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Oxygen radical induced alterations in polyclonal IgG

H. R. Griffiths, J. Lunec, C. A. Gee* and R. L. Willson+

*Departments of Biochemistry and Rheumatology, Selly Oak Hospital, Raddlebarn Road, Birmingham B29 6JD, *Department of Rheumatology, The Medical School, University of Birmingham, Birmingham B15 2TJ* and +*Department of Biochemistry, University of Brunel, Uxbridge, England*

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In the sera and synovial fluid of patients with rheumatoid arthritis, part of the IgG fraction is found in an aggregated and fluorescent form. Oxygen-free radicals have been implicated in this denaturation, although the precise radical species responsible is unknown. In this work, oxygen-free radicals generated radiolytically were allowed to attack polyclonal IgG in solution. $OH^{\cdot}$ radicals induced aggregation of the monomer and a new fluorescence appeared in the visible region (Ex 360 nm, Em 454 nm). The superoxide radical anion was found to be inert in both these respects, whilst peroxy radicals induced autofluorescence without concomitant aggregation. The results suggest that $OH^{\cdot}$ and/or peroxy radical attack may be an in vivo mechanism for IgG denaturation.

Oxygen-free radical; Fluorescence; IgG

1. INTRODUCTION

Nearly all known biomolecules are susceptible to oxygen radical attack [1,2]; lipids become peroxidised [3,4], carbohydrates degraded [5] and proteins oxidised [6–8]. The free radical oxidation of amino acids essential for biological function has been shown to alter the activities of α-1-antitrypsin [9], alcohol dehydrogenase [10] and lysozyme [11]. Similarly the function of caeruloplasmin as an antioxidant is reduced on oxidation, and the protein aggregates [12].

In vitro studies have shown that exposure of the antibody IgG to activated neutrophils, peroxidizing lipids or UV irradiation induces aggregation of the monomeric form and the appearance of a characteristic autofluorescence (Ex 360 nm, Em 454 nm), which is not present in the native molecule [13]. Such changes have been implicated in the perpetuation of rheumatoid inflammation. However, the actual damaging free radical species has not previously been identified.

Using steady-state radiation chemistry techniques to generate specific radical species, we have identified the free radicals responsible for aggregation and autofluorescence of polyclonal IgG.

Correspondence address: H.R. Griffiths, Departments of Biochemistry and Rheumatology, Selly Oak Hospital, Raddlebarn Road, Birmingham B29 6JD, England

2. MATERIALS AND METHODS

2.1. *Materials*

Lyophilised polyclonal human IgG, purified from Cohn fractions II and III, was supplied by Sigma. Buffer salts were obtained from either Sigma or British Drug Houses. All solutions were prepared in millipore filtered water.

2.2. *Irradiation procedures*

Free radicals were generated in air saturated solutions using a cobalt-60-gamma source [14,15]. Polyclonal IgG was irradiated at a concentration of 2.5 mg/ml in 40 mM phosphate buffer pH 7.4 in the presence and absence of either 200 mM formate or 10 mM phenylalanine. Doses of 1000 and 2000 Gy were used, at a dose rate of ~11 Gy per min. Tubes were agitated every 20 min to reoxygenate the solutions. All samples were subsequently put on ice and analysed within 24 h.

2.3. *HPLC analysis*

Control and irradiated IgG samples were applied to a TSK 3000 SW column and eluted at a flow rate of 1 ml/min with

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phosphate buffer (0.067 M KH_2PO_4, 0.1 M KCl, pH 5.4) as the mobile phase. The elution of native and free radical altered IgG was monitored using a Gilson fluorimeter with an *o*-phthalaldehyde filter, with maximum excitation and emission wavelengths of 360 and 454 nm, respectively. Simultaneous UV detection of protein peaks at 280 nm was performed by a Gilson variable wavelength detector. Calibration of the column was achieved using standards of known molecular mass (see fig.1).

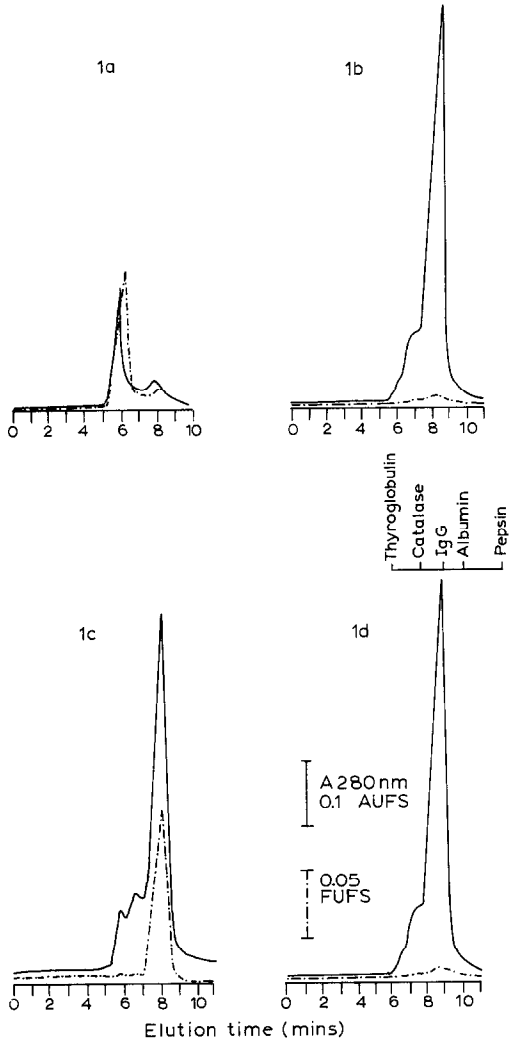

Fig.1. Effect of γ radiation on polyclonal IgG. This figure shows typical gel filtration chromatographs of IgG treated with (a) hydroxyl radicals in the presence of superoxide, (b) superoxide anions, (c) peroxy radicals and (d) the native protein. The IgG products were monitored by UV absorbance at 280 nm (——) and fluorescence (-----) at 454 nm following excitation at 360 nm. Molecular mass calibration of the column was achieved using thyroglobulin (670 kDa), catalase (240 kDa), IgG (150 kDa), albumin (60 kDa) and pepsin (30 kDa).

3. RESULTS

The effects of gamma irradiation on polyclonal IgG in air-saturated solution are shown in fig.1a. Under such conditions the predominant species present are the hydroxyl radical $OH^{\cdot}$ and the superoxide anion $O_2^{\cdot -}$ formed from the action of gamma rays on water molecules:

$$H_2O \longrightarrow OH^{\cdot} + e^-_{(aq)} + H_3O^+ \quad (1)$$

$$e^-_{(aq)} + O_2 \longrightarrow O_2^{\cdot -} \quad (2)$$

Gamma irradiation of polyclonal IgG in air saturated solution resulted in protein peaks corresponding to molecular masses of 10^6 Da, 3×10^5 Da and 1.5×10^5 Da following gel filtration chromatography. These protein fractions represent aggregates, dimers and the monomeric form of IgG. Gross aggregation seen at high doses rendered the protein insoluble, resulting in an equivalent loss of UV absorption at 220 nm (peptide bond absorption max.) and 280 nm (tryptophan absorption max.). Irradiation also induced a characteristic autofluorescence in all protein fractions with an emission maximum at 454 nm following excitation at 360 nm. Fragmentation of the monomer molecule into lower molecular mass components was not seen at the monitor sensitivities used.

The formation of fluorescent species and aggregates appeared to be induced in a dose-dependent manner (fig.2). After a dose of 1000 Gy 54% of the original monomer remained and after a further 1000 Gy only 16% was present in the monomeric form. The ratio of fluorescence to UV absorption similarly was found to be dose dependent.

When irradiations were carried out in the presence of 0.2 M formate, neither autofluorescence nor aggregation was observed (fig.1b). In this system the hydroxyl radicals formed in eqn 1 are scavenged by formate, forming carboxylate anions (eqn 3). These in turn react rapidly with oxygen, forming superoxide (eqn 4):

$$OH^{\cdot} + HCO_2^- \longrightarrow CO_2^{\cdot -} + H_2O \quad (3)$$

$$CO_2^{\cdot -} + O_2 \longrightarrow O_2^{\cdot -} + CO_2 \quad (4)$$

Hence the IgG molecules are exposed to twice the concentration of superoxide radicals as were pre-

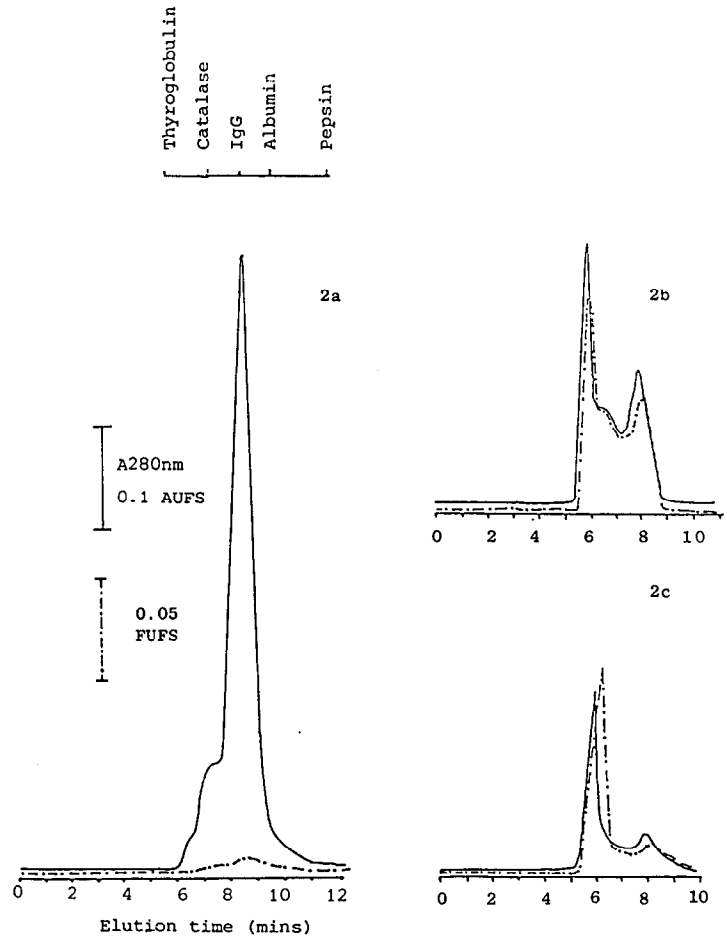

Fig.2. Dose-dependent changes in the fluorescence and aggregation of IgG induced by γ radiolysis. This figure shows the dose dependency of oxygen free radical treatment with respect to the induction of aggregation and fluorescence in IgG. In this case IgG was irradiated in aerated solution at doses of (a) 0 Gy, (b) 1000 Gy and (c) 2000 Gy.

sent in the previous system (see results in fig.1a). As no alteration of the native protein was observed under these conditions, the changes observed in fig.1a were attributed to the reactions of the hydroxyl radical.

The presence of 10 mM phenylalanine at the time of irradiation results in the formation of an oxygen-dependent phenylalanine radical, probably a peroxy radical [9]. Fig.1c shows that the attack by this species induces fluorescence in the monomer molecule but with no concomitant aggregation. Similarly, a thymine-derived peroxy species mimicked these effects i.e. fluorescence without aggregation (not shown). On the chromatogram (fig.1c), a low molecular mass peak can be observed corresponding to a phenylalanine aggregate formed on irradiation. However, this eluted 5 min after IgG and showed no interference with IgG analysis.

Fig.1d illustrates an HPLC profile which is representative of all controls performed in these experiments, indicating that there were no solute effects on the protein. IgG added to pre-irradiated solutions caused no physicochemical changes to the molecule, demonstrating that fluorescence and aggregation are not due to post-irradiation products such as hydrogen peroxide or organic peroxides.

4. DISCUSSION

In this work we have demonstrated that peroxy radicals induce fluorescence but not aggregation in IgG. The formation of fluorescent IgG aggregates is mediated by $OH^{\cdot}$ but not an $O_2^{\cdot -}$ under these conditions. This may be of particular importance during rheumatoid arthritis where the elevated concentrations of IgG produced by active synovial B cells [16] are a likely target for radicals released from invading polymorphs and macrophages. Fluorescent aggregates of IgG have been detected in the synovial fluids of rheumatoid patients and it is believed that the presence of circulating autoantibodies to IgG (rheumatoid factors), is a consequence of IgG denaturation in vivo [17,18]. Previously there has been no widely accepted mechanism for this observed denaturation, but here we have clearly demonstrated that the fluorescent aggregates are similar to those produced following $OH^{\cdot}$ attack. Using these techniques we are currently investigating whether $OH^{\cdot}$ mediated aggregation or fluorescence induction alone can induce antigenic changes in IgG. Such antigenic changes can facilitate immune complex formation with rheumatoid factor and thereby perpetuate the inflammatory process.

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