

Oxidative Cross-linking of Immune Complexes by Human Polymorphonuclear Leukocytes

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Abstract

Incubation of human serum albumin-anti-human serum albumin immune complexes bound to a plastic surface, with human polymorphonuclear leukocytes for 1 h at 37°C resulted in covalent cross-linking of $8.5\% \pm 0.5$ of the complexes, corresponding to a minimum rate of 700 antibody molecules per cell per minute. Similar results were obtained with IgG-anti-IgG and type II collagen-anticollagen II human antibodies. Cross-linking was defined as the antibody remaining attached to plastic-bound antigen after extraction with 3 M MgCl_2 and 0.1 N HCl solutions. The effects of addition of oxygen radical scavengers, heme-enzyme inhibitors, and omission of Cl^- indicated that the cross-linking process was mediated by the myeloperoxidase- H_2O_2 - Cl^- system. Cross-linking was also obtained with cell lysates, polymorphonuclear granules, and purified human myeloperoxidase in the presence of a steady flux of H_2O_2 provided by glucose oxidase-glucose. Cross-linking by the cell-free systems was also abolished by sodium azide or omission of chloride ions.

Cross-linked immune complexes were also generated by incubation with 20 to 50 μM solutions of freshly distilled hypochlorous acid. Addition of 10 mM hypochlorous acid to soluble IgG resulted in the formation of protein precipitates insoluble in 5 M guanidine, 0.1 N HCl, or boiling 2.3 M sodium dodecyl sulfate-1.4 M 2-mercaptoethanol. The remaining soluble IgG contained fluorescent high molecular aggregates (ex: 360 nm; em: 454 nm). Oxidative cross-linking of antigen-antibody molecules, and of immune complexes to connective tissue macromolecules may play a pathogenic role in acute and chronic inflammatory processes.

Introduction

Phagocytic cells activated by a variety of stimuli have the capacity to release large amounts of highly reactive oxygen-derived products that in conjunction with cell peroxidases and chloride ions play a major role in defense mechanisms and in the generation of tissue injury in acute and chronic inflammatory reactions (1-4). It is likely that many of the alterations induced by these processes are mediated by oxidative attack on protein molecules leading to deamination and decarboxylation with formation of aldehydes (5-9), sulfhydryl oxidation (5, 10), and covalent cross-linking (11) of the amino acid residues.

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We had previously shown that in an antigen-induced chronic arthritis model (12), and in rheumatoid articular tissues (13), long-lived immune complexes (IC)¹ sequestered in articular cartilage could only be solubilized completely after tissue digestion with bacterial collagenase. This observation suggested that a fraction of these IC may have established covalent interactions with the structural macromolecules of the articular tissues, and possibly play a role in the maintenance of the chronic inflammatory process. Since physiologic cross-linking of collagen fibers is mediated by a specific oxidase that is responsible for oxidative deamination of the ϵ -amino groups of lysine and hydroxylysine (14-16), it was of interest to examine similar biochemical processes that may lead to macromolecular cross-linking in inflammatory foci. Thus, we had previously shown that incubation of human IgG with myeloperoxidase, H_2O_2 , and a hydrogen donor such as catechol, resulted in the generation of large amounts of covalently cross-linked IgG aggregates that behaved as typical immune complexes (17). It was therefore deemed important to show that similar biochemical reactions could also be mediated by living cells.

In the present studies, we show that human polymorphonuclear neutrophils (PMN) incubated over IC attached to a solid phase, mediate covalent cross-linking of a significant proportion of these molecules. The cross-linking process requires the presence of a steady flux of H_2O_2 , the heme enzyme myeloperoxidase, and chloride ions. Evidence is presented demonstrating that cross-linking of IC is mediated by hypochlorous acid.

Methods

Materials. Crystalline HSA and rabbit anti-HSA serum were obtained from Miles Laboratories (Naperville, IL). Affinity-purified rabbit anti-HSA was obtained by absorption of the crude antiserum (Miles Laboratories) on crystalline HSA-linked Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, NJ). Antibodies crossreacting with BSA were first eliminated by absorption with Sepharose-BSA. Affinity-purified anti-human IgG and F(ab')_2 fragments were prepared and labeled with ¹²⁵I as previously described (18, 19). *Staphylococcus aureus* protein A was obtained from Pharmacia. Biotinylated affinity-purified anti-rabbit Ig antibody was purchased from Vector Laboratories, Inc. (Burlingame, CA). The specific leukocyte elastase inhibitor methoxysuccinyl-alanine-alanine-proline-valine-chloromethylketone (AAPVCMK) and the cathepsin inhibitor benzyloxy-carbonyl-glycine-leucine-phenyl-alanine-chloromethyl-ketone (GLPCK) were obtained from Enzyme Systems (Livermore, CA). Catalase, glucose oxidase, cytochrome c type VI, SOD, soy bean trypsin inhibitor, ϵ -amino caproic acid, phor-

1. **Abbreviations used in this paper:** AAPVCMK, methoxysuccinyl-alanine-alanine-proline-valine-chloromethylketone; DTNB, 5,5' dithiobis (nitrobenzoic acid); GLPCK, carbonyl-glycine-leucine-phenylalanine-chloromethyl-ketone; GO, glucose oxidase; IC, immune complexes; NRS, normal rabbit serum.

bol myristate acetate (PMA), were obtained from Sigma Chemical Co. (St. Louis, MO). Purified human myeloperoxidase was a gift from Dr. Isaac Ginsburg. This preparation had a specific activity of 28 U/mg protein. Hypochlorous acid was obtained by vacuum distillation of a 5% solution of sodium hypochloride, pH 7.5 (20). The resulting product was stored at -70°C . Concentrations were determined by spectrophotometric analysis ($\epsilon_{235} = 100 \text{ M}^{-1} \cdot \text{cm}^{-1}$) (21).

Preparation of plastic-bound immune complexes. Flat-bottomed flexible polyvinyl chloride 96-well trays (Falcon Plastics, Becton-Dickinson Co., Oxnard, CA) were used for all the experiments. The wells were first incubated with 100 μl of 1 mg/ml HSA in PBS pH 7.2 for 18 h at 22°C . The wells were washed twice with PBS, and twice with PBS containing 10% heat-inactivated normal rabbit serum (NRS). NRS was substituted with newborn calf serum in the experiments involving ELISA procedures. In preliminary experiments using ^{125}I -labeled albumin it was determined that the protein absorbed to the plastic wells could not be eluted by sequential treatment with 3 M MgCl_2 , 0.1 N HCl , and 5 M guanidine solutions.

The solid-phase IC were then generated by a second 3-h incubation step at 22°C and 1 h at 37°C with 50- μl vol of a ^{125}I -anti-HSA (17) solution in PBS-NRS containing $\sim 10^6$ cpm/ml and 6–8 $\mu\text{g}/\text{ml}$ antibody. The wells were washed twice with PBS at 22°C and twice with PBS-NRS, the last wash included a 1-h incubation step at 37°C . Each antigen-coated well retained 100–150 ng of anti-HSA. Control wells in which the first HSA coat was omitted did not retain any detectable antibody.

Purification of blood leukocytes. PMN were isolated from heparinized normal human blood by differential centrifugation on Ficoll-Isoopaque discontinuous gradients (22). The PMN were washed twice in Hanks' solution without phenol red, and the contaminating erythrocytes were lysed with 0.16 M ammonium chloride solution. After three more washes with Hanks' solution the PMN concentration was adjusted to $2.5 \times 10^6/\text{ml}$ unless otherwise specified in Results. The cell suspensions contained $> 90\%$ PMN, which were at least 97% viable as determined by trypan blue dye exclusion.

T lymphocytes were purified from the nonadherent cell population by rosetting with neuraminidase-treated sheep erythrocytes (18, 23).

Preparation of PMN granules. The PMN granules were isolated by the method of Chordirker et al. (24). Briefly, PMN were lysed in 0.2 M sucrose-heparin solution. The granules were sedimented by differential centrifugation at 25,000 g and washed once with 0.34 M sucrose solution. Most preparations contained 1 to 3 mg/ml protein (according to the method of Lowry), and a myeloperoxidase activity of 0.3–0.5 U/mg protein (17).

IC cross-linking protocol. Unless specified otherwise in Results, triplicate IC-containing wells received 5×10^5 cells in 200- μl vol. The trays were incubated for 1 h at 37.5°C in a water-saturated 95% air, 5% CO_2 atmosphere. Controls consisted of triplicate wells incubated with the cells previously killed by three cycles of freeze-thawing, wells with live PMN suspension containing 3 mM sodium azide, and wells incubated with Hanks' solution alone. In the experiments where oxygen radical scavengers and inhibitors were added, cell viability was assessed by trypan blue dye exclusion at the end of the incubation period. For the experiments in which chloride ions were omitted, the PMN were incubated in 0.02 M KH_2PO_4 –0.08 M Na_2HPO_4 , 10 mg/dl MgSO_4 , 100 mg/dl dextrose, pH 7.4, buffer.

The cultures were terminated by removing the cell suspension from the wells, which were then sequentially washed with 100- μl vol of 3 M MgCl_2 –10% NRS twice, and 0.1 N HCl –10% NRS, twice. In some experiments, further treatment with 5 M guanidine, 2 N H_2SO_4 , 0.05 M 2-mercaptoethanol or solutions containing detergents (Triton X or Tween 20) did not result in elution of additional radioactivity. Solubilization of bound antibody could be achieved, however, upon incubation of the wells with pepsin. Following the elution steps the wells were cut, and the radioactivity in the cell supernatants, 2 M MgCl_2 and 0.1 N HCl extracts, as well as the ^{125}I -anti-HSA bound to the wells was measured in an autogamma scintillation spectrometer (Packard Instrument Co., Inc., Downers Grove, IL). The results were expressed as

nanograms anti-HSA bound per well, or as the percentage of the radioactivity remaining, corrected for the bound radioactivity measured in wells incubated with dead PMN. Wells incubated without cells retained from 3.5 to 5.5% of the radioactive antibody after extraction, a value not significantly different from the control wells incubated with dead PMN.

In selected experiments, the rabbit anti-HSA bound was measured by ELISA. After incubation of the cells and extraction, the washed wells were sequentially incubated for 1 h with biotinylated goat anti-rabbit immunoglobulin, and a mixture of avidin and biotinylated horseradish peroxidase (ABC, Vector Laboratories). After washing with PBS, hydrogen peroxide and *O*-phenylenediamine were added. The reaction was stopped with 4 N H_2SO_4 after 5–15 min incubation. The optical density for each well was read at 492 nm with a Titertek Multiskan reader (Flow Laboratories, McLean, VA). Background readings were obtained from wells in which the anti-HSA coat was omitted. The wells were subsequently cut and the bound radioactivity was measured as described above.

Measurements of release of superoxide ion. Release of O_2^- by PMN was determined by SOD-inhibitable reduction of cytochrome *c* (25). PMN were incubated in quadruplicate in the absence of cytochalasin B for 1 h as described above, in Hanks' solution containing cytochrome *c*, 0.05 mM with or without SOD, 0.03 mg/ml. At the end of the incubation period SOD was added to the experimental supernatants before optical density measurements.

Fluorescence measurements. Fluorescence measurements were performed on a fluorescence spectrophotometer (MPF-44B; Perkin-Elmer Co., Norwalk, CT). The instrument settings were as follows: excitation wavelength 360 nm, emission 454 nm; excitation and emission slits, 10 nm; gain ranged from 10 to 50x.

Statistics. Analysis of significance was performed using unpaired Student's *t* test. Chi-square analysis was performed using the formula with Yates' correction for chi-square with one degree of freedom.

Results

Cross-linking of immune complexes by live PMN. Incubation of plastic-bound IC with live PMN for 1 h resulted in significant cross-linking of ^{125}I -anti-HSA after extraction with strong chaotropic solutions. Table I shows the results of a group of experiments demonstrating the basic observation. Mean net

Table I. Polymorphonuclear Leukocyte-mediated Cross-linking of Immune Complexes

	^{125}I -anti-HSA		ng/ 10^6 PMN/h
	Percent bound/well	Net percent bound*	
PMN (8) [‡]	12.9 \pm 0.5 [§]	8.5 \pm 0.5	12.0
Dead PMN (8)	4.2 \pm 0.2	—	—
PMN + Na azide (8)	4.4 \pm 0.2	0.1 \pm 0.07	0.2
Medium (7)	4.8 \pm 0.4	0.4 \pm 0.3	0.8
PMN 4°C (3)	5.2 \pm 0.2	0	0
Dead PMN 4°C (3)	5.3 \pm 0.1	—	—

Immune complex-containing sextuplicate wells were incubated with 5×10^5 PMN or Hanks solution for 1 h at 37°C . PMN were killed by three cycles of freeze-thawing. After removal of cells, the wells were sequentially extracted twice with 3 M MgCl_2 and 0.1 N HCl solutions.

* Net percent bound = percent experimental (live PMN) – percent control (dead PMN).

[‡] Numbers in parentheses represent number of separate experiments.

[§] $P < 0.0001$ with respect to control, PMN + Na azide, and wells incubated with medium alone.

cross-linking of 8.5% of the IC was obtained, corresponding to a minimum rate of 700 IgG molecules/cell per min. Addition of sodium azide to the wells resulted in a decrease of ^{125}I -anti-HSA remaining after extraction to levels seen in control wells containing dead PMN or medium alone. Metabolic activity was needed for the cross-linking process since the wells incubated at 4°C failed to show increased retention of antibody. Similar experiments using purified T lymphocytes failed to show increased cross-linking (results not shown).

The cross-linking rate of 700 IgG molecules/cell minute was considered to be a minimum estimate since close to 50% of the IC was digested by the overlying cells during the incubation step. Addition of sodium azide did not decrease the rate of digestion appreciably while the wells incubated with dead cells showed a 40% decrease in solubilized radioactivity. However, after extraction of the remaining radioactive antibody with MgCl_2 and HCl solutions only the wells containing live PMN showed significantly increased retention of IC over the control values.

The impression that the observed cross-linking was the end result of two opposing processes, cross-linking and breakdown of IC, was corroborated in experiments that included addition of the specific elastase inhibitor AAPVCMK to the PMN (Table II). This oligopeptide reduced IC digestion ~ 25% while net cross-linking showed a 50% increase when compared to similar wells incubated with cells in the absence of inhibitor. Similar experiments involving the addition of other protease inhibitors such as GLPCK, pepstatin, soy bean trypsin inhibitor, and ϵ -amino caproic acid failed to increase cross-linking significantly (results not shown).

In view of the extensive PMN-mediated digestion of the plastic-bound IC it was important to show that the cross-linked immunoglobulin contained intact Fc domains. Experiments were carried out with two reagents known to react with the Fc fraction of IgG: rheumatoid factor and *Staphylococcus aureus* protein A. Wells containing plastic bound HSA-anti HSA or HSA alone were incubated with PMN, extracted with 3 M MgCl_2 and 0.1 N HCl and further incubated with a monoclonal rheumatoid factor (200 ng/ml) or ^{125}I -protein A. The results shown in Table III indicate that both reagents were bound in much greater amounts to the wells containing the cross-linked IC thus demonstrating the presence of intact Fc domains within them.

Table II. Effect of Neutrophil Elastase Inhibitor on Cross-linking of Immune Complexes

	^{125}I -anti-HSA		
	AAPVCMK	Percent bound/well	Net percent bound
	μM		
Experimental (3)*	0	13.0±1.5	8.1±1.6
Control (3)	0	4.9±0.5	—
Experimental (2)	10	16.3±0.9	12.2±0.7
Control (2)	10	4.1±0.2	—
Experimental (3)	20	18.4±1.4	13.0±1.5
Control (3)	20	5.4±0.1	—

See Table I for experimental protocol.

* Numbers in parentheses represent number of experiments.

Table III. Presence of Fc Determinants in the Cross-linked Immune Complexes

Wells	Rheumatoid factor	Protein A
	cpm/well	cpm/well
HSA-anti HSA	5,272±36	818±32
HSA	1,439±36	32±2

Sextuplicate wells containing plastic-bound HSA-anti HSA or HSA alone were incubated with PMN and subsequently extracted with 3 M MgCl_2 and 0.1 N HCl followed by incubation with a monoclonal rheumatoid factor (200 ng/ml) or ^{125}I -protein A (1.25 $\mu\text{g}/\text{ml}$). The wells with rheumatoid factor were developed with affinity purified F(ab')_2 ^{125}I -anti IgM (18).

Experiments were carried out to rule out the possibility that the PMN-mediated increase in radioactivity bound to the wells was merely the result of a transiodination reaction (26, 27). To ascertain that the increase in bound radioactivity represented a true increase in cross-linked rabbit anti-HSA, rabbit IgG bound to each well was quantitated by ELISA before measurement of radioactivity. The results in Table IV show an excellent correlation between radioactivity and ELISA values ($r = 0.97$).

Additional experiments were carried out to rule out the possibility that the increase in cross-linked IgG was due to direct binding of the antibody to the plastic surface. When anti-HSA was bound directly to plastic, omitting the antigen, over 80% of the antibody remained bound to the wells, and no increase in binding was detected between experimental and control wells. The possibility that incubation with PMN may have denuded the plastic of HSA with subsequent replacement by ^{125}I -anti-HSA, which would then be resistant to extraction was also addressed. In these experiments, wells coated with HSA alone were incubated with live PMN, PMN with PMA, 10 ng/ml, PMN with sodium azide, or medium, before addition of the ^{125}I -anti-HSA coat. After extraction of the radioactive antibody 2.6%±0.4 remained in the wells incubated with PMN, 2.7%±0.3 in wells incubated with PMN plus PMA, 2.0%±0.2 in wells incubated with PMN plus Na azide, and 1.9%±0.2 in wells incubated with medium. These experiments indicated that cross-linking took place between antigen and

Table IV. Detection of Cross-linked IgG by ELISA

	^{125}I -antiHSA	OD Units	r
	% bound		
PMN	12.0±0.9	1.013±0.017	0.97
PMN + NA azide (3 mM)	5.8±0.4	0.727±0.007	
Dead PMN	4.0±0.2	0.579±0.005	

Experimental protocol as in Table I. After elution with MgCl_2 and HCl solutions, the wells were sequentially incubated with biotinylated goat anti-rabbit IgG, avidin, and biotinylated horse radish peroxidase. Color was developed with H_2O_2 and *O*-phenyldiamine and read at 492 nm before measurement of ^{125}I -anti-HSA bound in individual wells. Background OD in control wells was 0.471. r was calculated using the values obtained for each individual well.

antibody, rather than between the tagged antibody and the plastic surface.

Cross-linking of IC was shown to take place with antigen-antibody systems other than HSA-anti HSA. Table V shows the results of a group of experiments involving IC composed of human IgG-rabbit anti-IgG. The magnitude of PMN-mediated cross-linking measured was similar or greater than that found with the standard system. Inquiry into possible protein cross-linking mechanisms in inflammation was prompted by our original observation that long-lived, difficult to extract IC were found sequestered in superficial areas of articular cartilage in an antigen-induced arthritis rabbit model (12) and in rheumatoid arthritis (13). Therefore, IC were also generated with type II collagen-human anti-collagen II as detailed in Table VI. In three separate experiments, incubation with live PMN resulted in a twofold increase in cross-linking compared to wells incubated with dead cells. The aggregate of the data presented indicated that short-term incubation of PMN with a variety of IC results in covalent cross-linking of a proportion of the antibody molecules to the plastic-bound antigen.

The next series of experiments was designed to determine the optimal conditions for cross-linking of IC. Experiments were carried out to explore the relationship between the number of cells added per well and the magnitude of cross-linking. Maximal cross-linking was obtained with 2.5 and 5.0×10^5 PMN per well. The latter number, resulting in the formation of a confluent monolayer on the bottom of the microwells, was chosen for the remainder of the experiments included in this work.

The time-course of cross-linking was also explored (Fig. 1). When a suspension of PMN was placed in the wells before the incubation step there was a lag period of at least 15 min before cross-linking became detectable. However, if the cells were centrifuged down to the bottom of the wells before incubation, significant cross-linking of IC was detected in the first 15 min, suggesting that the lag was due to the time taken by the cells to sediment to the bottom of the wells and interact with the IC. In either case, maximal cross-linking was achieved after 1 h incubation.

Role of oxygen metabolites in cross-linking of IC. The observation that polymorphonuclear phagocytes mediated protein cross-linking, and that this process was almost completely inhibited by sodium azide, suggested that oxygen products secreted by the cells activated by the insoluble IC may be involved in the cross-linking process. Experiments were carried out to determine which class of reactive oxygen metabolites was responsible for cross-linking. Table VII shows the results obtained when various oxygen radical scavengers and inhibitors were added to the IC-containing wells during incubation

Table V. Cross-linking of Human IgG-anti IgG

	Percent bound	Net percent bound
PMN (3)	23.2	12.4±2.4
PMN + Na azide (3 mM) (3)	11.9	1.0±0.2
Dead PMN (3)	10.9	—

Experimental protocol as in Table I. Numbers in parentheses represent number of experiments.

Table VI. Cross-linking of Immune Complexes Composed of Collagen II-Human Anticollagen Antibody

	Exp. I	Exp. II	Exp. III
	cpm bound/ well±SEM	cpm bound/ well±SEM	cpm bound/ well±SEM
PMN	133±29*	163±9‡	102±14§
Dead PMN	51±2*	88±4‡	53±5§

Immune complexes were generated by incubation of microtiter wells coated with bovine collagen II with a rheumatoid synovial fluid known to contain anticollagen II antibodies (13). After incubation with PMN and sequential elutions, triplicate wells were developed with affinity purified F(ab)₂ fraction of ¹²⁵I-anti-human IgG-Fc.

* $P < 0.05$; ‡ $P < 0.003$; § $P < 0.03$.

with PMN. As previously shown, sodium azide was a very efficient inhibitor of cross-linking. Sodium cyanide was also effective, indicating that heme enzymes were involved in the process. Of interest was the fact that catalase was able to inhibit cross-linking only partially. The relative inefficiency of catalase to inhibit cross-linking may have been due to partial exclusion of this macromolecule from the cellular microenvironment involved in interactions with the solid-phase IC (28). The remaining thiol reagents listed in Table VI also inhibited cross-linking probably by competing with the IC as substrates for oxidation. Additional experiments involving addition of Fe²⁺, desferrioxamine, Fe-saturated desferrioxamine, or NH₄⁺ failed to enhance or inhibit cross-linking (results not shown). In the light of the negative results obtained with scavengers of O₂⁻ and OH[•] radicals it is unlikely the hydrogen peroxide is the oxygen product involved in the cross-linking process. Support for the suggestion that hydrogen peroxide was necessary for cross-linking was obtained in experiments designed to investigate the role of Cl⁻ ion in this process. Incubation of PMN in a chloride-free medium resulted in complete inhibition of cross-linking (Table VIII). Addition of taurine as a competing substrate for myeloperoxidase-mediated chlorination (29, 30) also resulted in significant inhibition of cross-linking. These results strongly suggest that the myeloperoxidase-H₂O₂-Cl⁻ system may be responsible for the cross-linking process.

Cellular-IC interactions involved in cross-linking. A series of experiments was carried out to investigate the state of activation of the PMN incubated over IC, and the interactions between cells and IC during the cross-linking process. Addition of phorbol myristic acetate, and ionomycin, alone or combination, during the incubation period increased cross-linking only marginally (results not shown) suggesting that

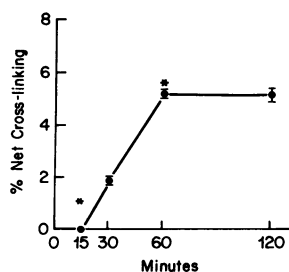


Figure 1. Time-course of immune complex cross-linking. Cell suspension incubated at 37°C without prior centrifugation (●). Cells sedimented by centrifugation prior to the incubation step *, 15 min = 1.1%±0.06 net cross-linking ($P < 0.01$ when compared to control); 60 min = 5.1%±0.3 ($P < 0.0005$).

Table VII. Effect of Oxygen Radical Scavengers and Inhibitors on Cross-linking of Immune Complexes

Addition	Concentration	Percent inhibition*
Na azide	3 mM	90.4±2.0
NaCN	1 mM	75.3±12.8
Catalase	4,000 U/ml	57.5±6.0
Superoxide dismutase	50 µg/ml	17.0±1.5
DMSO‡	10 mM	6.8±0.03
DMSO	50 mM	-8.5±0.7
Mannitol	10 mM	-10.2±0.05
Mannitol	50 mM	-8.5±0.05
Benzoate	1.0 mM	-40.7±2.5
Methionine	1.0 mM	100.0±13.1
Cysteine	1.0 mM	89.3±1.0
Cysteine	10.0 mM	76.7±2.3
DMTU‡	0.1 mM	84.5±1.1
DMTU	1.0 mM	100.0±5.1
DMTU	10.0 mM	94.3±6.0

PMN incubated with IC in the presence of the reagents listed.

* Minus signs represent increase in cross-linking over controls.

‡ DMSO, dimethylsulfoxide; DMTU, dimethylthiourea.

interaction of the PMN with the insoluble IC resulted in near maximal activation. This finding was supported by measurements of superoxide ion production carried out in identical experimental conditions (Table IX). When PMN were incubated over antigen alone, there was little or no stimulation of O_2^- production. Of interest was the observation that addition of PMA to the cells incubated with IC increased O_2^- production only by 30% indicating that the solid-phase IC were able to induce near-maximal cell activation. Moreover, incubation of the IC with fresh human serum as a source of complement before cell incubation also failed to increase cross-linking (results not shown).

The role of cellular thiol groups in the cross-linking process was also investigated. When PMN were incubated with *n*-ethyl maleimide there was profound inhibition of cross-linking. This process appeared to be irreversible, since washing the cells after a brief incubation period failed to restore activity. However, similar experiments carried out with DTNB, a thiol reagent that does not cross the cell membrane (31), failed to inhibit cross-linking after its removal by washing suggesting that external thiol groups were not involved in the interactions between IC and cells leading to activation and the oxidative burst.

Table VIII. Role of Chloride Ions in Cross-linking of Immune Complexes

	Net percent cross-linking	Percent inhibition
PMN	6.5±0.3	—
PMN-Cl ⁻ *	0.4±0.03	93.8±2.0
PMN + Taurine (1 mM)	2.5±0.2	62.3±5.3

* PMN incubated in Na-K phosphate buffer, pH 7.4 containing $MgSO_4$, 10 mg/dl, and glucose, 100 mg/dl.

Table IX. Quantitation of Superoxide Ion Production by Polymorphonuclear Neutrophils Incubated with Insoluble Immune Complexes

Experiment	O_2^- nmol/10 ⁶ PMN	Percent inhibition*
1 PMN	6.1	—
PMN (-anti-HSA)‡	0.7	88.5
Dead PMN	0.3	95.1
2 PMN	12.4	—
Dead PMN	1.1	91.1
PMN + PMA (10 ng/ml)	16.4	-32.2
Dead PMN + PMA (10 ng/ml)	0.6	95.2

* Minus sign denotes increase over control.

‡ PMN incubated over plastic-bound human serum albumin only.

That cell activation appeared to be a prerequisite for activation was supported by experiments where activation was inhibited by modification of the Fc fraction of the anti-HSA molecule. Enzymatic removal of the Fc fragment of the antibody moiety reduced cross-linking by > 60%. Similarly, reduction and alkylation of the immune complexes resulted in a 91.6% reduction in cross-linking. The decrease in cross-linking appeared to be due to a decrease in cell activation since addition of phorbol ester resulted in partial recovery of the activity.

Immune complex cross-linking by subcellular fractions. The data obtained with PMN suggested that cross-linking of IC was mediated by the myeloperoxidase- H_2O_2 -Cl⁻ system. If this were the case, it should be possible to reproduce the experiments using myeloperoxidase-containing subcellular fractions incubated with a source of H_2O_2 . Fig. 2 shows the results of a group of experiments using PMN lysates obtained by freezing and thawing, incubated with IC in the presence or absence of glucose oxidase-glucose as a source of a steady flux of H_2O_2 . As little as 2.5×10^4 lysed cells per well were capable of significant IC cross-linking in the presence of glucose oxidase. Omission of this enzyme from the incubation mixture resulted in insignificant amounts of cross-linking. Cross-linking was observed over a wide range of concentrations, however, as the number exceeded 5×10^5 PMN per well there was a decline in insignificant values probably because the soluble proteins and amino acids contained in the cell lysates competed with the IC as substrate for oxidation. As previously shown for live cells, addition of sodium azide brought down the values to baseline levels in this system.

Cross-linking activity was also obtained with isolated PMN

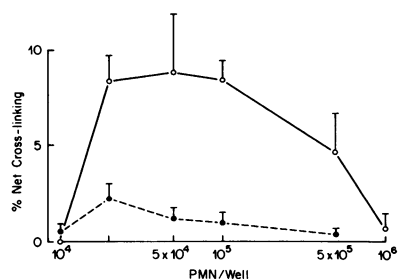


Figure 2. Cross-linking of immune complexes by PMN lysates. Cell lysates obtained with three cycles of freeze-thawing incubated in the presence (○), or in the absence (●), of glucose oxidase.

granules (Table X). When IC-containing wells were incubated with 10 μ l of granule suspension, equivalent to a peroxidase activity of 0.05 U/ml, in the presence of Cl^- , glucose, and glucose oxidase, brisk cross-linking was observed. As previously shown for live cells, the cross-linking process was inhibited by the addition of Na azide, the omission of Cl^- , or glucose oxidase. Moreover, inclusion of the elastase inhibitor in the incubation mixture resulted in a significant increase in cross-linking. IC cross-linking was also demonstrated in experiments using highly purified human myeloperoxidase (Table XI). Cross-linking of a large proportion of IC was achieved with 10 $\mu\text{g}/\text{ml}$ of the enzyme in the presence of glucose oxidase concentrations of 5 mU or more. Using concentration of 0.5 mU did not yield significant cross-linking. As shown previously for live cells and subcellular fractions, addition of sodium azide or omission of chloride ions resulted in complete inhibition of cross-linking. Similar experiments using horseradish or lactoperoxidases failed to yield detectable cross-linking (results not shown).

Cross-linking of IC by hypochlorous acid. The aggregate of results presented indicated that hypochlorous acid generated by the action of myeloperoxidase on H_2O_2 may be the oxidizing agent responsible for cross-linking. Fig. 3 shows the results of experiments in which plastic-bound IC were incubated with increasing concentrations of freshly distilled hypochlorous acid. Cross-linking amounting to almost 50% of the IC was obtained at 20- to 40- μM concentrations. The brisk decrease in cross-linking observed at higher concentrations was due to fragmentation of the IC, since incubation with a 50- μM solution of HClO , for instance, resulted in solubilization of 75% of the radioactivity initially present in the wells. Cross-linking and fragmentation was totally abolished by addition of 10 mM solution of sodium thiosulfate before the hypochlorous acid. Addition of 1 mM taurine also decreased cross-linking significantly.

Table X. Cross-linking of Immune Complexes with Polymorphonuclear Neutrophil Granules

Experiment	Cl^-	Na azide	GO*	AAPVCMK	Percent cross-linking
1	+	-	+	-	15.3 \pm 2.8
	+	+	+	-	2.8 \pm 0.3
	+	-	-	-	2.6 \pm 0.1
	+	+	-	-	3.0 \pm 0.1
	+	-	+	+	28.4 \pm 1.4
	+	+	+	+	3.9 \pm 0.4
	+	-	-	+	3.3 \pm 0.6
	+	+	-	-	2.6 \pm 0.2
2	+	-	+	-	23.9 \pm 1.0
	+	+	+	-	3.5 \pm 0.7
	-	-	+	-	3.7 \pm 0.3
	-	+	+	-	3.3 \pm 0.6

Immune complex-containing wells incubated for 1 h with PMN granules, 14.0 $\mu\text{g}/\text{protein}/\text{well}$. Where indicated, Hanks' solution was replaced with Na-K phosphate buffer containing MgSO_4 and glucose. Glucose oxidase was added at a final concentration of 0.1 U/ml, Na azide 3 mM, and AAPVCMK 10 μM .

* GO, glucose oxidase.

Table XI. Cross-linking of Immune Complexes by Human Myeloperoxidase

Cl^-	GO	MPO*	Na azide	Net % cross-linked†
	U	$\mu\text{g}/\text{ml}$	mM	Mean \pm SEM
+	0.5	0	0	0
+	0.5	10	0	38.7 \pm 1.0
+	0.5	10	3	0
+	0	10	0	0
+	0.05	0	0	0
+	0.05	10	0	33.6 \pm 0.7
+	0.05	10	3	0
-	0.05	0	0	0
-	0.05	10	0	0
-	0.05	10	3	0
+	0.005	0	0	0
+	0.005	10	0	39.5 \pm 2.4
+	0.005	10	3	0

* MPO, myeloperoxidase.

† Corrected for the percentage ^{125}I -anti-HSA bound to wells incubated with medium alone.

The brisk cross-linking of plastic-bound IC obtained by relatively small concentrations of HClO suggested that it may be possible to obtain large amounts of the cross-linked product using proteins in solution. Indeed, when a solution of 10 mg/ml IgG was reacted with 10 mM HClO , 30% of the protein was irreversibly precipitated. The protein aggregates could not be solubilized by sequential addition of 5 M guanidine, 0.1 N HCl , or boiling 2.3 M Na dodecyl-sulfate-1.4 M 2-mercaptoethanol solutions. The resulting supernatant contained significant amounts of high-molecular weight IgG when fractionated by Sepharose A5M column chromatography (Fig. 4). The high molecular weight soluble aggregates were shown to be fluorescent (ex: 360 nm; em: 454 nm).

Discussion

These studies show that cross-linking between antigen and antibody takes place when living phagocytic cells are incubated over solid-phase IC. This process requires that the molecules to be cross-linked be in close proximity to each other because experiments involving incubation of activated PMN with soluble IgG have failed in our hands to induce significant aggregation (Jasin, H. E., unpublished observations). A second re-

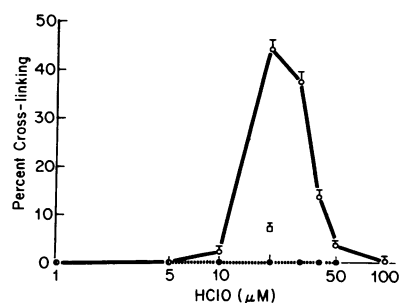


Figure 3. Cross-linking of immune complexes by hypochlorous acid. Freshly distilled hypochlorous acid solutions were incubated with plastic-bound immune complexes for 1 h at 37°C. Experimental (○); 1 mM sodium thiosulfate added (●); 1 mM taurine added, (□).

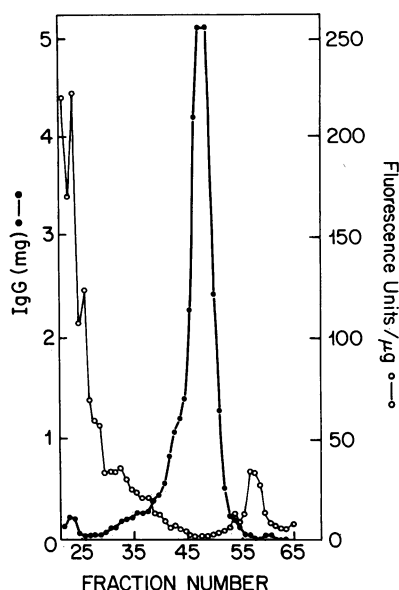


Figure 4. Gel chromatography of HClO-treated IgG. A solution of 10 mg/ml IgG, was incubated with HClO to a final concentration of 10 mM. After removal of the protein precipitate, the supernatant was fractionated by gel filtration on a Sepharose A5M column. The protein concentration of the individual fractions was measured at 280 nM and their fluorescence measured (ex: 360 nm; em: 454 nm).

quirement appears to be that of a steady flux of H_2O_2 ; addition of preformed H_2O_2 to PMN lysates incubated with IC have also failed to yield significant cross-linking. The possibility existed that the PMN-mediated increase in radioactive ^{125}I -antibody bound to the microtiter wells are not due to a true increase in bound IgG but to a peroxidase-mediated transiodination reaction (26, 27). This was excluded by the simultaneous detection of an increase in "antigenic" IgG by ELISA in the same wells showing the increase in ^{125}I radioactivity (Table IV).

The possibility that incubation with PMN may have resulted in an increase in the availability of binding sites for IgG on the plastic surface was also ruled out. Experiments in which the plastic-bound antigen was incubated with resting or activated PMN prior to addition of the antibody failed to show a significant increase in irreversibly bound ^{125}I -anti-HSA. It should be pointed out that the protein-binding sites on the plastic were saturated with large excess of protein provided by the 10% NHS used in the washings before and after the cell incubation step, thus minimizing the possible exchange of antigen for ^{125}I -antibody on the plastic. The increase in IC cross-linking demonstrated when the specific PMN elastase inhibitor AAPVCMK was added to the incubated cells indicated that the calculated rate of 700 antibody molecules cross-linked per cell/minute must have been a minimum estimate. It is likely that in our experiments the final yield of cross-linked IC was the end-result of a dynamic process in which IC cross-linking, breakdown by the oxidative attack, and digestion by cell proteases took place simultaneously. Neutrophil elastase appeared to be the enzyme responsible for the proteolytic attack, since addition of several cathepsin and serine protease inhibitors failed to decrease solubilization of radioactive antibody and to increase cross-linking.

In view of the evidence indicating that the IC were partially digested by the overlying PMN, it was important to show that the FC portion of the Ig molecules remained intact in the cross-linked proteins. Two lines of evidence indicated that this was the case; (a) in the experiments using human antibodies

against type II collagen, the plates were developed with a monospecific anti-IgG-Fc antibody; and (b) the cross-linked IC were shown to interact with rheumatoid factor and *Staphylococcus aureus* protein A, two reagents known to interact with the Fc domain of IgG.

Our studies indicate that the myeloperoxidase- H_2O_2 - Cl^- system may be directly involved in the cross-linking process. Whereas polymorphonuclear phagocytes were able to mediate cross-linking, purified T lymphocytes, which do not have the capability to generate oxygen radicals, and are devoid of peroxidase, were unable to do so. Moreover, the heme-enzyme inhibitors, sodium azide and cyanide, abolished the cross-linking process efficiently. Additional evidence for the role of myeloperoxidase in our studies was provided by the experiments involving the use of subcellular fractions. Both whole cell lysates and purified PMN granules containing measurable myeloperoxidase activity were able to cross-link IC only in the presence of H_2O_2 -generating system and chloride ions. In either case, the process was completely inhibited upon addition of sodium azide. Direct evidence for the role of myeloperoxidase in the cross-linking process was also obtained, and as shown previously with PMN lysates and isolated granules, cross-linking mediated by the purified enzyme required a source of H_2O_2 and chloride ions (Table XI).

Experiments carried out with purified horseradish and lactoperoxidase failed to mediate cross-linking supporting our conclusion that Cl^- ion may play a key role, since these enzymes exert their oxidative action with halide ions other than chloride (32). The aggregate of the evidence presented in these studies also point out to H_2O_2 as the key oxygen product involved in cross-linking. The roles of O_2^- and OH^\cdot were ruled out by the failure of superoxide dismutase and the various radical scavengers listed in Table VII to inhibit cross-linking. The relative inefficiency of catalase to do so can be ascribed to the possibility that this macromolecule may be excluded from the microenvironment where interaction between the IC and the phagolysosomes take place (28). Similar considerations may apply to preexisting cytosol H_2O_2 scavengers that may be unable to penetrate the IC-phagosome interphase. The important role of H_2O_2 is also underscored by the experiments involving subcellular fractions, since omission of the H_2O_2 -generating system resulted in abolition of cross-linking.

Many of the profound effects mediated by the action of PMN-derived reactive oxygen products are believed to be the result of oxidative attack of hypochlorous acid or chloraminated byproducts generated by the myeloperoxidase- H_2O_2 system (1, 4, 6–10). The data presented appears to point to the same mechanism with regard to cross-linking of IC. Omission of Cl^- completely inhibited cross-linking by PMN. It may be argued that in the absence of Cl^- some of the many cell functions involved in activation of phagocytosis may be altered. However, the inhibitory effects of excess taurine, and the failure to induce cross-linking with PMN granules and myeloperoxidase in the absence of Cl^- strongly suggest that this ion is involved in the generation of hypochlorous acid. That this reactive molecule may be directly responsible for the oxidative attack on the IC is indicated by our experiments showing brisk cross-linking on exposure of the IC to dilute solutions of hypochlorous acid. However, the possibility that chemical interactions between hypochlorous acid and the target proteins may have generated chloramines that in themselves may be

oxidants, cannot be ruled out (33) even though addition of NH_4 in the experiments with live PMN failed to enhance cross-linking.

Our ability to readily detect cross-linking of IC may be due to the experimental design used in our studies. Previous work in other laboratories indicate that solid-phase IC may be much more efficient stimuli for PMN activation than IC in the fluid phase (34). In addition, allowing the cells to spread over solid surfaces prepares them for maximal responses to subsequent stimuli (35). These points were born out by our experiments suggesting that the PMN incubated with IC were optimally activated in terms of O_2^- secretion, and by our failure to increase cross-linking significantly when PMA or cytochalasin B were added. Finally, the interaction between PMN and IC attached to a solid phase ensures regurgitation of large amounts of lysosomal contents through the process of "frustrated phagocytosis." A combination of these factors, and the close proximity between the molecules to be cross-linked (36) probably yielded optimal conditions for cross-linking. This scenario is directly relevant to the pathogenic mechanisms operative in IC-induced tissue injury in vivo. In this group of diseases, IC frequently localize in vessel walls, basal membranes, and cartilage.

The importance of PMN activation as a requisite for IC cross-linking is highlighted by the experiments involving chemical modification of the antibody moiety. Both removal of the Fc portion of the antibody, and reduction and alkylation of S-S bonds, inhibited cross-linking markedly. This inhibition was probably due to the lack of interaction between the antibody and the Fc receptors. Barnett-Foster et al. (37) had previously shown that reduction and alkylation of IgG reduced its affinity for the granulocyte Fc receptors by roughly 50-fold. Bypassing this initial activation step by the addition of the secretagogue PMA resulted in partial recovery of cross-linking activity indicating that sulfhydryl groups may not be involved in the cross-linking process itself.

The chemical modifications of proteins oxidized by peroxidases are numerous but only a few well characterized reactions may lead to covalent cross-linking. Although the nature of the chemical bonds responsible for PMN-mediated cross-linking has not been elucidated, the failure to solubilize the plastic-bound IC and the HClO -induced insoluble IgG aggregates with strong chaotropic solutions, detergents, or thiol reagents, strongly suggests that the chemical alterations induced by the PMN resulted in the establishment of intermolecular covalent bonds between the protein molecules. Moreover, the ability to establish cross-linking bonds within reduced and alkylated IC, and the failure to solubilize the cross-linked antibody with thiol reagents suggest that formation of intermolecular S-S bridges is not the mechanism responsible for cross-linking. In addition, cross-linking was achieved between human and rabbit IgG, two molecular species lacking free sulfhydryl groups (38). Studies on the effects of H_2O_2 oxidation on amino acids have shown that the main chemical reactions that take place involve decarboxylation and deamination yielding aldehyde derivatives of the original amino acids (5, 6). The same reactive groups generated by the action of lysyl oxidase on lysine and hydroxylysine are responsible for covalent cross-linking of collagen fibers (14, 15). In this situation, close apposition of the molecules is a precondition for successful cross-linking (39). In addition, cross-linking of lysine-contain-

ing proteins by a similar oxidative mechanism has been achieved with hydrogen peroxide, myeloperoxidase, and catechol (17, 40, 41). In this case, the resulting quinone mediates the oxidative deamination of lysine with the formation of aldehydes that react with other aldehydes by aldol condensation, and with amino groups to form Schiff bases (41). Thus, it is likely that when proteins are exposed to concentrations of hypochlorous acid that do not result in excessive peptide bond breakage, deamination of the ϵ -amino groups of lysine may lead to covalent cross-linking by the same chemical mechanism discussed above. As a matter of fact, the optimal hypochlorous acid concentration range capable of mediating optimal in vitro IC cross-linking approaches to concentrations of H_2O_2 (~ 0.2 mM) that activated PMN can achieve in vitro (42). Moreover, a significant proportion of the H_2O_2 generated may be used to generate hypochlorous acid (43) suggesting that the in vitro experiments probably reproduced closely the conditions extant in activated PMN.

Another major consequence of the oxidation of proteins is the modification of tryptophan, methionine (44), and tyrosine residues (5). Oxidation of tyrosine to a quinone by specific or nonspecific oxidases could also mediate cross-linking of proteins (17). This mechanism has been shown to be responsible for the hardening of the cuticle of insects and crustaceans before pupation or molting (45), and for the hardening of sea urchin egg membrane after fertilization (46). In the latter case, a peroxidase from cortical granules, in conjunction with H_2O_2 , react to generate di- and tri-tyrosine linkages that may contribute to the process of hardening (47). Amino acid analysis of cross-linked IgG is in progress in our laboratory.

Recent work by Lunec et al. (48) has shown that when human IgG was exposed to ultraviolet irradiation or activated PMN, characteristic changes in autofluorescence could be detected. In addition, ultraviolet light exposure resulted in polymerization of a fraction of the IgG molecules. These workers concluded that the hydroxyl radical was responsible for the chemical alterations observed. We have been unable to show evidence of polymerization when activated PMN were incubated with IgG monomers in solution (unpublished observations). However, irreversible aggregation of soluble IgG was readily obtained with hypochlorous acid. In view of our results suggesting that cross-linking is mediated by hypochlorous acid and not by the hydroxyl radical, it is likely that the fluorescent compounds detected by Lunec et al. may be generated by both mechanisms, whereas polymerization may be mediated by alternative chemical reactions such as the ones discussed above.

Nonspecific aggregation of immunoglobulins and cross-linking of IC to themselves and to other structural proteins may play important pathogenic roles in the establishment and maintenance of acute and chronic inflammatory reactions. It may help explain the presence of circulating IC-like material in a large number of diseases without a readily apparent immune pathogenesis (49). It may also clarify the chemical interactions between IC and autoantibodies directed against structural macromolecules such as collagen and basal membranes. Our studies should pave the way to unravel the chemical reactions responsible for the protein cross-linking. Once the chemical markers of oxidative cross-linking are known, it should be possible to obtain direct evidence for the biological importance of this process.

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