phagocytosis, control of inflammatory response (by modulating cytokine release from monocytes) andpromotion of apoptosis (demonstrated by its binding to apoptotic T-cells)[15]. In complement activation MBL acts together with MASPs in a similar fashion to the C1q, C1r and C1s components of the classical pathway[16]. To date three MASPs have been identified; MASP-1, MASP-2 and MASP-3 as well as a non-enzymatic protein known as the MBL-associated protein of 19 kDa (MAp19). Of these four proteins it is MASP-2 that has been identified as combining with MBL to play a role in the activation of complement components C4 and C2[17]. The activity of the MBL-MASP-2 complex is triggered by contact with carbohydrate moieties found on microbial surfaces. This leads to the activation of the MASP-2 portion of the complex, which goes on to cleave C4 and C2 in a similar fashion to the $C1qr_2s_2$ complex of the classical pathway, forming C4bC2a (C3 convertase). The subsequent steps in the MBL pathway up to the cleavage of C5 to C5b and beyond are identical to the classical pathway[14,18].

The complement system is activated after ischaemia-reperfusion as evidenced by studies that have shown localisation of various complement components within the ischaemic tissue and that protection from ischaemia-reperfusion injury can be achieved by complement depletion[15,19]. Moreover it has also been demonstrated that blocking the MBL pathway by using monoclonal antibodies against MBL protects the heart against ischaemia-reperfusion injury[20]. Other more recent studies involving MBL depletion have supported the above observations by showing that the lectin pathway also initiates complement activation after hypoxia-reoxygenation in an experimental model using human endothelial cells[21].

Despite this evidence it is still not clear whether ROS and components of the blood complement pathways interact during ischaemia-reperfusion injury. No studies have so far assessed whether there is some specific role of ROS in generating molecular modifications in the key components of the pathways of complement activation that may affect overall complement reactivity, a feature that can have implications on the overall inflammatory process.

In this study we investigated whether:

- Specific ROS radicals induce any molecular modifications to the key complement components C3, C4 and whether there is any significant effect on the MBL-mediated and classical pathways of complement activation in human deficient and sufficient serum.
- The molecular modification on key complement components including C3 and C4 by ROS affects the reactivity of the MBL-mediated and classical pathways of complement activation in whole blood using a model of circulating blood.

MATERIALS AND METHODS

Purified C3 and C4 proteins, C4c monoclonal biotinylated antibody and anti-human C5 monoclonal antibody were purchased from Quidel (San Diego, CA, USA). MBL-deficient serum was a generous gift from Dr Nick Lynch, University of Leicester. Xanthine, xanthine oxidase, hydrogen peroxide, normal human serum, Mannan, Human Serum Albumin (HSA), p-Nitrophenyl Phosphate Substrate (pNPP), Gelatin Veronal Buffer (GVB^{2+}), antibody sensitised sheep erythrocytes and N-acetyl-D-Glucosamine (GlcNAc) were obtained from Sigma-Aldrich (Poole, UK). Peroxynitrite was purchased from CN Biosciences (Nottingham, UK). Polyvinylidene difluoride (PVDF, Hybond-P™) membrane and ECL Plus™ western blotting detection system were purchased from Amersham Biosciences (Bucks., UK). Mouse monoclonal anti-nitrotyrosine antibody and nitrotyrosine immunoblotting control purchased from Upstate Biotechnology (Lake Placid, NY, USA). Precision *Plus Protein All Blue* Standards were purchased from Bio-Rad Laboratories. Donkey anti-mouse IgG HRP conjugate were purchased from Affinity Bioreagents. Purified human IgG and streptavidin conjugated with alkaline phosphatase were purchased from Serotec (Oxford, UK).

30 healthy volunteers (male: female, 19:12; mean age 34.1± 12.3 years) each donated 50 mL of blood for the recirculation model experiments. The study protocol was approved by the local medical ethics committee and informed consent was obtained by all participating individuals.

Detection of modification of c3 and c4 purified proteins by specific ROS

Treatments: Protein samples were prepared by diluting purified C3 and C4 proteins (1.0 mg mL^{-1}) in phosphate buffered saline (PBS, pH 7.4 at 1:10 dilutions). Xanthine (10 μM) and xanthine oxidase (1-1000 μM) were used as part of Xanthine/Xanthine Oxidase system (X/XO) to generate superoxide anions whereas purified peroxynitrite ($ONOO^-$, 0.01-1mM) and hydrogen peroxide (H_2O_2) (0.1-100 μM) were used. Protein modification was detected by determining changes in electrophoretic mobility and presence of nitrated residues.

Determination of the effect of ROS on classical and MBL-dependent human serum complement reactivity: Normal human serum and human serum deficient of MBL were also treated with ROS generators at different concentrations. Classical and MBL-dependent complement activity were determined by measurement of the ability to induce C4b deposition when treated serum was incubated on wells coated with IgG or mannan as shown below. To establish whether C3 treatment affects complement activity, the experiments were performed using C3- and C4-deficient human serum supplemented with untreated and ROS-treated C3 and C4 before determination of C4b deposition. All the samples were incubated at 37°C for 30 min and then stored at -80°C until analysis.

Blood treatment with ROS and analysis of complement activity following recirculation in an *In vitro* model

Blood sampling: 50 mL of blood from healthy volunteers were recirculated at normothermia (37°C) in the model of extracorporeal circulation for 30 min previously described[22]. Blood samples apart from controls were treated with free radical generators or GlcNAc prior to circulation in the presence of X/XO, $ONOO^-$, H_2O_2 and GlcNAc. Five groups consisting of one control, three different OFR generators and GlcNAc were investigated and each group comprised of six volunteers. Baseline blood samples were taken before circulation in the circuit and after 30 min of recirculation. All samples were collected into sterile tubes and left to clot on the bench for 1 h. Serum was separated by centrifugation at 2000 g for 5 min and immediately snap-frozen in liquid nitrogen and stored at -80°C until analysis. The activity of the MBL and classical complement pathways in separated serum was measured using a C4b-ELISA and a complement haemolytic assay respectively, as described below.

SDS-polyacrylamide gel electrophoresis: Protein profiles were obtained after the electrophoresis of 4 μg of protein of the eight protein samples (C3 control, C3 + X/XO, C3 + $ONOO^-$, C3 + H_2O_2, C4 control, C4 + X/XO, C4 + $ONOO^-$, C4 + H_2O_2) plus one sample of pre-stained protein standards. The samples were diluted (4:1) in SDS-PAGE loading buffer containing â-mercaptoethanol and denatured at 85°C for 5 min. They were loaded onto 5% polyacrylamide gels and the electrophoresis was performed at 100 volts for 60 min in tris-glycine-SDS (TGS) buffer. Following electrophoresis the gel was stained with coomassie brilliant blue dissolved in destaining solution (acetic acid: methanol: water, in the ratio 1:1:10). After several washes with destaining solution the gel was rinsed and scanned and the image stored in a computer.

Western blot analysis: To determine the presence of 3-nitrotyrosine residues on the proteins, the samples of untreated and treated complement proteins as were electrophoresed as described above. The gel was subsequently electro-blotted onto a hydrophobic PVDF membrane at 15 volts for 50 min. Non-specific protein binding sites were blocked by a 60-minute incubation in milk solution (5% milk powder w/v, tris buffered saline (TBS), 0.05% v/v Tween 20). After several washings with dilution buffer (TBS, 0.05% v/v Tween 20) the blot was incubated with the primary antibody (2 μg mL^{-1} mouse monoclonal anti-nitrotyrosine antibodies in milk solution) overnight at 4°C with agitation. After several washes with dilution buffer the secondary antibody (HRP conjugated donkey anti-mouse IgG: dilution buffer, ratio 1:4000) was added and the blot incubated for one h at room temperature. The proteins were detected using the ECL Plus™ western blotting detection system by following the manufacturer's instructions (Amersham Biosciences UK).

Complement activity determination using C4b Deposition Enzyme-Linked Immunosorbent Assay: Complement activation via the MBL-pathway was measured by quantifying the level of C4b deposition using an enzyme linked immunosorbent assay (C4b-ELISA). However, the complement component C4b is a product of both MBL-pathway and classical pathways activation. Therefore to distinguish between the two, the assay was performed first on Mannan-coated plates to determine the activity of MBL-pathway and then on IgG-coated plates in place of Mannan to detect only the classical pathway as previously described[23]. Briefly, microtiter wells (Maxisorb, Nunc, Kamstrup, Denmark) were coated with 1 μg normal human IgG for classical pathway or 1 μg mannan in 100 μL of 15 mm Na_2CO_3, 35 mM $NaHCO_3$, 1.5 mM NaN_3, pH 9.6 (coating buffer) for MBL-dependent pathway. After an overnight incubation at room temperature, residual protein-binding sites were blocked by the addition of 200 μL 0.1% (w/v) HSA in 10 mM Tris-HCl, 140 mM NaCl, 1.5 mM NaN_3, pH 7.4 (TBS) for 1 hour. After washing using TBS with 0.05% (v/v) Tween 20 and 5 mM $CaCl_2$ (TBS/tween/Ca^{2+} buffer), the wells received MBL-deficient or sufficient serum samples diluted twice with assay buffer (4 mM barbital, 145 mM NaCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 3.8 mM NaN_3, pH 7.5). For MBL activity high ionic conditions were preferred for the assay to prevent interference from classical pathway activation. Therefore the following high ionic buffer was used instead: 20 mM Tris-HCl, 10 mM $CaCl_2$, 1 M NaCl, 0.05% (v/v) Triton X-100, 0.1% (w/v) HSA, pH 7.4 (MBL-binding

buffer). Wells containing only buffer were used as negative controls. The addition of high ionic buffer prevents the activation of endogenous C4 whose quantity is subject to inter-individual variability thus eliminated the possibility of detecting differences in MBL-activity arising from this. All dilutions were added in duplicates. Following overnight incubation with diluted serum and washing with TBS/tween/Ca^{2+} buffer, C4d-depositing capacity was assessed by the addition of 0.5 µg C4 in 100 µL TBS/tween/Ca^{2+} buffer. After incubation at 37°C and a wash as described above, deposited C4b was detected by the addition of alkaline phosphatase (AP)-conjugated chicken anti-human C4 diluted 1000 fold in TBS/tween/Ca^{2+} buffer followed by addition of 100 µL of substrate (1 mg *p*-nitrophenyl phosphate per mL of 1 M diethanolamine, 1 mM $MgCl_2$, 3 mM NaN3, pH 9.6). Colour development was determined by reading the absorbance at 405 nm on a microtiter plate reader.

Complement haemolytic assay: Cell lyses as a result of Membrane Attack Complex (MAC) production following complement activation was measured by a haemolytic assay[24]. Determination of haemolytic activity arising from the MBL activity was not performed because it was felt that existing published protocols were not sufficiently specific. For the determination of the classical pathway haemolytic activity, the sheep red blood cells (SRBC) were at first sensitised using rabbit anti-SRBC antibodies (Antibody-coated erythrocytes (EA)). A total number of 7×10^9 EA diluted in dextrose gelatin Veronal buffer/Ca2+ (0.5 x VBS, 0.05% gelatin, 167 mM glucose, 0.15 mM CaCl2, 0.5 mM MgCl2 (DGVB++; volume 50 µL) were mixed with ROS-treated serum (final dilution 1/10 in DGVB++, final volume 100 µL) for 30 min at 37°C. For the analysis of the alternative pathway haemolytic activity, rabbit erythrocytes (7×10^9) suspended in DGVB++ containing 10 mM MgEGTA were incubated in a 1:1 ratio with human serum, final volume of 100 µL, for 30 min at 37°C. In both assays after the addition of 1.5 mL of PBS and centrifugation, haemolysis was assessed by measuring absorbance using a benchmark microplate reader at 415 nm. The lytic activity of a sample was expressed as a percentage = (Mean sample OD-mean OD of negative control/mean OD of 100% haemolysis with H_2O-mean OD of negative control) x 100.

Statistical analysis: The Mann-Whitney test was used to compare data judged to be non-parametric. Statistical significance was set at a 5% level using the SPSS statistical package (SPSS Inc. Chicago).

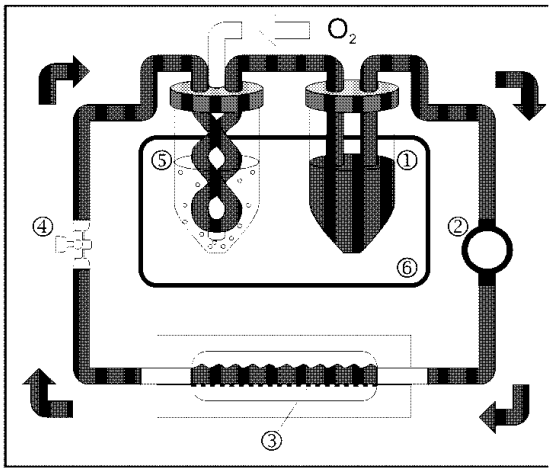

Fig. 1: The schematic diagram of the *in vitro* recirculation model showing; 1) blood reservoir; 2) pump; 3) filtration membrane; 4) sampling point; 5) oxygenator; (6) water bath at 37°C

RESULTS

Oxidative modifications: Figure 2A and 2B show electrophoretic mobility patterns for C3 and C4 proteins with and without ROS treatment. In the presence of ROS treatment C3 and C4 appear to generate distinctly darker bands with reduced mobility, suggesting an alteration on protein residues. Fig. 3-A shows the results of the western blot analysis for the key complement protein C3. The dark bands demonstrated proteins with nitrotyrosine residues, which are recognised as sites of intense nitration (Fig. 3 B and 3 C).

The Effect of ROS on Classical and MBL-dependent C4b deposition in Human Serum: Complement activation was measured by quantifying the level of C4b deposition by C4b-ELISA using IgG- and mannan-coated plates. As shown in Fig. 4A, in the IgG-coated plates C4b deposition was detectable in both normal human serum and MBL-deficient serum almost to the same level. For the mannan-coated plates however even greater levels of C4b deposition were seen in the normal human serum, but was almost eliminated in the MBL-deficient serum. In addition, GlcNac which is a competitive ligand that blocks MBL binding to its substrate mannose almost completely eliminated complement activities in serum but did not affect the IgG-dependent C4 activation. Interestingly, as shown in Fig. 4 B-4 D, treatment of both normal human

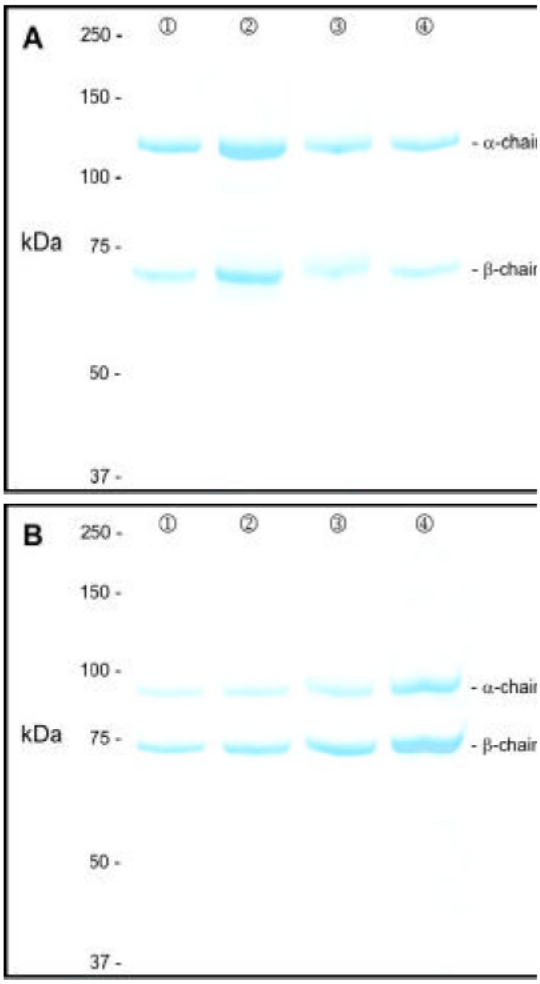

Fig. 2: Electrophoretic mobility of complement proteins C3 (Fig. A) and C4 (Fig. B) separated by SDS/PAGE and stained Coomassie brilliant blue stain. Treatments: untreated (lane 1), superoxide (lane 2), peroxynitrite (lane 3) and hydrogen peroxide (lane 4)

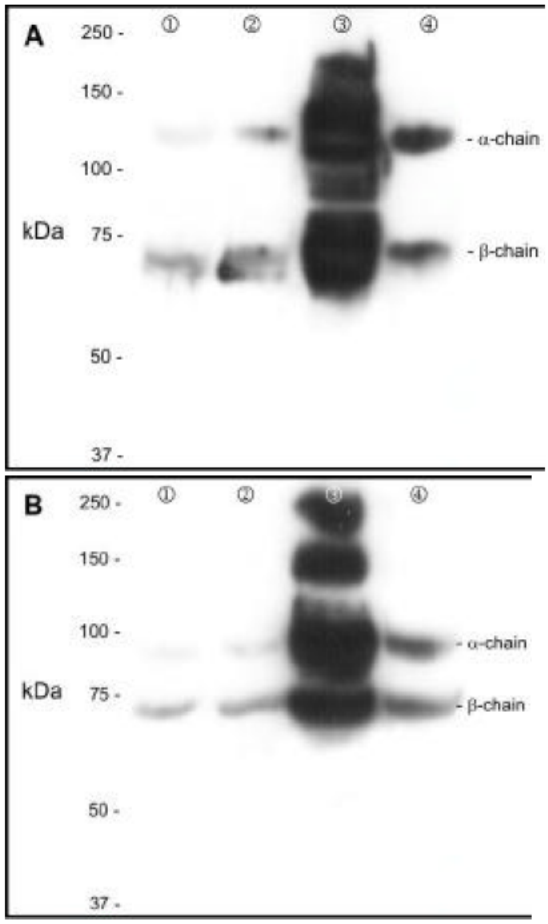

Fig. 3: Detection of nitration of complement proteins C3 (Fig. A) and C4 (Fig. B) by western immunoblotting. Treatments: untreated (lane 1), superoxide (lane 2), peroxynitrite (lane 3) and hydrogen peroxide (lane 4)

and MBL-deficient serums with ROS almost completely eliminated IgG-mediated and MBL-dependent C4b deposition in all the groups. Fig. 4E, shows that untreated C3-deficient serum induced significantly reduced C4b deposition compared with normal and C4-deficient serums. In addition, Fig. 4F shows that supplementation with ROS-treated purified C3 proteins significantly improved the induction of C4b deposition. On the other hand, ROS-treated C4 supplementation had no additional effect compared with the untreated samples.

Complement Mediated Haemolysis: Fig. 5A shows that treatment of serum with ROS significantly increased the alternative pathway-induced haemolysis by about 3-fold. In contrast, although ROS treatment significantly increased the classical pathway mediated haemolytic activity (Fig. 5B), the overall increase was on the average only about 1-fold. In addition, Fig. 5C shows that whilst supplementation with untreated C3 increased haemolytic activity, ROS-treated C3 not effective at inducing increased haemolytic activity.

Effect of Pre-treatment of blood with ROS on Complement Activation using an *In Vitro* blood Recirculation model classical and MBL-pathway mediated C4b deposition: C4b-ELISA was used to measure the levels of complement activation in plasma extracted from blood treated with or without ROS or GlcNAc during recirculation in the *in vitro*

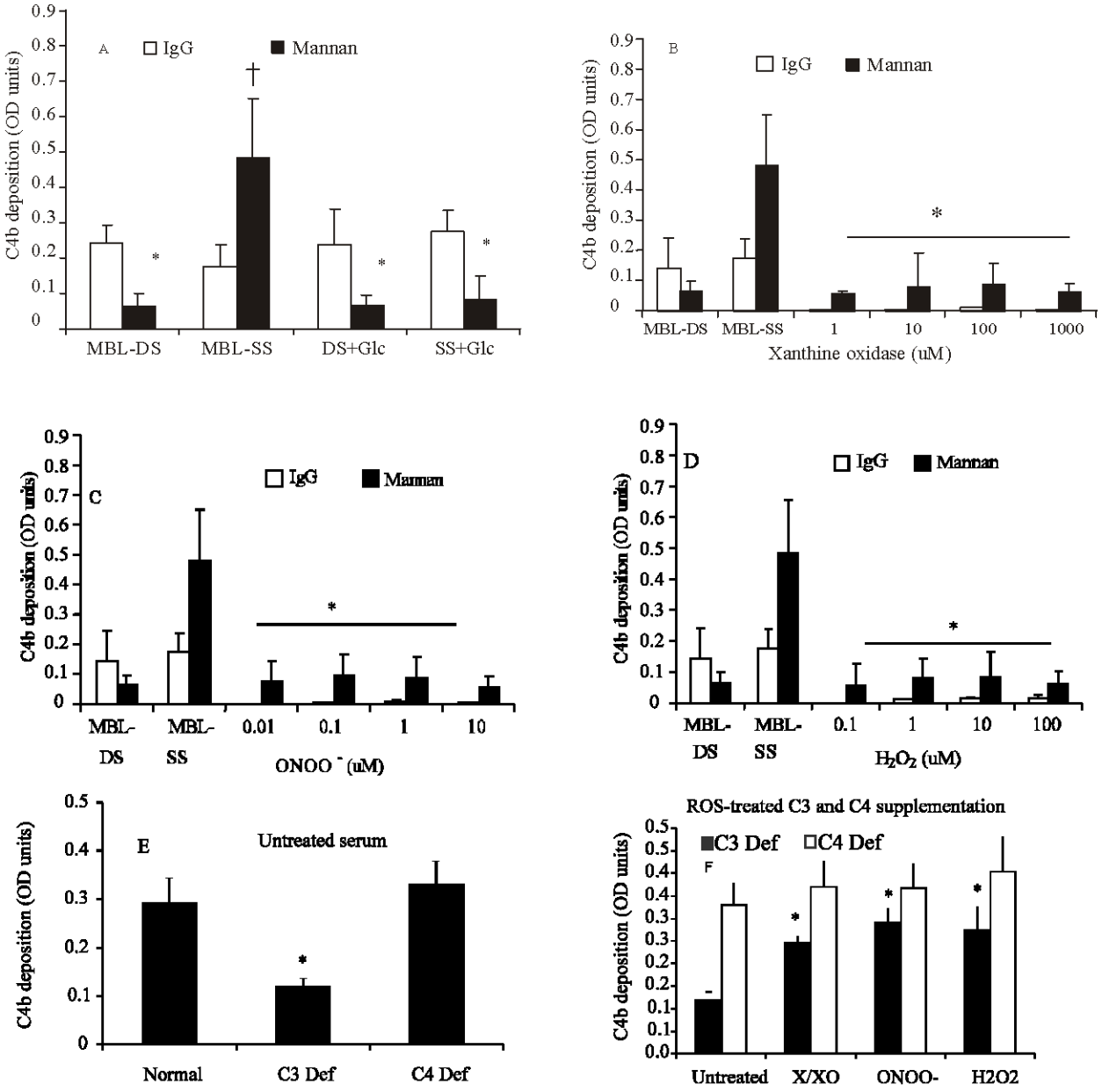

Fig. 4: Mannan-coated detected significantly greater C4b deposition by MBL-pathway as compared with IgG-coated plates († indicates $p<0.05$), respectively as shown in Fig. 4A. Fig. 4B-4D also show significantly reduced (* indicates $p<0.05$) C4b deposition in MBL sufficient and deficient human serum compared with untreated samples. In addition, Figure 4E shows that there was a significantly reduced (* indicates $p<0.05$) C4b deposition from C3 deficient serum as compared with C3 sufficient serum. Interestingly, as shown in Figure 4F supplementation of C3 deficient serum with ROS-treated C3 significantly increased (*indicates $p<0.05$) C4b deposition

model. Control blood was circulated without any prior treatment (untreated samples). Results were corrected for baseline readings. As shown on Fig. 6A and 6B, C4b deposition in the control group was significantly greater than the ROS-treated blood samples as assessed by both MBL-dependent and classical pathway activation. However, ROS treatment in induced much more reduced C4b deposition in the Classical pathway as compared with the MBL-dependent pathway (Fig. 6B). In contrast, blood treated with GlcNAc demonstrated a significant decrease in C4b deposition only in the MBL-dependent pathway measurements (Fig. 6A).

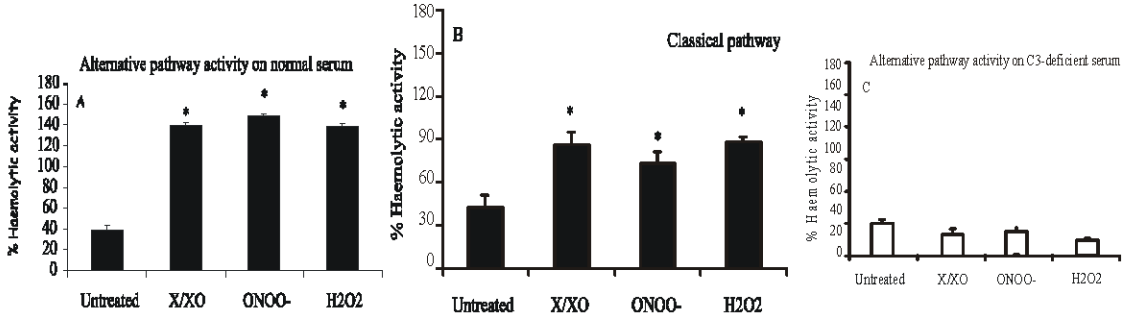

Fig. 5: Up to 3-4-fold increased (*indicates $p<0.05$) haemolytic activity in serum treated with ROS was detected by the alternative pathway method as compared with untreated control (Fig. 5A). Fig. 5B shows only a 1-fold increase in haemolytic activity compared with untreated control as detected by the classical method. In contrast, there was very little haemolytic activity as detected by the classical pathway method in C3-deficient serum which was not affected by ROS treatment (Fig. 5C)

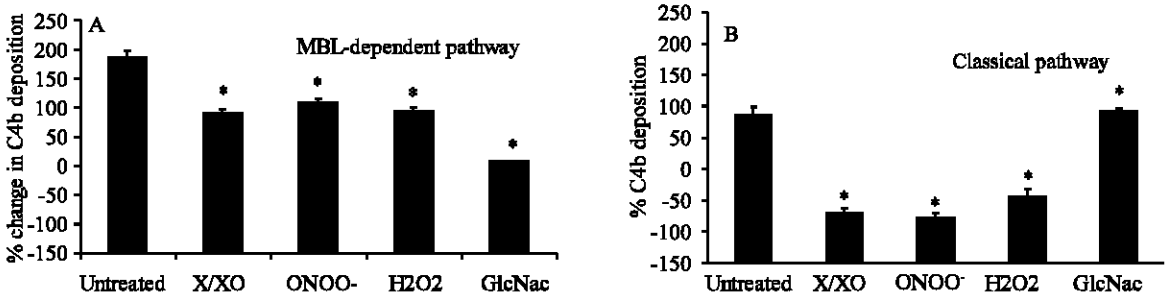

Fig. 6: Fig. 6A shows a significant reduction in C4b deposition induced by the MBL pathway during recirculation of ROS-treated blood as compared with untreated sample (*indicates $p<0.05$), values that were further inhibited by GlcNac treatment. Interestingly, ROS-treatment almost completely eliminated C4b deposition induced by the classical pathway (Fig. 6B) but not the blood samples treated with GlcNac alone

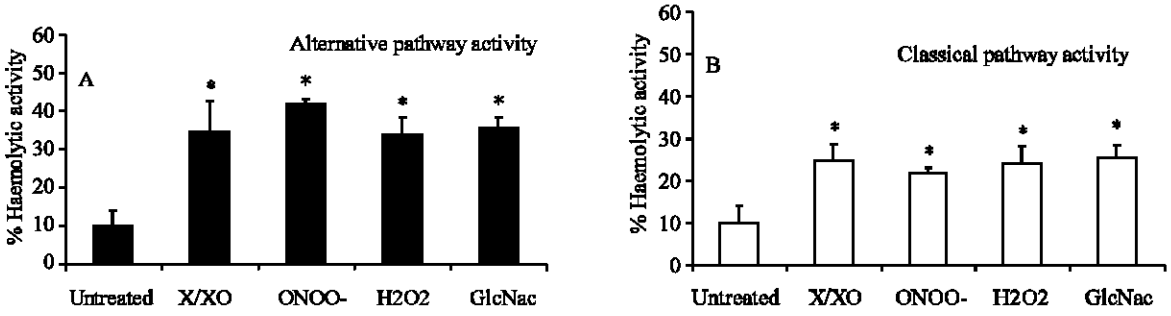

Fig. 7: Fig. 7A shows that there is up to 4-fold increase in haemolytic activity in ROS-treated blood during recirculation as compared with untreated blood (*indicates $p<0.05$) as detected by the alternative pathway method. In contrast, only up to 1-fold increase in haemolytic activity was detected by the classical pathway method

Complement Mediated Haemolysis: The alternative pathway-induced haemolytic activity in all treatment groups throughout the circulation period up to 3-4 fold increase as shown in Fig. 7A. Although a significant increase in classical-mediated haemolytic activity was observed as shown in Fig. 7B this amounted only to an overall moderate increase of 1-fold.

DISCUSSION

Inflammation and complement activation mediated by oxygen free radicals have been implicated in the pathophysiology of cardiovascular disease and in the development of Systemic Inflammatory Response Syndrome (SIRS)[22]. It is also known that oxygen free

radicals, in particular $ONOO^-$ act on protein residues to modify their structure and possibly their function[25]. Clearly the activation of complement during disease process has major clinical implications and therefore warrants investigating to dissect the mechanisms involved.

This study was conducted in three parts to investigate the role of oxygen free radicals on the MBL-pathway as compared with the classical pathway of complement activation. In the first instance we studied the effect of ROS on purified proteins, in terms of effect on electrophoretic mobility and molecular modifications to protein residues. We then studied the effects of these molecular modifications on the functional complement activity. Finally, an in-vitro blood recirculation model was utilised to study the effects of ROS on the classical, alternative and MBL-pathways of complement activation. These observations are clearly of clinical and scientific interest and therefore are discussed in greater detail below.

Although different theories have been proposed to explain protein oxidation, it is generally agreed that there is a correlation between protein oxidation and the accumulation of oxidatively damaged proteins, lipids and nucleic acids. Studies revealed an inflammation related increase in the level of protein carbonyl content andprotein hydrophobicity and as well as the accumulation of less active enzymes that are more susceptible to heat inactivation and proteolytic degradation[25].

Although the chemistry of ROS-mediated protein modification is still not clear, it is fair to speculate that the accumulation of oxidatively modified proteins may reflect deficiencies in one or more parameters of a complex delicate balance between the presence of pro-oxidants, antioxidants, repair, replacement or elimination of biologically damaged proteins[26] .

In this study we observed that when purified C3 and C4 proteins were treated with superoxide and peroxynitrite generators, physical changes in the complement proteins occurred (Fig. 2A and 2B). MBL-mediated and classical pathways activity of complement was measured by quantifying the level of C4b deposition by C4b-ELISA on mannan-coated and IgG-coated plates respectively. When analysing the complement deposition in ROS-treated serum samples a significant drop in C4b deposition was observed despite the presence of significant haemolytic activity predominantly via the alternative pathway. This observation was also observed in serum taken from ROS-treated blood undergoing recirculation for 30 min.

Whilst earlier reports have demonstrated that $ONOO^-$ formed by the reaction of nitric oxide and superoxide is released predominantly by inflammatory cells at the site of injury in several inflammatory pathologies[26-28] there is still no information as to whether this is related to complement activation in blood. $ONOO^-$ is highly reactive towards all classes of biomolecules, including proteins, lipids and DNA and is a potential candidate for effecting the production of proinflammatory cytokines. In proteins, $ONOO^-$ modifies residues such as cysteine, methionine, tryptophan andtyrosine[29]. The irreversible modification of protein residues by $ONOO^-$ such as by nitration of critical tyrosine amino acids exhibits functional consequences like inactivation of a wide variety of enzymes[33-34] and may also affect structural proteins[30,32].

It has been demonstrated that the potential role of ROS may be regulated by intracellular ROS-induced activation of nuclear factor-kappa B, new protein synthesis and activation of the classical pathway of complement activation during Ischaemia-Reperfusion (IR)[35]. Both ROS and complement activation can injure tissue and that studies have also determined that local complement activation might represent a mechanism by which ROS mediate tissue injury[36]. Our observation were in part in agreement with the earlier report[35,36] of an observed increased haemolysis in serum treated with ROS, a feature we have shown to be strongly related to the alternative pathway and partly via the classical pathway. Interestingly, we observed that ROS treatment had an overall inhibitory effect on the MBL pathway. This is the first study that has demonstrated that oxidative stress leads to a decrease in the C4b deposition and a reduction in MBL-pathway activation. Together, this in-vitro data demonstrated an increase in total complement haemolytic activity when compared to control, suggesting that inhibition of MBL pathway does not halt the other complement pathways particularly the alternative pathway leading to membrane attack complex (MAC) formation. It is also not clear from this study which proteins of the alternative pathway are acted upon by ROS to induce MAC formation.

Although the observations from this study using the extracorporeal blood circulation model cannot provide information on the in-vivo relationship between ROS and the MBL-pathway of complement activation, the model does provide a platform for simulating the effect on blood alone particularly if patients are exposed to the artificial surfaces of the extracorporeal circuit and other experimental conditions. Other several in-vitro and in vivo studies have provided conflicting results with regards to

the effect of blood recirculation on complement activation, there may be as many reasons for the discrepancies [37,38]. This may simply be due to differences in experimental conditions and the fact that the in-vitro studies utilised blood from healthy human subjects. Other differences such as the effect of surgical trauma, whole blood response and protamine administration cannot be simulated in-vitro. Also the ability of blood to retain its antioxidant capacity during a period of oxidative insult is severely affected by chronic heart disease,[39] a feature that may play a role in the modulation of the inflammatory process. However, it is not clear whether it is the depletion of the antioxidant capacity seen in blood from the heart disease patients or the observed increase in ROS production that is responsible for the altered complement response. The findings by Shingu *et al.*,[37] that have showed that oxygen radicals produced by activated neutrophils may be one of the mechanisms by which complement is activated in human immune complex diseases is in agreement with results from this study.

CONCLUSION

In conclusion, protein modifications due to ROS generation may result in covalent cross-linking of C3 chains, that leads to loss of protein function of C4, however, the pathways to this reaction are as yet not clear and may warrant further studies. This data suggests that complement activation is independent of the MBL-pathway in extracorporeal mediated oxidative stress. These findings may have important implications for the application of extracorporeal circulation of blood in patients with an underlying inflammatory disease process.

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