Self-perpetuating Mechanisms of Immunoglobulin G Aggregation in Rheumatoid Inflammation

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Abstract

When human IgG is exposed to free radical generating systems such as ultraviolet irradiation, peroxidizing lipids, or activated human neutrophils, characteristic auto-fluorescent monomeric and polymeric IgG is formed (excitation [Ex], 360 nm, emission [Em], 454 nm). 1 h ultraviolet irradiation of IgG results in the following reductions in constituent amino acids; cysteine (37.0%), tryptophan (17.0%), tyrosine (10.5%), and lysine (3.6%). The fluorescent IgG complexes, when produced in vitro, can stimulate the release of superoxide from normal human neutrophils. In the presence of excess unaltered IgG, further fluorescent damage to IgG occurs. Measurement and isolation of fluorescent monomeric and polymeric IgG by high performance liquid chromatography, from in vitro systems and from fresh rheumatoid sera and synovial fluid, indicates that identical complexes are present in vivo; all these fluorescent complexes share the property of enhancing free radical production from neutrophils. The results described in this study support the hypothesis that fluorescent monomeric and aggregated IgG may be formed in vivo by oxygen-centered free radicals derived from neutrophils, and that in rheumatoid inflammation this reaction may be self-perpetuating within the inflamed joint.

Introduction

Oxygen-centered free radicals (i.e. O_2^- and $OH^\cdot$) can be released by activated neutrophils in response to cell surface stimulation, by a variety of particulate and nonparticulate substances (1–5). Such highly reactive chemical species have the potential to denature proteins (6), oxidize lipids (7), damage DNA, and denature virtually all types of biomolecules (8, 9).

Ever since free radical reactions were first implicated in such processes, their relevance to the development of human pathological states has been sought (for review see reference 10). McCord and others (11, 12) have suggested that in rheumatoid inflammation, which is characterized by a large infiltration of phagocytic cells into the inflamed joint, neutrophils may release oxygen radicals into extracellular fluid and damage its macromolecular components. Such elements of the synovial fluid are normally unprotected by intracellular antioxidant enzymes such as superoxide dismutase (SOD),[1] catalase, and glutathione peroxidase (11).

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1. *Abbreviations used in this paper:* Em, emission; Ex, excitation; HPLC, high performance liquid chromatography; PMA, phorbol myristate acetate; SOD, superoxide dismutase.

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We have shown previously that human IgG undergoes fluorescent and sulphhydryl-related damage when exposed to free radical reactions (13, 14). The fluorescence formation is thought to be related to selective hydroxylation and destruction of aromatic amino acid constituents of the protein (15, 16). In this report we have characterized the nature of the fluorescent changes in IgG that are induced by free radical reactions in vitro and identified similar fluorescent products in fresh human sera and synovial fluid, using high performance liquid chromatography (HPLC).

Methods

Chemicals. Human IgG (Cohn fraction II) phorbol myristate acetate (PMA), mannitol, thiourea, catalase, horse ferricytochrome *c* and cytochalasin B were obtained from Sigma Chemical Co., St. Louis, MO. Protein molecular weight markers for chromatography were obtained from Pharmacia Fine Chemicals, Piscataway, NJ. Desferrioxamine was obtained from Ciba Geigy Corp., Pharmaceuticals Div., Summit, NJ. All other chemicals were of Analar grade and purchased from British Drug Houses Chemicals Ltd., Poole, Dorset, United Kingdom.

Fluorescence measurements. All fluorescence measurements were performed on an MPF-3L-spectrofluorimeter (Perkin Elmer Corp., Beaconsfield, Herts, United Kingdom). The instrument settings were as follows: excitation and emission slits, 12 and 14 nm, respectively; sensitivity settings ranged from 1 to 30×; wavelength calibration was performed with quinine sulfate (10 mmol/liter in 100 mmol/liter H_2SO_4); fluorescence intensity calibration was made with a polymer block standard (block 5 compound 610, Perkin-Elmer Corp., approximate concentration 5×10^{-5} mol/liter). Filters of 310, 190, and 430 nm were used as appropriate and all measurements were made at ambient temperature.

Preparation of free radical altered IgG. In all experiments, human IgG was dissolved in 40 mmol/liter phosphate buffer (KH_2PO_4/K_2HPO_4), pH 7.4, to give a concentration of 2.5 mg/ml. 3-ml vol were irradiated (366- + 254-nm source) in matched quartz cuvettes, 1 cm^2 in cross-section, or in open Petri dishes at a distance of 6 cm from the light source. Fluorescent alteration of IgG was also induced in separate experiments as follows: (*a*) 100 μl of a mixture of copper sulfate (0.5 mmol/liter) plus hydrogen peroxide (100 mmol/liter) was added to 3 ml of IgG (2.5 mg/ml) and incubated at 25°C for 30 min (13). (*b*) Arachidonic acid was sonicated (Rapidus Ultrasonics, Shipley, Yorks, United Kingdom) in phosphate-saline buffer (KH_2PO_4/K_2HPO_4, 40 mmol/liter; NaCl 150 mmol/liter) pH 7.4, to give a suspension of 3 mmol/liter. 5 ml of the suspension was then removed and added to an equal volume of 2.5 mg/ml IgG and incubated at 37°C for 1 h.

Neutrophil experiments. Neutrophils were isolated from peripheral blood of healthy human volunteers by employing standard techniques of Ficol-Hypaque sedimentation and ammonium chloride lysis of erythrocytes. To induce free radical damage to IgG, neutrophils (4 ml of 4×10^6 cells/ml suspended in phosphate-buffered saline plus 2 mM glucose) were incubated with an equal volume of 2.5 mg/ml IgG. Activation of cells was performed by adding PMA to a final concentration of 10 μg/liter. Cells with and without PMA were then incubated with pure IgG for up to a period of 1 h. In one series of experiments, the cells (4 ml of 4×10^6 cells/ml) were incubated with 500 μl of ultraviolet-altered IgG in the presence of excess unaltered IgG (4 ml of 2.5 mg/ml IgG) at 37°C in order to test the hypothesis that free radical alteration of IgG might result in a perpetual production of fluorescent IgG mediated by the neutrophil. At various time periods, 200-μl aliquots were removed, the cells were separated by centrifugation, and the supernatant assayed for fluorescence (Ex 360 nm, Em 454 nm).

Neutrophil superoxide production. A continuous assay of superoxide generated by activated neutrophils was performed according to the method of Cohen and Chovaniec (17). Neutrophil suspensions (1 ml of 2×10^6 cells/ml) were added to an equal volume of phosphate-buffered saline (40 mM KH_2PO_4/K_2HPO_4/150 mM NaCl; pH 7.4) containing 2 mM glucose and 100 μM ferricytochrome *c*. After time was allowed for temperature equilibration (37°C), 500 μl of the test IgG material (i.e., free radical altered IgG isolated by HPLC) was added.

A second identical solution, containing in addition 100 μg/ml of SOD, was also prepared. The mixture was placed in the blank compartment of a double beam spectrophotometer. In this way, continuous assay of superoxide-dependent cytochrome *c* reduction could be monitored in the test sample at a constant temperature of 37°C for the duration of the reaction.

High performance liquid chromatography. Separation of samples of synovial fluids, sera, and IgG samples were performed on a TSK 3000 SW column. The proteins were eluted at 1 ml/min with phosphate buffer as mobile phase (0.067 M KH_2PO_4 + 0.1 M KCl). The elution of free radical altered IgG products was monitored with a fluorimeter (Gilson Instruments Ltd., Villiers le Bel, France). An *o*-pthaldehyde filter allowed detection of protein peaks with maximum excitation and emission of 360 and 454 nm, respectively. Detection of proteins at 280 nm was also performed simultaneously using a Uvicord spectrophotometer (Pye Unicam).

Amino acid and carbohydrate analysis. A Locarte amino acid analyzer was used for the measurement of amino acids. Samples of IgG were hydrolyzed in sealed tubes at 6 M HCl at 110°C for 24 h. Oxygen was removed from the sample by degassing. For tryptophan, which is entirely destroyed by acid hydrolysis, high yields were recovered from proteins by hydrolysis of protein in 4 M $Ba(OH)_2$. The sample, at low pH, was injected into a column of strong cation exchange resin by means of an automatic loader. The amino acids were then eluted from the column by a stepwise gradient of increasing pH and ionic strength. The column eluent was mixed with a stream of ninhydrin reagent, reacted at 100°C, then detected at 570 nm (440 nm for proline). The amino acids were identified by their elution position and quantified by integration of the chromatogram.

Neutral sugars were separated by ion exchange chromatography of their borate complexes on a strong anion exchange resin. The mixture of sugars to be analyzed were obtained from IgG preparations after hydrolysis (as described above), and injected on the resin bed in a solution of 0.13 M boric acid. A series of borate buffers of increasing pH and ionic strength then eluted the sugar-borate complexes. The sugars were visualized by mixing the eluent with a stream of orcinol/concentrated sulfuric acid reagent, heating at 95°C for 15 min followed by detection in a flow cell at 425 nm in a modified Jeol amino acid analyzer.

Clinical samples. Sera and synovial fluids were obtained for diagnostic or therapeutic purposes from patients (age range 27–85; mean 49) with classical or definite rheumatoid arthritis (according to American Rheumatism Association criteria) and from patients with other forms of arthritis (nonrheumatoid) presenting at the Department of Rheumatology, The Medical School. Normal control serum specimens were obtained from laboratory staff (age range: 35–60 yr; mean 43 yr).

For the purpose of the present study, synovial fluids were divided into two groups: (*a*) a nonrheumatoid series that was characterized mainly by clinical and/or radiological evidence of degenerative arthritic lesions, but also included three fluids from patients with inflammatory (one ankylosing spondylitis, two psoriatic arthritis) seronegative arthritis; (*b*) a rheumatoid group classified according to American Rheumatism Association criteria. No attempt was made to score the activity of the disease at the time of sampling. All patients were receiving conventional analgesic/anti-inflammatory therapy.

Statistical methods. The statistical analysis of significance for paired data was performed using a *t* test.

Results

Changes in the physical and chemical nature of IgG induced by free radicals. When human IgG was exposed to ultraviolet irradiation, the protein became characteristically fluorescent (excitation [Ex] 366 nm, emission [Em] 454 nm) and aggregated. Fig. 1 describes the molecular weight and interrelationship of fluorescent IgG protein complexes produced by irradiating IgG for up to 60 min. The fluorescence (Ex 360 nm, Em 454 nm) and ultraviolet (280 nm) elution profiles in this figure describe the successive production of fluorescent monomeric IgG, intermediate aggregates (dimers), and high molecular weight polymers ($>10^6$ [one million] mol wt) as identified by HPLC. After 90 min ultraviolet irradiation, fluorescent monomer formation plateaued, while fluorescent aggregates continued to rise. Low molecular weight fragments consistent with production of heavy and light chains of IgG rather than proteolytic fragments could also be distinguished at higher monitor sensitivities. The relative changes in the proportion of amino acids, measured in IgG over this time period, are illustrated in Fig. 2. The amino acids found most susceptible to free radical attack were cysteine, tryptophan, tyrosine, and lysine; reduction of these amino acids after a total

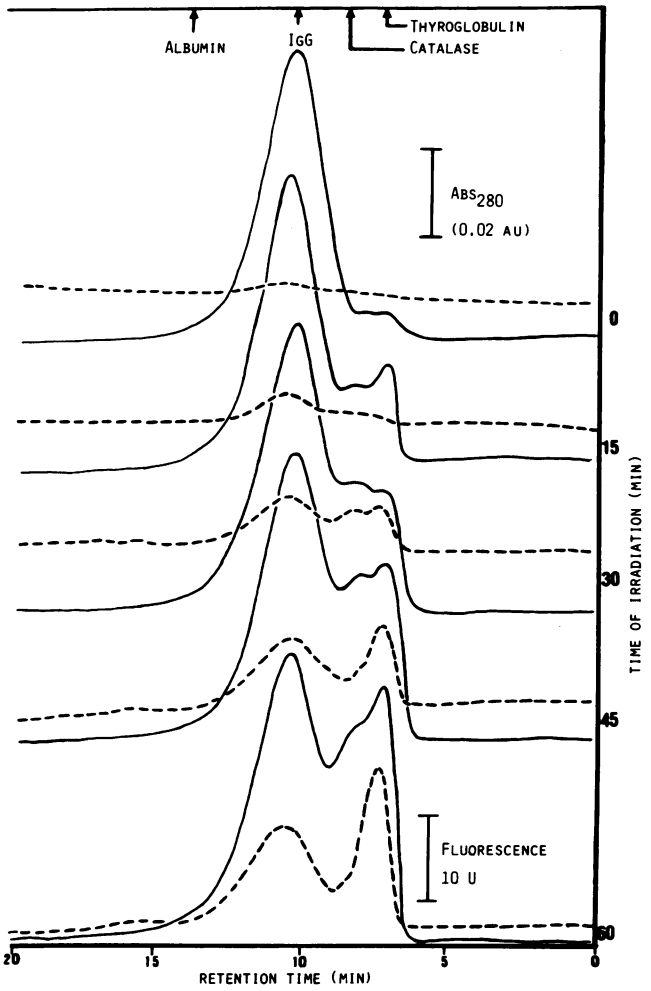

Figure 1. Time relationship between fluorescent monomeric and polymeric free radical reaction products of human IgG during timed intervals of exposure of IgG to ultraviolet light (366- + 254-nm source). Elution of IgG products was monitored simultaneously for absorbance at 280 nm (——) and fluorescence (-----; Ex 360 nm, Em 454 nm). The fluorescence detector was set at range 20×: the maximum setting available was 100×. Molecular weight calibration of the TSK 3000 column was performed by monitoring the elution time of pure standards of albumin (60,000 mol wt), IgG (150,000 mol wt), catalase (230,000 mol wt), and thyroglobulin (600,000 mol wt).

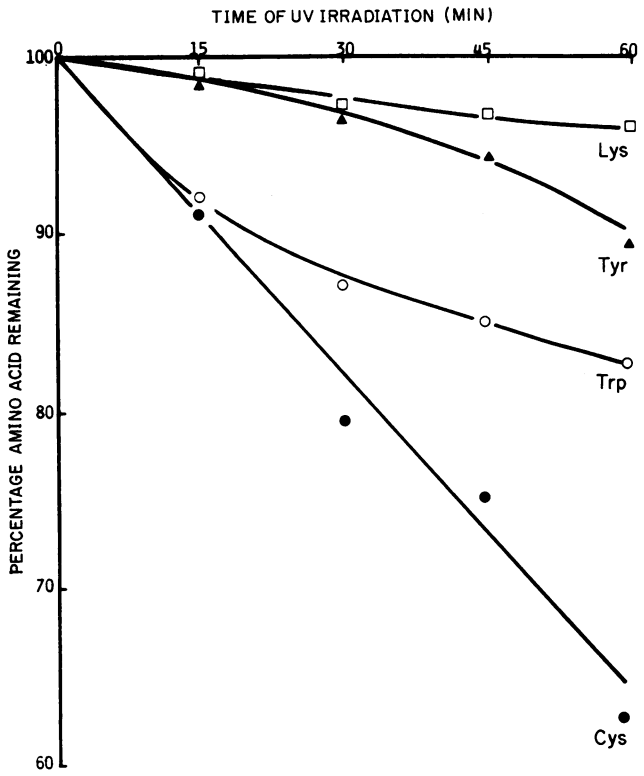

Figure 2. Relative changes in the cysteine (●), tryptophan (○), tyrosine (▲), and lysine (□) content of human IgG following ultraviolet irradiation (Results represent the mean of duplicate analyses.). All other amino acids were not significantly altered over the 60-min duration of ultraviolet irradiation.

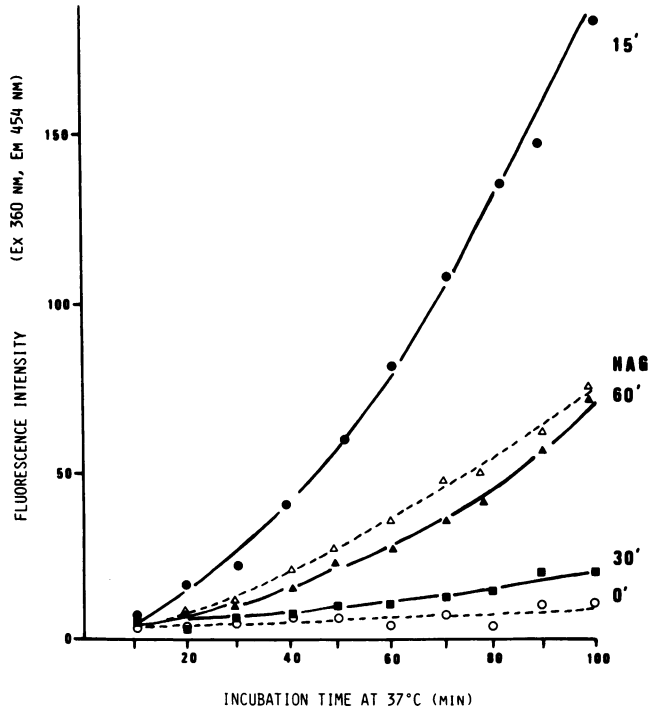

Figure 3. Generation of fluorescent IgG (Ex 360 nm, Em 454 nm) by human neutrophils (2.0×10^6 cells/ml phosphate-buffered saline) incubated at 37°C with normal human IgG that had been irradiated with ultraviolet light (366 + 254 nm) for 0 min (○), 15 min (●), 30 min (■), and 60 min (▲) in the presence of excess unaltered human IgG (final concentration 2.5 mg/ml). Heat-aggregated IgG (15 mins, 63°C) is shown for comparison (△). The fluorescence changes represent fluorescence increases over control values (i.e., identical system incubated without cells). The results are expressed as the mean of four separate experiments.

of 1 h ultraviolet irradiation of IgG was calculated as 37.0, 17.0, 10.5, and 3.6%, respectively.

Self-perpetuation of fluorescent alteration to IgG. Fig. 3 illustrates the relationship between time of irradiation and fluorescent IgG production in the supernatant from activated neutrophils. The results show that irradiation of IgG generates two optima for inducing the further production of fluorescent IgG by activated neutrophils; one after 15 min irradiation of IgG, corresponding to very little aggregate formation, and one at 60 min of irradiation that represented predominantly aggregates of the molecule that had molecular weights in excess of 10^6. In a matched series of experiments (Fig. 4), normal human neutrophils were incubated with IgG that had been exposed to identical time periods of ultraviolet radiation. Superoxide-dependent cytochrome *c* reduction was measured during its incubation with irradiated IgG and cells. Production of superoxide by these neutrophils in response to stimulation by IgG reached a maximum after ~45 min of irradiation. Cells that had been preincubated with the fungal metabolite cytochalasin B, to inhibit vacuole formation, increased their ability to generate superoxide in the presence of free radical altered IgG. This was consistently the case throughout the 90-min period of irradiation, although superoxide production from these cells correlated best with altered fluorescent monomer activity.

In PMA-stimulated neutrophils incubated with native IgG (Table I), subsequent fluorescent IgG formation could be inhibited by SOD (9.5%), catalase (71.0%), thiourea (20%), desferrioxamine (32.8%), and mannitol (4.7%). Catalase was by far the most effective at inhibiting fluorescence formation.

Measurement and isolation of free radical altered IgG by HPLC. Fig. 5 compares the fluorescence HPLC elution profile of free radical altered IgG with typical samples of rheumatoid sera and synovial fluids. Both fluids and sera had significant fluorescence associated with the IgG fraction. This encouraged us to look at a small series of 10 nonrheumatoid and 10 rheumatoid synovial fluids (Table II). Rheumatoid synovial fluids were found generally to have significantly more fluorescence associated with IgG than nonrheumatoid fluids. This was also the case in rheumatoid sera vs. control sera. In both instances this fluorescence remained significantly increased even after correction for IgG concentration. Fluorescent complexes that were indistinguishable by HPLC from those generated by free radical reactions were isolated from both rheumatoid sera and synovial fluid. In order to see whether these individual fractions further resembled in vitro generated products, they were tested for their ability to stimulate neutrophils to generate the superoxide radical. Table III shows the relative activities of fractions separated and isolated by HPLC. Heat-aggregated IgG had lower activity than IgG that had been irradiated, mixed with peroxidizing lipid, or reacted with a copper-hydrogen peroxide mixture. Whole rheumatoid sera and synovial fluids were also able to stimulate neutrophils to generate superoxide; however, the total activities of the fluid and sera were consistently of the order of 50% of the activity of some of the individual monomeric fluorescent protein fractions isolated by HPLC.

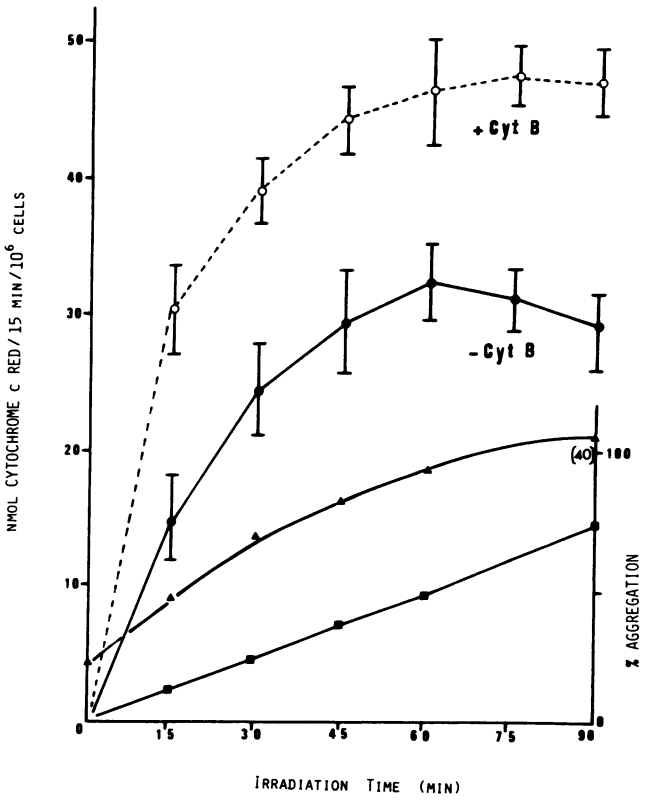

Figure 4. Superoxide (O_2^-) production by human neutrophils activated by IgG that had been ultraviolet irradiated for various time intervals, with (○) and without (●) preincubation with cytochalasin B. Results are ±SEM of 10 experiments. A correlation with the production fluorescent monomer (▲) and aggregates (■) is also shown. Fluorescence scale is shown in parenthesis. Percentage aggregation was determined by calculation of the ratio of aggregate peak (eluting within the void volume [500,000 mol wt] to the ultraviolet absorbance [280 nm]) of untreated monomeric IgG.

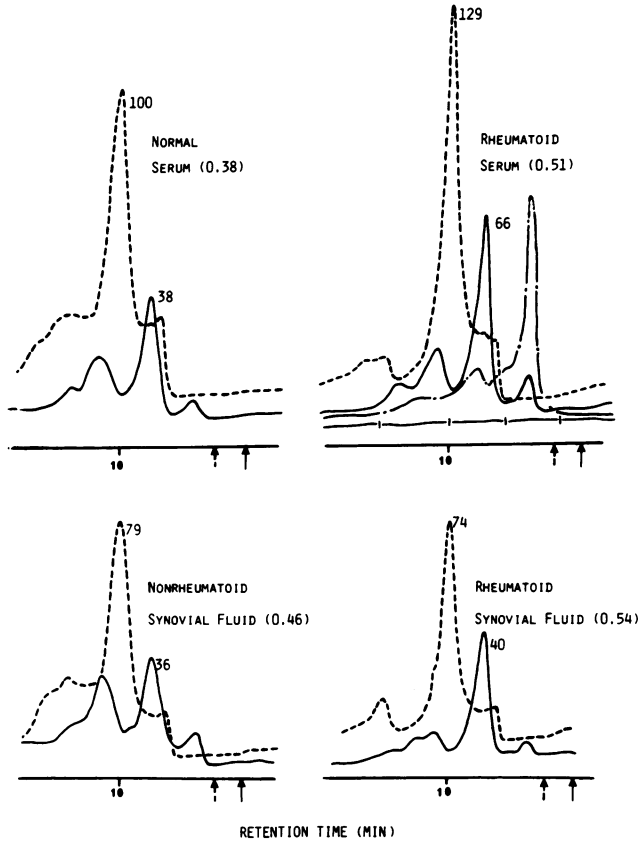

Figure 5. A comparison of the HPLC profiles of rheumatoid synovial fluid, corresponding serum, nonrheumatoid fluid, and normal serum. Fluorescence of ultraviolet irradiated IgG (–··–) and heat-aggregated IgG (–|–) are shown for comparison. Sera and synovial fluids were diluted with mobile phase before injection onto a TSK 3000 SW column and eluted at 1.0 ml/min with buffer (0.067 M KH_2PO_4 + 0.1 M KCl; pH 6.8). Fluorescence monitoring (——) of eluent was carried out at 450–460 nm and ultraviolet monitoring (-----) at 280 nm. Fluorescence and ultraviolet intensity measurements are indicated at the top of IgG peaks. The corresponding fluorescence-ultraviolet ratios are shown in parenthesis. ↑ Indicates injection point. Ultraviolet profiles are displaced by 2 min for the sake of clarity.

Discussion

Previously we have reported that fluorescence (Ex 360 nm, Em 454 nm) and aggregation, which accompanies free radical damage to IgG, occurs at the site of the aromatic amino acids on the IgG molecule as well as at the surface intramolecular disulfide bonds (14). In this report we confirm and extend these findings, and show that the thiol containing amino acid cysteine is the most susceptible to free radical attack. This is consistent with the oxygen radical-dependent reduction and breaking of disulfide

Table I. Fluorescence Generation in Native IgG Caused by PMA Activation of Neutrophils

Inhibitors	Phosphate buffer	SOD	Cat	Thio	Mannitol	DFX
PMA + neutrophils	6.3±2.5	7.5±3.1	15.5±5.3	8.5±2.4	6.3±3.2	10.5±2.1
PMA + IgG	7.5±3.0	10.0±3.1	21.8±4.9	7.5±3.7	7.9±3.5	8.5±2.5
PMA + IgG + neutrophils	63.5±3.9	57.5±4.1	18.7±4.3	50.7±4.8	60.5±3.6	42.7±5.6
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Fluorescence intensity measurements were made on supernatants after 1 h incubation of IgG with neutrophils (Ex 360 nm, Em 454 nm). In parallel experiments, netrophils were preincubated with inhibitors plus PMA before addition of IgG. The final concentrations of each inhibitor were as follows: SOD, 100 μg/ml; catalase, 500 μg/ml; thiourea, 50 mmol/liter; mannitol, 50 mmol/liter, and desferroximine (DFX), 0.5 mmol/liter. The results are expressed as the mean±1 SD of four separate experiments. * $P < 0.05$. ‡ $P \ll 0.001$. § $P < 0.001$. ‖ NS. ¶ $P \ll 0.001$.

Table II. Fluorescent (FL) Monomeric IgG in Normal Sera, Rheumatoid Sera, and Matched Synovial Fluids (SF)

	SF (nonrheumatoid) (n = 10)	SF (rheumatoid arthritis) (n = 10)	Rheumatoid sera (n = 10)	Normal sera (n = 15)
Fl	13.1±8.3*	25.4±6.2	47.1±38.8*	21.4±10.6
Ultraviolet	29.2±19.1	39.0±10.4	97.8±33.9	56.4±8.9
Fl/ultraviolet	0.46±0.26‡	0.52±0.15	0.49±0.36‡	0.38±0.11

Samples were diluted 1/20 with mobile phase, and 500 μl of this was injected onto a TSK 3000 column. The mobile phase used for elution of fluorescent IgG was 0.67 M KH_2PO_4 + 0.1 M KCl. Ultraviolet monitoring of eluent was at 280 nm, and fluorescence monitoring was at 454 nm when excited at 360 nm. The fluorescence of the monomeric IgG was expressed as a ratio of fluorescence to its ultraviolet absorbance (280 nm) so as to take into account variability in the IgG content of the samples. * $P < 0.001$. ‡ $P < 0.01$.

bonds and the formation of thiol groups (13). The reductions that we observe in the aromatic amino acids tryptophan and tyrosine are in agreement with our previous findings that aromatic amino acids, in particular tryptophan and tyrosine, undergo fluorescence formation when they are irradiated independently of the protein molecule (15, 16). Several of the major fluorescent oxidation products of tryptophan, which have been generated by free radical reactions, have been tentatively identified, and their structures are shown in Fig. 6. The oxidized derivatives of tryptophan are shown as they might occur in the free radical altered IgG molecule. The structures of the main components of the free radical action on tryptophan include a fluorescent hydroxylated derivative (5-hydroxy tryptophan); compounds that result from the bond breaking of the pyrrole structure of tryptophan are also found. The hydroxylation of tryptophan residues on IgG is consistent with our experimental findings since (*a*) hydroxyl radicals are produced by human neutrophils when they are metabolically activated, (18), and (*b*) the fluorescent generation of fluorescent IgG can be inhibited by desferrioxamine, catalase and hydroxyl radical scavengers. (In our experiments, mannitol was less effective than thiourea, probably because of its relatively low reactivity with hydroxyl radical.) Further support for this mechanism of damage comes from the fact that the hydroxylation of aromatic compounds is an established procedure for the detection and measurement of the hydroxyl free radical (19).

We believe that fluorescence formation (Ex 360 nm, Em 454 nm) specifically characterizes free radical-induced damage to proteins. This is also implied by other workers (20) who have observed identical visible fluorescence changes occurring in a variety of proteins when they have been stored or artificially aged. The fluorescence alteration (Ex 360 nm, Em 454 nm) is therefore not specific to IgG, but generally can reflect free radical (in particular hydroxyl-free radical) damage to any protein. The extent of the fluorescent damage would seem to be related to the periodicity and content of aromatic amino acids within the protein.

Table III. Superoxide Production from Neutrophils after Stimulation with Fractions of Fluorescent IgG Isolated by HPLC

Fraction	Origin	nmol O_2^-/15 min/10^6 cells
	Total serum	0.85
Fluorescent monomer		1.85
Fluorescent aggregate		0.50
	Total synovial fluid	1.30
Fluorescent monomer		2.75
Fluorescent aggregate		1.70
	IgG/Cu/H_2O_2	1.25
	PAG mixture	0.60
	HAG mixture	0.35
	Human IgG	0.10
	Human IgG (15′ UV)	0.95
Fluorescent monomer		0.85
Fluorescent aggregate		0.30

Total IgG concentrations of fractions were standardized at ~2.5 mg/ml when samples were added (200 μl) to 2 ml of a suspension of normal human neutrophils (final cell concentration 1×10^6/ml). Superoxide production was calculated using the following extinction coefficient for reduced cytochrome *c*: 21.1×10^3 m^{-1} cm^{-1}. These results are the mean of four separate experiments. PAG is a peroxidized arachidonic acid IgG mixture; HAG is heat aggregated IgG (63°C, 15 min). UV, ultra-violet irradiation.

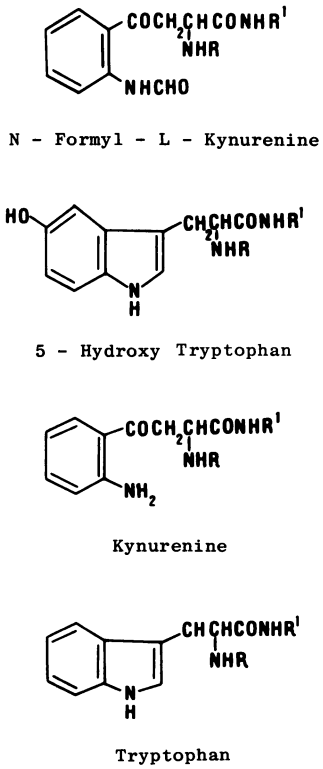

Figure 6. A structural comparison of the main products of the free radical oxidation of tryptophan. NHR and (CONHR¹) represent positions of peptide link.

It appears likely that the lysis of surface disulfide bonds alone can result in a significant conformational change in the IgG molecule; however, hydroxylation and damage to the aromatic amino acids may also give rise to specific areas of damage in the protein that cause it to polymerize. Alteration in amino acid structure is considered an important prerequisite for the formation of IgG aggregates and the subsequent production of rheumatoid factors (21). This mechanism of aggregation is supported by our own results, since the relationship and distribution of fluorescence between the monomeric, intermediate, and polymeric forms of IgG suggest that a critical concentration of altered monomer fluorescence is required for the subsequent polymerization of the molecule. This situation contrasts with the widely used heat aggregation model (63°C, 15 min), which often, but not always reproducibly, results in the generation of dimers and polymers, neither of which are fluorescent.

The activation of neutrophils by polymeric IgG was anticipated, as similar results have also been described for heat-aggregated IgG. However, we were surprised to find that free radical denatured monomeric IgG activated neutrophils to generate superoxide. There appears to be no apparent specificity in the mode of induction of the respiratory burst in neutrophils, since several different substances can interact and perturb the plasma membrane. We would suggest that, in the case of our altered monomeric IgG, the binding to the cell occurs probably through Fc receptors on the cell surface. For this to happen efficiently, the molecule of IgG needs to denature or unfold. Aggregation could enhance this reaction, at least until either the aggregation is such as to conformationally restrict Fc binding, or, cause saturation of receptors that in turn reduces the availability of the receptor as a trigger for free radical production. Either mechanism would explain the eventual falloff in O_2^- production from neutrophils as aggregation of IgG progresses with increased ultraviolet exposure.

In rheumatoid disease it appears that there is little if any inherent difference between the ability of rheumatoid neutrophils to generate free radicals compared with normal control neutrophils (22, 23). However, recently Gale and his co-workers (24) have observed that rheumatoid sera and synovial fluids can stimulate neutrophils to generate oxygen-centered free radicals. They have also shown that this is a function of the immune complexes present. IgG aggregation is thought to be a stimulus for the formation of immune complexes with rheumatoid factor antibody (25, 26). These complexes are thought to be important in amplifying and perpetuating rheumatoid inflammation (27). For several years now, physical and chemical methods for denaturing IgG have served as models for the observed alteration of IgG found in rheumatoid sera and synovial fluid (26); however, heat aggregation of IgG has never been convincingly related to biochemical or pathological mechanisms occurring in the rheumatoid joint. In this paper we have shown that neutrophils, activated during inflammation, have the potential to alter IgG so that it becomes fluorescent and aggregates. Alternative means of generating free radicals also results in identical conformational changes to IgG. Recently other workers have shown that hydrogen peroxide can aggregate IgG via the myeloperoxidase-hydrogen peroxide system (28), but only in the presence of catechol or orthoquinone. We and others (13, 29) have shown previously that hydrogen peroxide, together with cupric salts or catalytic iron, will promote fluorescence formation and aggregation that can be inhibited in vitro by hydroxyl radical scavengers and metal chelators such as desferrioxamine. This is presumably due to the following generalized Fenton reaction that could occur within synovial fluid. $M^{n+} + H_2O_2 \rightarrow M^{(n+1)+} + OH^{\cdot} + OH^-$. In support of this mechanism, Gutteridge et al. (30) have described the presence of a form of iron in synovial fluid that could act as a catalyst for this reaction.

In this report we have distinguished other proteins by HPLC, apart from IgG, which are also fluorescent when isolated from biological material. We suggest that these fluorescent proteins (approximate Ex 360 nm, Em 460 nm) isolated from sera and synovial fluid, also reflect the extent of free radical reactions induced by polymorphonuclear leukocytes during chronic inflammation. Although the changes initiated in other as yet uncharacterized proteins in human sera and synovial fluid may also be of importance in the pathogenesis of rheumatoid inflammation, we have studied here mainly the possible implications of altering the IgG, because its free radical denaturation may be the key to the production of rheumatoid factors in rheumatoid arthritis.

We have demonstrated that fluorescent monomer and aggregates are present in synovial fluid and sera, and have found a significant difference between the fluorescence to ultraviolet ratios of monomer in nonrheumatoid vs. rheumatoid fluids despite the fact that (*a*) some of the nonrheumatoid fluids were taken from patients diagnosed as having an inflammatory arthropathy, and (*b*) no account was taken of whether the disease was in remission or active at the time of sampling.

Although we have shown that catalase is by far the most potent at inhibiting fluorescence damage to IgG, H_2O_2 itself is known not to be damaging (at these concentrations) unless it is reacting in the presence of catalytic amounts of transition metal ions (30). We would therefore suggest hydroxyl (generated by a Fenton reaction)-mediated damage to IgG as a cause of its aggregation in vivo. The inhibition by the iron chelator desferrioxamine strongly supports this conclusion. IgG complexes produced by free radical reactions are fluorescent and can activate resting neutrophils to produce further free radicals. When cytochalasin B is added to neutrophils, fluorescent IgG aggregates may still engage appropriate receptors, but they are not endocytosed. It appears, therefore, from our results, that free radical altered IgG potentially can stimulate neutrophils to generate further free radicals by a process that is, at least in part, independent of phagocytosis. Similar results have also been obtained by workers using the heat aggregation model (31–33).

Finally, perhaps the most important implication of this work is that free radical damage to IgG could be perpetuated, provided that there is a suitable supply of substrate IgG at the site of inflammation. This is certainly the case in rheumatoid synovial fluid, where up to 50 mg of IgG can be synthesized per day (34). From our HPLC and in vitro studies, we would suggest that fluorescent monomeric and aggregated IgG present in synovial fluid can activate human neutrophils in an identical manner to their in vitro-generated counterparts. Taken together, these observations may be of considerable importance, since they describe a possible self-perpetuating mechanism of free radical release and tissue damage mediated by the neutrophil. Conversion of complement by free radical altered IgG might further amplify this destructive mechanism, which would be consistent with findings of complement activation and depletion in rheumatoid sera and synovial fluid (35).

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