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Inflammatory Properties of IgG Modified by Oxygen Radicals and Peroxynitrite

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In inflammatory arthritis, there is evidence indicating that the affected tissues produce large amounts of oxygen-free radicals and NO. Herein, we examine the biologic effects of exposure of IgG to hypochlorous acid (HOCl) and peroxynitrite (ONOO). The concentrations of IgG modified by chlorination and nitrosation were measured in synovial fluids from inflammatory and noninflammatory arthritis. Human IgG was exposed to increasing concentrations of HOCl and ONOO, and the resulting products were tested for complement component binding; binding to FcγRI; activation of polymorphonuclear neutrophils; effect on the Ab-combining site of Abs; and in vivo inflammatory activity in a rabbit model of acute arthritis. Rheumatoid synovial fluids contained significantly greater concentrations of nitrosated and chlorinated IgG compared with ostearthritic specimens. In vitro exposure of human IgG to HOCl and ONOO resulted in a concentration-dependent decrease in C3 and C1q fixation. The decrease in Fc domain-dependent biologic functions was confirmed by competitive binding studies to the FcγRI of U937 cells. HOCl-treated IgG monomer was 10 times less effective in competing for binding compared with native IgG, and ONOO-treated IgG was 2.5 times less effective. The modified IgGs were also ineffective in inducing synthesis of H$_2$O$_2$ by human PMN. The Ag-binding domains of IgG also showed a concentration-dependent decrease in binding to Ag. The ability of the modified IgGs to induce acute inflammation in rabbit knees decreased 20-fold as gauged by the intensity of the inflammatory cell exudates. These studies clarify the modulating role of biological oxidants in inflammatory processes in which Ag-autoantibody reactions and immune complex pathogenesis may play an important role. 

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Materials and Methods

Materials

Human IgG was purified from normal serum by affinity chromatography with Hitrap Protein A columns (Pharmacia Biotech, Piscataway, NJ). Mouse monoclonal anti-nitrotyrosine was purchased from Upstate Biotechnologies (Walther, MA). Affinity-purified rabbit anti-human C1q antiserum was obtained by immunoabsorbing IgG with purified human C1q (11). Rabbit anti-BSA Abs were purified as described previously (12). Affinity-purified goat anti-human IgG, anti-rabbit IgG, and anti-mouse IgG Abs were purchased from BioSource International (Camarillo, CA) and labeled with [125$I$]Na with IODO-beads (Pierce, Rockford, IL). Rabbit anti-HOCI-treated IgG antiserum was prepared by immunization of rabbits with human IgG treated four times with 0.6 mM HOCl in CFA. The Abs were purified sequentially by affinity chromatography using Sepharose 4B-IgG and Sepharose 4B-IgG columns. The resulting Abs were shown to react only with HOCl-IgG by RIA. No reactivity was detected against normal IgG or HOCI-treated BSA (results not shown). HOCl was distilled from 5% sodium hypochlorite (Sigma, St. Louis, MO) as previously described (5). ONOO was synthesized from NaNO$_2$ and hydrogen peroxide (H$_2$O$_2$) by the method of Rakesh (13).

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3 Abbreviations used in this paper: HOCl, hypochlorous acid; PMN, polymorphonuclear leukocytes; ONOO, peroxynitrite; H$_2$O$_2$, hydrogen peroxide; RA, rheumatoid arthritis.
Chlorination and nitrosation of IgG

HOCl solutions (20–100 mM) were prepared by diluting HOCl with distilled water to final concentrations of 0.2–1.0 mM. The HOCl solutions were added to affinity-purified human IgG, 1.0 mg/ml. The HOCl-IgG solutions were incubated at 37°C for 1 h. Nitrosation was performed in a similar fashion by adding ONOO solutions of different concentrations to human IgG.

Quantitation of nitrosated IgG in synovial fluids

Synovial fluid IgG was purified by absorption of 1 ml volumes onto protein A affinity columns (Hitrap Protein A) following brief incubation with testicular hyaluronidase (100 μg/ml). Monoclonal anti-nitrotyrosine Ab at 1:500 dilution was coated on polystyrene microtiter 96-well plates. The wells were washed and blocked with PBS-10% FCS. Triplicate wells were then incubated with the synovial fluid IgGs, washed, and incubated with 125I-labeled goat anti-human IgG. After washing, the bound radioactivity was measured with a COBRA-2 gamma scintillation spectrometer. After washing and blocking with PBS containing 0.1% BSA, 100 μM CaCl2 were added to each well. After 1 h incubation at 37°C, H2O2 was added to some wells as a positive control. Standard curves were constructed with seven dilutions of 30% H2O2. As a rule, plastic-bound normal rabbit IgG was used for each synovial fluid as background controls. Total IgG concentration in each fluid was measured by RIA.

Polymerized leukocytes (PMN) isolation

PMN were isolated from healthy donors by density gradient centrifugation with PolymorphPrep (Accurate Chemical and Scientific, Westbury, NY). Contaminating erythrocytes were lysed with 0.2% NaCl. The PMN were washed and resuspended in a solution containing 140 mM NaCl, 10 mM KH2PO4, 5.5 mM dextrose, 0.28 mM phenol red, HRP 8.5 U/ml, pH 7.0, and used immediately after isolation. PMN viability was >95% by trypan blue staining.

Colorimetric assay for H2O2

Microtiter plates were coated with normal or oxidized IgGs as described above. Preliminary experiments using 125I-labeled anti-human IgG Ab indicated that the amounts of treated IgG attached to the plastic wells was identical with that found for native IgG (results not shown). After twice washing and blocking with PBS containing 0.1% BSA, 100 μl of 1.0 × 108/ml PMN suspension in the buffer described above containing 1.0 mM CaCl2 were added to each well. After 1 h incubation at 37°C, H2O2 was measured colorimetrically (oxidized phenol red) (14). PMA (10 ng/ml) was added to some wells as a positive control. Standard curves were constructed with seven dilutions of 30% H2O2. As a rule, plastic-bound normal human IgG resulted in H2O2 secretion of ∼3.0 nmol/1.0 × 106 cells; about 50% of the H2O2 secretion induced by PMA stimulation.

C1q and C3 binding to normal and oxidized IgG

Normal and oxidized IgG-coated microtiter plates were washed and blocked with PBS containing 0.2% Tween 20. After incubation with 50 μl 1:10 dilutions of normal or heat-inactivated normal human serum for 1 h at 37°C, followed by washing, 50 μl of 2 μg/ml rabbit anti-C3 or C1q were added and incubated for 2 h at room temperature. After washing, 125I-labeled goat anti-rabbit IgG was added and incubated for 2 h. After washing, bound radioactivity was measured in a gamma spectrometer.

Fc receptor (FcrRI) binding assay

Experiments to gauge the capacity of the oxidized IgG monomers to compete for binding to FcRI by normal IgG were conducted by a modified method of Crabtree et al. (15). Briefly, normal and oxidized IgG monomers were obtained by gel filtration on Sephrose CL-6B columns. 125I-labeled normal IgG was added at saturation (50 nM) to 1.5 × 106 1937 cells (CRL-1593; American Type Culture Collection, Manassas, VA) in medium containing 5 μg/ml cytochalasin B with increasing concentrations (0–500 nM) of unlabelled normal, HOCl, or ONOO-treated IgG monomers. After incubation at 37°C for 1 h, the free IgG was separated from the bound molecules by centrifugation of the cells through a mixture of 16% paraform oil (Fisher Scientific, Pittsburg, PA) and 84% silicon oil (Sigma) at 9000 rpm for 1 min. An aliquot of the supernatant and the tips of the conical tubes containing the cell pellets were cut off, and the free and cell-bound radioactivities were measured in a gamma scintillation spectrometer.

Ag binding of oxidized Ab

Oxidation of 1.0 mg/l affinity-purified rabbit anti-BSA with increasing concentrations of HOCl or ONOO was performed as described above for human IgG. BSA (20 μg/ml) was added to microtiter wells and incubated for 2 h. After washing and blocking, 50 μl of normal or oxidized rabbit anti-BSA Abs (100 ng/ml) were added to the plates and incubated for 2 h. After washing and blocking, 125I-labeled goat anti-rabbit IgG was added and incubated for 2 h. After washing, bound radioactivity was measured in a gamma spectrometer.

Acute arthritis in rabbits

New Zealand White rabbits of either sex (2.0–2.5 kg) were used to induce acute arthritis by intraarticular injection in the knee joints of 100 μg of heat aggregated, HOCl (0.8 mM), or ONOO (0.8 mM)-treated IgG in 1 ml sterile saline solution or vehicle alone. Aggregates from the three types of IgG of identical molecular masses were isolated by gel chromatography using Sepharose CL-6B columns (Pharmacia). The fractions containing high molecular mass aggregates were pooled, concentrated, and sterilized by filtration. Preliminary experiments using heat-aggregated IgG in increasing concentrations (10, 100, and 1000 μg) indicated that near maximal acute inflammatory responses were obtained with a concentration of 100 μg per joint. The animals were sacrificed 48 h after the intraarticular injection, the knee joints were opened, and the suprapatellar pouches were flushed with 1 ml PBS containing heparin (16). The cells were counted manually in a modified Neubauer chamber.

Statistical analysis

Statistical analysis was performed using the unpaired Students t test (StatView version 4.5; SAS Institute, Cary, NC). Values of p ≤ 0.05 were considered significant.

Results

Chlorinated IgG in synovial fluids

HOCl-IgG was quantitated in 27 rheumatoid, 18 osteoarthritic, and 9 gouty synovial fluids. As shown in Fig. 1A, HOCl-IgG concentration was higher in synovial fluids of rheumatoid arthritis than in synovial fluids of osteoarthritis or gout. The levels of HOCl-IgG were positively correlated with the level of C1q in synovial fluids of RA patients. A, Concentrations of chlorinated IgG in synovial fluids of 29 patients with RA, 18 patients with osteoarthritis, and 9 patients with gout. * , p < 0.002 with respect to osteoarthritis and gout. B, Concentrations of nitrosated IgG in synovial fluids of 29 patients with RA and 19 with osteoarthritis. * , p < 0.002.
tations were highest in the rheumatoid fluids (80.5 ± 3.1 μg/ml) compared with osteoarthritic (66.6 ± 1.8 μg/ml; p < 0.002), and gouty fluids (63.1 ± 3.4 μg/ml; p < 0.005 with respect to RA). Of interest was the finding that when the values were corrected for IgG concentrations in each synovial fluid, the mean percentages of HOCl-IgG in rheumatoid and osteoarthritic fluids were similar (0.9 ± 0.08 vs 1.0 ± 0.08%; p > 0.2), with the mean percentage in gouty fluids being significantly lower (0.6 ± 0.1%).

**Nitrosated IgG in synovial fluids**

Measurements of nitrosated IgG were carried in 29 rheumatoid and 19 osteoarthritic synovial fluids. Fig. 1B shows the results obtained without correction for the IgG concentration in each synovial fluid. The rheumatoid fluids contained a mean of 12.1 ± 1.4 μg/ml, whereas the osteoarthritic specimens had 5.8 ± 0.8 μg/ml (p < 0.002). When the results were expressed as percent of the total IgG in each fluid the difference was still statistically significant (p < 0.04), albeit not as considerable as that seen with the uncorrected values.

**Complement C3 and C1q binding to modified IgGs**

The capacity of HOCl-treated IgG to fix the complement components C3 and C1q was measured by incubation of fresh human serum as a source of complement with plastic-bound IgG incubated with increasing concentrations of HOCl or ONOO. Preliminary experiments were conducted to show that the degree of binding of the modified IgGs to the plastic wells was not decreased with treatment (results not shown). The results in Fig. 2A indicate that incubation of IgG with HOCl resulted in a significant concentration-dependent decrease in C3 fixation, which became almost negligible at 0.6 mM concentration. Treatment of IgG with ONOO also resulted in a decrease in C3 binding, albeit not as pronounced as that shown for HOCL (Fig. 2B). Similar results were obtained in experiments where C1q binding to IgG was studied (Fig. 3, A and B). In the case of ONOO treatment, the decrease in C1q binding was more pronounced at low concentrations, and it did not decrease further as the ONOO concentration was increased.

**Monomer IgG binding to FcRI**

The results described above suggested that oxidant attack altered the physicochemical characteristics of the Fc domain of IgG. Many of the important cell-modifying functions of IgG are mediated by its binding to membrane-bound Fc receptors present in a variety of inflammatory and immune cells (17). Therefore, we conducted experiments to study the binding properties of the modified IgGs to FcRI in the myelo-monocytic cell line U937. In these studies, we measured the ability of the modified IgG monomers to compete with native IgG for binding to the cell receptor. As shown in Fig. 4, A and B, 1:1 mixing of labeled and unlabeled native IgG reduced 125I-IgG binding by an expected 50%, whereas it took 10-fold more HOCl-IgG and 2.5-fold more ONOO-treated IgGs to achieve the same degree of competition.

**H₂O₂ production by PMN**

The significant reduction in FcR binding by the modified IgGs suggested that they may also have a decreased capacity to induce cell activation. Normal human PMN were incubated with plastic-bound normal and modified IgGs. H₂O₂ production was measured as an indicator of activation. Preliminary experiments showed that the magnitude of native and modified IgGs binding to the plastic wells was identical. As shown in Fig. 5, A and B, there was a dose-dependent decrease in H₂O₂ production as the oxidants’ concentrations increased, which in the case of HOCl reached baseline levels with IgG treated with 0.8 mM.

**FIGURE 2.** A, Complement component C3 binding to human IgG treated with increasing concentrations of HOCl. C3 binding to chlorinated IgG is expressed as the mean percent binding compared with native IgG, which is taken as 100%. *, p < 0.0001; ***, p < 0.003; n = 3. B, Complement component C3 binding to human IgG treated with increasing concentrations of ONOO. *, p < 0.0001; **, p < 0.002; ***, p < 0.0005; n = 3.

**FIGURE 3.** A, Complement component C1q binding to human IgG treated with increasing concentrations of HOCl. *, p < 0.02; **, p < 0.002; n = 3. B, Complement component C1q binding to human IgG treated with increasing concentrations of ONOO. *, p < 0.0001; **, p < 0.0005; n = 3.
Ag binding to oxidized Ab

The experiments described above probed the consequences of oxidative modification of the Fc portion of the IgG molecules. Additional experiments were conducted to study the effects of oxidative attack on the Ag-binding domain of affinity-purified rabbit anti-BSA IgG. Exposure of the affinity-purified Ab to HOCl resulted in a concentration-dependent loss of binding capacity (Fig. 6A). Of particular interest was the observation that the rabbit Ab was particularly vulnerable to attack by ONOO. As seen in Fig. 6B, exposure to as little as 0.4 mM concentrations reduced binding capacity by almost 90%.

Acute arthritis in rabbits

Taken as a whole, the in vitro results described thus far strongly suggested that the modified IgGs may have significantly reduced capacity to induce inflammation. However, it was deemed necessary to study the proinflammatory properties of the modified IgGs in an in vivo model of inflammation, and in particular a model of inflammatory arthritis. Fig. 7 shows the results obtained using the magnitude of cellular inflammatory responses of rabbit knee joints as an index of the intensity of inflammation. Extra care was taken to isolate aggregates of similar molecular masses for the heat-, HOCl-, or ONOO-treated IgGs. Whereas injection of heat-aggregated IgG induced a mean response of 4.5 ± 1.2 × 10⁵ cells per joint, injection of the oxidized aggregates or vehicle resulted in an inflammatory response that was 20 times less intense than the positive control joints. In each case, over 95% of the cells recovered were PMN.

Discussion

There is ample evidence that oxygen free radicals are generated within the joint in RA (1) and that these reactive molecules play a role in the generation of tissue injury (2). Work in our laboratory had shown that in vitro oxidative attack on IgG resulted in the generation of covalent cross-linked aggregates that behaved as immunocomplexes in that they were able to activate the complement system and precipitate with rheumatoid factor (5). Further work showed that activated human PMN or monocytes induced similar changes in native IgG and that generation of HOCl by these cells

![FIGURE 4. A, Binding of HOCl-treated human IgG to FcR. Competitive inhibition assay of native vs treated IgG using U937 cells as a source of FcR. *, p < 0.02; **, p < 0.003; n = 3. B, Binding of ONOO-treated human IgG to FcR. *, p < 0.03; **, p < 0.05; n = 3.](http://www.jimmunol.org/)

![FIGURE 5. A, H₂O₂ production by PMN incubated on plastic-bound native and HOCl-treated IgG. The amount of H₂O₂ secreted by PMN stimulated with native IgG was taken as 100%. PBS, PMN incubated on uncoated wells. *, p < 0.002; **, p < 0.0001; n = 3. B, H₂O₂ production by PMN incubated on plastic-bound native and ONOO-treated IgG. The amount of H₂O₂ secreted by PMN stimulated with native IgG was taken as 100%. PBS, PMN incubated on uncoated wells. *, p < 0.005; **, p < 0.0003; n = 3.](http://www.jimmunol.org/)
was responsible for cross-linking (4, 18). Finally, analysis of IgG obtained from inflammatory synovial fluids showed evidence of covalent cross-linking and oxidative modification (6).

In related work, previous studies by other investigators indicated that in inflammatory arthritis there was evidence of large increases in the concentration of another powerful oxidant, NO (3), and that both the synovial membrane and cartilage were responsible for synthesis of this molecule (19). Further studies by us and others revealed that the activated chondrocytes in the superficial layer of cartilage were responsible for much of the NO produced by this tissue (8, 9). These findings, coupled to previous studies showing the presence of immune complexes trapped on the surface of rheumatoid cartilage (20), suggested that in this disease, large concentrations of oxygen radicals and NO may coexist at the cartilage surface. Finally, recent studies in our laboratory indicated that H$_2$O$_2$, and the product of oxidation of NO, nitrite salts, as well as ONOO, were able to cross-link and nitrosate immunocomplexes (10). The above-mentioned studies raised the question of whether the chemical modification of IgG by these oxidants resulted in an enhancement or a decrease of the well known proinflammatory properties of this molecule. Thus, in the present studies we have systematically investigated the biologic properties of IgG pertinent to the generation of inflammation.

The pertinence of this work was highlighted by the confirmation of previous data indicating that the synovial fluids contained significant amounts of nitrosated and chlorinated IgG species. Previous data using mAbs specific for nitrotyrosine had detected this modified amino acid in whole synovial fluids (21). The present work used the purified IgG obtained from the fluids to ascertain that the nitrosated amino acid was present within this molecule. In the case of chlorinated modification, it was not necessary to isolate the protein because detection was conducted by an independent methodology using specific Abs reacting only against IgG modified by HOCl. The results obtained indicated the presence of higher absolute amounts of the modified IgGs in rheumatoid fluids than in osteoarthritic specimens, although the differences were less distinct when the values were corrected for the IgG concentrations in each fluid. These results reflect levels of the modified proteins at one time point; they do not take into account the rates of formation, catabolism, ingress, and egress from the joint cavity, which may be quite different in inflammatory and noninflammatory arthritides. As a matter of fact, the concentrations of the modified IgGs in each synovial fluid did not correlate with the cell concentrations measured at the time of joint aspiration.

The Fc domain of IgG mediates many of the biological properties of Abs once the Ab site binds to Ag and two or more molecules attached to neighboring epitopes are placed in close proximity to each other. Activation of the complement cascade mediates many of these properties mainly through the action of the C3 convertase of the classical pathway on the third complement, C3. In the present work, we show that brief exposure of the IgG molecule to increasing concentrations of either HOCl or ONOO results in a dose-dependent inhibition of complement activation, as reflected by binding to the modified IgGs of the first component of the cascade, C1q, and C3, the molecule responsible for many of the proinflammatory properties of complement. With small variations, treatment of the IgG with both oxidants at 0.2 mM or greater resulted in a significant decrease in complement component binding. In this respect, Griffiths and Luneq (22) showed that in vitro treatment of IgG with HOCl concentrations as high as 0.2 mM did not decrease C1q binding to that molecule. This apparent discrepancy may be explained by the fact that these investigators used smaller concentrations of HOCl, and also by the very nature of the solid-phase C1q immunoassay used, which did not take into account the degree of aggregation of the IgGs tested. Thus, the treated molecules may have had decreased binding capacity for C1q and increased aggregation simultaneously, leading to the negative results obtained. Moreover, they did show a decrease in C1q binding when the IgG was exposed to activated PMN presumably containing myeloperoxidase, a situation where the cells produce significant amounts of HOCl.

The Fc domain of IgG is also responsible for the mediation of inflammatory cell activation and the secretion of many proinflammatory factors. Oxidative modification of this domain with concomitant inhibition of binding to the membrane receptor and significant decrease in cell activation as demonstrated by H$_2$O$_2$ secretion by PMN would also contribute importantly to a reduction of the severity of the inflammatory process. It should be pointed out that the present experiments were designed to distinguish the effects of the chemical modification of the IgGs from the proinflammatory effects resulting from intermolecular cross-linking and aggregation. In the experiments conducted to gauge IgG binding to the FcR, for instance, it was important to use purified monomers, because the cooperativity effects of two or more binding domains in close proximity may increase the apparent binding affinity by as much as 10$^2$.

Are the concentrations of oxidants used in these experiments physiologically relevant? The concentrations used in our experiments approach the concentrations of H$_2$O$_2$ achieved by PMN in vitro ($\sim$0.2 mM) (23). Moreover, a significant proportion of the H$_2$O$_2$ made may be used to generate HOCl (24), and it is likely that the concentrations achieved within phagolysosomes are even higher.

Synovial tissue-derived autoantibodies present within the joint cavity in RA are thought to contribute significantly to the chronic inflammatory process (1). Thus, the studies described here involving the effects of the oxidants on the business end of the Ab molecules, the Ag binding domains, suggest that one of the possible beneficial effects of NO and oxygen radicals may reside in their ability to decrease the formation of immunocomplexes. Pertinent to this discussion is the observation that rodents with absent inducible NO synthase developed inflammatory arthritis of similar magnitude to their wild-type counterparts (25, 26), although other pathologic processes such as vasculitis were ameliorated (26).

Finally, the in vitro studies suggesting inhibition of the proinflammatory properties of IgG by the biological oxidants used were confirmed by the in vivo studies in rabbit knees. In these studies, equal amounts of oxidant-modified aggregated IgG injected intraarticularly induced an acute inflammatory response that was significantly less severe than that induced by heat-aggregated IgG of similar molecular mass. Hewitt et al. (27) had previously shown that UV-treated IgG injected into an already inflamed rat air pouch
induced worsening of inflammation when compared with the native molecule. It is difficult to compare our results to those of Hewitt et al. We introduced IgG in a normal joint cavity and gauged the results 2 days later, whereas they injected the IgGs into an already inflamed air pouch and found differences 7 days after injection. They used UV light-modified IgG, whereas Hewitt et al. injected 5 mg in the air pouches. Although these authors stated that UV treatment did not result in cross-linking, if only 2% of the irradiated IgG became aggregated, it could account for the proinflammatory effects observed.

It is clear that although our results are suggestive, confirmation of the biologic importance of these observations would ideally involve testing the inflammatory capacity of modified IgG aggregates obtained from rheumatoid synovial fluids. Unfortunately, it may be very difficult, if not impossible, to purify enough oxidatively modified aggregates from aggregates generated by other mechanisms, i.e., true immunocomplexes, complexes formed by partial enzymatic digestion of IgG, etc., to compare their inflammatory activities.

Both reactive oxygen species and NO and derivatives have been shown to have protective or proinflammatory effects, depending on the concentrations and the models of inflammation used (1, 7, 28).

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