

The C1q binding activity of IgG is modified in vitro by reactive oxygen species: implications for rheumatoid arthritis

H.R. Griffiths*, J. Lunec

Division of Chemical Pathology, Hodgkin Building, P.O. Box 138, Leicester LE1 9HN, UK

Received 19 April 1996; revised version received 7 May 1996

Abstract IgG can be denatured in vitro by reactive oxygen species (ROS). Native IgG activates the complement cascade through C1q. Using a modified ELISA, C1q binding activity of rheumatoid IgG has been compared to IgG denatured by neutrophil-derived ROS. The C1q binding activity of rheumatoid synovial fluid IgG is greater than the corresponding serum IgG ($P < 0.01$). Denaturation of IgG by activated polymorphs or the Fenton reaction decreased its C1q binding activity ($P < 0.01$). In vitro exposure of IgG to OH^\bullet and ROO^\bullet increased its interaction with C1q ($P < 0.01$). Hypochlorous acid had no effect. ROS-induced alteration to IgG–C1q binding activity may promote the inflammatory response in rheumatoid arthritis.

Key words: IgG; Reactive oxygen species; C1q binding activity; Rheumatoid arthritis

1. Introduction

During the initial acute phase inflammatory response, neutrophils are recruited to the site of injury and activated to release proteolytic components and generate reactive oxygen species (ROS) [1,2]. These include hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), the hydroxyl radical (OH^\bullet), the superoxide anion radical ($\text{O}_2^{\bullet-}$) and peroxy radicals derived from ROS-induced lipid peroxidation (LOO^\bullet). ROS are highly reactive with biomolecules and can induce autoaggregation and molecular changes within proteins, where the target protein is defined by its size, concentration and constituent amino acids [3,4]. Within the rheumatoid joint, IgG levels are raised as a result of de novo synthesis in situ [5]. It has previously been shown that IgG from rheumatoid synovial fluids exhibits macromolecular and molecular changes typical to those seen following ROS-induced denaturation in vitro [6].

The activation of complement is also an important feature of the acute inflammatory response against bacteria. The triggering event in the activation of complement via the classical pathway involves the binding of the Fc region of aggregated IgG1, 2, 3 or IgM to the C1q component of C1, and culminates in the formation of a membrane attack complex that mediates target cell lysis. The multivalency of C1q, each of six subunits possessing its own Fc binding site, has been proposed to explain the strong enhancement of binding to complexed IgG [7]. The motif on IgG that binds C1q involves a number of charged residues: Glu 318, Lys 320 and Lys 322 [8]. In close proximity to this binding motif lies a fold at proline 332 which also contributes to the IgG–C1q binding site [9]. Furthermore, studies on the role of the glycoform using a mouse/human chimeric antibody have demonstrated that the

absence of the carbohydrate moiety leads to deficiencies in C1q binding and activation [10].

Physicochemical abnormalities have been reported in IgG isolated from rheumatoid patients, and we have previously shown that ROS can induce such modifications [11]. Herein we have studied IgG isolated from rheumatoid biofluids for differences in C1q binding capacity compared to IgG isolated from non-inflammatory control sera. In addition, we have investigated the effects of neutrophil-derived oxidants on the capacity of IgG to bind C1q in order (a) to examine the effects of different ROS and (b) to investigate the possibility of ROS-induced functional modifications during inflammation. Such a denatured IgG molecule may act in a proinflammatory manner in rheumatoid arthritis through increased activation of complement.

2. Materials and methods

2.1. Materials

All biochemical and immunochemical reagents were of the highest grades available from Sigma (Poole, UK) unless otherwise stated. Polyclonal IgG, and C1q were from CalBiochem (Nottingham, UK). Phosphate-buffered saline pH 7.2 (PBS) containing 0.05% Tween 20 (PBST) was used as a washing buffer between all ELISA steps. Concentrated sulphuric acid and buffer salts were of Analaar grade from BDH (Poole, UK). Nunc-Immuno plates were supplied by Gibco-BRL.

2.2. Patients

Specimens used in this study were obtained from patients with classical rheumatoid arthritis (as defined by the American Rheumatism Association) attending rheumatology clinics at Selly Oak Hospital, Birmingham (mean age = 58, range 37–81). Patients receiving steroids were excluded from the study. Elderly control sera were obtained from informed volunteers (mean age 67 years) from orthopaedic patients within the South Birmingham Health Authority.

2.3. Isolation of IgG from biological fluids

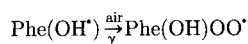
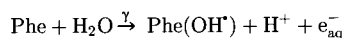
For the isolation of IgG from rheumatoid synovial fluids, the viscosity of the fluid was initially reduced by incubation overnight with hyaluronidase. (This procedure had no effect on the C1q binding activity of hyaluronidase-treated serum samples when compared to untreated control sera.) The resultant samples were then extracted as for serum samples. The isolation method has been described by Perosa et al. [12], and briefly involves the caprylic acid-induced precipitation of IgG from serum. The purity of the IgG preparation was confirmed by immunoelectrophoresis.

2.4. Denaturation of IgG in vitro

In order to generate defined radical species in solution, IgG (1 mg/ml in 40 mM phosphate buffer, pH 7.2) was exposed to steady-state γ radiolysis as previously described [13], for doses of 0, 250, 500 and 1000 Gy at a rate of 11 Gy/min. In aerated solution, a mixture of hydroxyl radical (OH^\bullet) and superoxide anion radical ($\text{O}_2^{\bullet-}$) are produced in a 2:1 ratio. The incorporation of 100 mM formate into the irradiation buffer results in the generation of the $\text{O}_2^{\bullet-}$ alone. The presence of 10 mM phenylalanine leads to the scavenging of all OH^\bullet and $\text{O}_2^{\bullet-}$ to generate the peroxy radical (ROO^\bullet) according to

*Corresponding author. Fax: (44) (116) 252-5593.

the equations below [14]:



To mimic the site-specific generation of OH^\bullet at metal ion binding sites on a protein backbone, 20 μM copper sulphate was incorporated into 0.01 M PBS containing IgG (1 mg/ml) followed by the addition of increasing doses of H_2O_2 ($\text{Cu}/\text{H}_2\text{O}_2$).

To simulate the primary source of radicals at the inflammatory site, polymorphonuclear leukocytes (PMNs) were isolated from the peripheral venous blood of normal healthy volunteers, and activated in vitro using phorbol myristate acetate (PMA). Briefly, PMNs were isolated by differential centrifugation using mono-poly resolving medium and contaminating erythrocytes were lysed using Tris-ammonium chloride buffer pH 7.2. Leishman's stained smears showed greater than 90% of cells to be PMNs. Following three washes in RPMI 1640, PMNs were resuspended to a final concentration of 2×10^6 viable cells per ml in the presence of 20 $\mu\text{g}/\text{ml}$ cytochalasin B. The cell suspensions were preincubated at 37°C for 15 min prior to activation with 10 ng/ml PMA. To denature IgG, PMNs were activated as described in the presence of 1 mg/ml IgG, whilst control experiments were carried out with IgG and PMNs in the absence of a stimulus. The amount of $\text{O}_2^{\bullet -}$ generated (in nmol/ 10^6 cells) was calculated as the superoxide dismutase inhibitable reduction of 160 μM cytochrome *c* from PMA-activated cells in the absence of IgG, given that the latter has an extinction coefficient of $21 \times 10^3 \text{ mol}^{-1} \text{ cm}^{-1}$ at 550 nm. IgG was also denatured using hypochlorous acid (HOCl) freshly prepared from sodium hypochlorite in water. HOCl concentration was determined spectrophotometrically using the molar extinction coefficient of 350 M^{-1} at 290 nm, and IgG was treated with 0–200 μM HOCl for 1 h at 37°C.

2.5. C1q binding assay

Using a modified ELISA procedure, 50 μl human C1q (10 $\mu\text{g}/\text{ml}$) was coated onto Nunc microtitre plates at pH 9.6 in carbonate/bicarbonate buffer for 1 h at 37°C. After 3 washes in PBST, unoccupied sites on the plates were blocked for 1 h at 37°C using 1% bovine serum albumin. IgG (1 mg/ml in PBS) was allowed to interact with the immobilised C1q ligand, and the degree of binding was assessed using a peroxidase-labelled anti-human IgG diluted 1:1000 in PBST. No reaction was observed between the peroxidase-labelled anti-human IgG and the immobilised C1q. After washing, the reaction of peroxidase with 50 μM H_2O_2 and 20 mg *ortho*-phenylene diamine was allowed to proceed at room temperature for 30 min, and was stopped by the addition of 2 M H_2SO_4 . The plate was then read at 492 nm in a Titertek multiscan.

2.6. Statistical analysis

Statistical analysis was carried out using Student's *t*-test for in vitro studies, and a parametric distribution of results was assumed. A non-parametric paired comparison test, Wilcoxon matched pairs, was used for assessing the differences between synovial fluid IgG and paired serum IgG.

3. Results

3.1. C1q binding activity of IgG isolated from biofluids

The interaction of isolates of IgG from rheumatoid serum

and synovial fluid (SF) with C1q is shown in Fig. 1. Rheumatoid SF IgG was found to bind more effectively to C1q than its paired serum IgG, but no significant difference was observed between rheumatoid serum IgG and normal serum IgG.

3.2. The effects of steady-state γ radiolysis on IgG–C1q interaction

The effects of different radiolytically generated ROS on the ability of IgG to interact with C1q are shown in Fig. 2. Superoxide radical treatment had no significant effect on the C1q binding capacity of IgG. However, in the presence of OH^\bullet , there was a significant increase in the ability of IgG to bind to C1q up to a dose of 500 Gy. After a dose of 1000 Gy, this effect was less marked and binding was only increased by 20% above control levels. In contrast, ROO^\bullet induced a dose-dependent linear increase in the C1q binding capacity of IgG at all doses studied, giving a 30% increase after exposure to 1000 Gy. $\text{O}_2^{\bullet -}$ had no effect on IgG binding to C1q at any of the doses studied.

3.3. The effects of site-specific generation of ROS by $\text{Cu}/\text{H}_2\text{O}_2$ on IgG–C1q interaction

Following the addition of H_2O_2 to IgG in the presence of Cu, a significant loss in reactivity with C1q was observed (see Table 1). The loss of 50% of C1q binding activity occurred after exposure to 200 μM H_2O_2 , and further increasing the H_2O_2 concentration had no effect (data not shown).

3.4. The effects of HOCl on IgG–C1q binding

The treatment of IgG with HOCl had no effect on its subsequent interaction with C1q (see Table 1).

3.5. The effects of activated PMNs on IgG–C1q interaction

The binding of PMN-denatured IgG to C1q was found to be significantly lowered by 26% compared to that of the native molecule ($P < 0.01$, $n = 4$) using paired comparison analysis (see Table 2).

4. Discussion

One of the consistently observed features of rheumatoid arthritis is the local consumption of complement by joint fluids, with the corresponding appearance of C3 activation products [15,16]. Indeed, complement fixation by immune complexes has been considered to play a central role in the pathogenesis of rheumatoid inflammation. The presence of high concentrations of aggregates could partially explain this observation. Another possibility is the occurrence of a denatured form of IgG with a higher affinity for C1q, and we have

Table 1
Effects of denaturation of IgG by ROS on the subsequent interaction of IgG with C1q: the effects of hypochlorous acid and hydrogen peroxide on IgG interaction with C1q

ROS	IgG bound to C1q—absorbance at 492 nm \pm SEM $\times 10^3$				
	Concentration (μ M)	0	20	50	200
Hydrogen peroxide with copper (20 μ M)		115 \pm 8	94 \pm 14*	65 \pm 6**	56 \pm 11**
Hypochlorous acid		99 \pm 10	101 \pm 15	95 \pm 2	96 \pm 2

The results show the amount of IgG bound to immobilised C1q determined spectrophotometrically at 492 nm following treatment of IgG with HOCl and H_2O_2 .

* $P < 0.05$, ** $P < 0.01$ compared with untreated controls ($n = 3$).

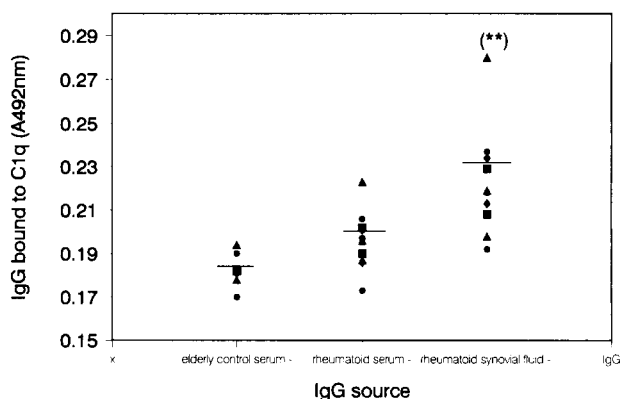


Fig. 1. The ability of IgG isolated from elderly and rheumatoid sera, and rheumatoid synovial fluid to bind C1q in the modified ELISA. Results are the means of triplicate determinations for each isolate, and the mean for each group is indicated by a horizontal bar. Paired comparison analysis was used to statistically analyse the paired sera and SFs, where 80% of SF IgGs gave higher values than serum; ** $P < 0.01$.

investigated whether ROS-induced damage to IgG can mediate such changes.

When a comparison is made between isolated IgG from rheumatoid serum and paired synovial fluids, an increase in binding capacity is seen in the latter. This may be due to the presence of IgG rheumatoid factors in the isolated samples, which lead to autoaggregation [17] and thus an increase in the valency of the IgG molecules involved in C1q binding. Conversely, the higher binding activity of SF IgG compared to serum may reflect isotypic variation between the samples; IgG3 is secreted at higher levels in the SF [18] and this isotype has a much higher affinity constant for C1q than the major paraprotein IgG1 [19]. However, increases in autofluorescence in synovial fluid IgG are observed characteristic of ROS-induced damage, supporting the hypothesis that ROS may be important in modifying the physicochemical properties and the binding activities of IgG [6].

The results have shown that OH^\bullet exposure, which we have previously reported as inducing autofluorescence and aggregation of IgG [19], increases the avidity of IgG for C1q up to a dose of 500 Gy and this may be due to the increase in valency of the IgG aggregate. At a higher dose (1000 Gy) this effect was reduced, where binding was only enhanced by 20%, and this may reflect a gross disruption in the conformation caused by many molecular changes and the total aggregation of the monomeric form [20]. Exposure of IgG to the longer lived ROO^\bullet [14], which has similar properties to peroxy moieties generated during lipid peroxidation, induced a linear dose-dependent increase in C1q binding. Our studies have pre-

Table 2

Effects of denaturation of IgG by ROS on the subsequent interaction of IgG with C1q: the effect of activated polymorphonuclear leukocytes on IgG and its interaction with C1q

IgG treatment	Mean C1q binding activity—absorbance at 492 nm \pm SEM
IgG + PMNs	0.217 \pm 0.014
IgG + PMNs + PMA	0.183 \pm 0.09*

The C1q binding activity of IgG which has been exposed to activated and resting PMNs is shown as mean absorbance readings at 492 nm \pm SEMs generated in the modified ELISA.

* $P < 0.05$ ($n = 4$).

viously shown that ROO^\bullet attack results in autofluorescence but not aggregation. The proposed C1q binding site lies in the following sequence: glu-tyr-lys-cys-lys, and the observed elevation in reactivity with C1q may be due to cleavage of disulphide bridges or increases in hydrophobicity following ROO^\bullet treatment. Increases in hydrophobicity are common following ROS damage to proteins [21], and such modifications increase the avidity of protein–protein interactions. Exposure of IgG to $\text{O}_2^{\bullet-}$ did not affect IgG and C1q interaction. Previously we have reported that $\text{O}_2^{\bullet-}$ is unable to induce any changes in the macromolecular structure of IgG [19]. Chemical and physicochemical studies have shown that $\text{O}_2^{\bullet-}$ is rarely damaging per se and is damaging indirectly in aqueous solution only through the more reactive radical species, OH^\bullet [22].

Catalytic metal ion availability has long been implicated in ROS generation in situ. Site-specific generation of OH^\bullet on IgG by H_2O_2 in the presence of Cu via the Fenton reaction reduced the subsequent interaction of IgG with C1q. This effect was not seen when IgG was incubated with copper in the absence of H_2O_2 (data not shown). Altered binding may reflect ROS-induced changes in critical amino acid residues. A recent report has described increased levels of copper in the synovial fluid of rheumatoid patients [23]. This is largely complexed to albumin providing a sink for site-specifically generated ROS. A comparison between our data on the C1q binding activity of SF IgG and Cu- H_2O_2 -treated IgG supports the observation that copper chelation with subsequent local OH^\bullet production on IgG is unlikely to play a role in the altered C1q binding observed in rheumatoid arthritis.

Exposure of IgG to HOCl does not affect C1q binding capacity, but at concentrations > 1 mM, HOCl can induce monomeric autofluorescence in IgG in a similar manner to that described for ROO^\bullet [20]. This indicates that whilst fluorescence can result from exposure to an oxidative flux, changes to the residues involved in fluorescence (e.g. tryptophan, tyrosine) are unlikely to account for altered C1q binding.

Isolated polymorphs activated in vitro to produce $\text{O}_2^{\bullet-}$ induce denaturative changes in IgG similar to those induced by the Fenton system. H_2O_2 is the secondary ROS generated from the dismutation of $\text{O}_2^{\bullet-}$, and its increased hydrophobicity and half-life when compared with other ROS facilitate its reactivity with metal ions at distant sites.

We have previously shown that OH^\bullet can cause a loss in

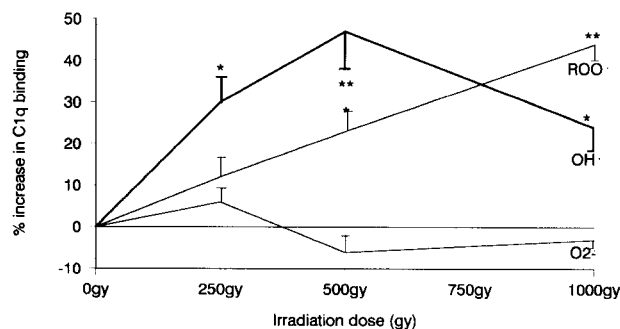


Fig. 2. The percentage change in binding of modified IgG to C1q is shown for OH^\bullet , ROO^\bullet , and $\text{O}_2^{\bullet-}$ treated IgG. Steady-state γ radiolysis generates specific radicals in solution, where an increase in irradiation dose is paralleled by an increase in ROS concentration. The results are expressed as the mean \pm SEMs of three experiments, and compared with the unirradiated control; * $P < 0.05$, ** $P < 0.01$.

galactose on the IgG glycoform [12], a phenomenon also observed in rheumatoid IgG. Whilst a study of an aglycosylated mouse/human chimeric antibody has confirmed a loss in C1q binding and complement activation [10], it remains to be elucidated whether the deletion of a single galactose moiety could modulate C1q binding effects.

Further studies on the appearance of complement products are required before implications can be made about the pathological consequences of ROS denaturation of IgG and the interaction of denatured IgG with the complement cascade. However, this study has shown that IgG has increased C1q binding activity in rheumatoid synovial fluid. We suggest that specific residues in the CH2 domain of IgG are affected by ROS, the nature of the effect being governed by the radical species.

Acknowledgements: The authors gratefully acknowledge financial support from the ARC for this work. Specimens were kindly provided by Dr P. Emery.

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