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# REACTION OF PROTEASE-TREATED IgG WITH GLUTATHIONE

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Molecular sieving in Sepharose 6B of rabbit IgG first treated with human leukocyte neutral protease and then incubated with 1 mM reduced glutathione or 1 mM reduced glutathione/0.1 mM oxidized glutathione showed different elution patterns when compared to each other or when compared to untreated protease-reacted IgG. Different products were also seen when analysis was carried out by electrophoresis or when the differently treated IgG samples were used to immunize rabbits and the resulting antisera reacted with human IgG coated latex particles. It was suggested that the different products are formed by sulfhydryl-disulfide exchange reactions in the IgG molecule.

## Introduction

Like all proteins IgG has a discrete half-life and is ultimately degraded by proteases to its constituent amino acids. Sites of its catabolism include liver, lung, kidney, endothelial cells, and phagocytic blood cells. IgG's many disulfide bonds must be reduced by GSH*, the major cell thiol, before complete proteolysis to amino acids takes place. Because protein disulfide bonds are generally not available for reaction with GSH (1), limited proteolysis probably precedes disulfide bond reduction which is followed by more proteolysis. In the present work IgG treated with a leukocyte neutral protease was reacted with glutathione and the products examined in various ways. The purpose of the work was to investigate the possibility that novel species of IgG are formed by such treatment. In this connection Gerber (2) has proposed that the putative altered IgG in rheumatoid arthritis results from sulfhydryl-disulfide interchange reactions in the molecule. Leukocyte protease has been used as leukocytes are present in large numbers in inflammatory lesions where altered IgG is thought to play a pathophysiologic role.

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## Materials And Methods

Rabbit IgG and yeast glutathione reductase were purchased from Sigma Chemical Co., St. Louis, MO. Glutathione insulin transhydrogenase was purified from bovine liver through the CM-Sephadex step according to the procedure of Carmichael et al. (3).

Leukocytes were prepared from washings obtained during preparation of human leukocyte-poor erythrocytes at the University Hospital blood bank. The washings to which were added 1000 units of heparin per 100 ml were centrifuged at 1000 xg for 15 min and the buffy coat removed. Further separation of leukocytes from contaminating erythrocytes was obtained by sedimenting the erythrocytes at 1 xg in 6% dextran-0.15M NaCl and by hypotonic lysis. Protease was separated from granules prepared from these cells by low salt extraction as described by Feinstein and Janoff (4) for elastase preparation. Protease-reacted IgG was prepared by incubating 15 mg of rabbit IgG in 1.5 ml of 0.1 M Tris-HCl buffer, pH 7.4, with protease for 1 hr at 30°C. The amount of enzyme used liberated 0.25 $\mu$-mole of p-nitrophenol from p-nitrophenyl-N-tert-butyloxycarbonyl-L-alanine in 5 min in the assay described by Visser and Blout (5). In addition to elastase activity the protease showed some activity toward a cathepsin G substrate, N-benyoyl-DL-phenylalanine 2-napthyl ester. The protease was separated from the IgG by passing the mixture through a Sepharose 6B column (2 x 16 cm) with 0.02 M K phosphate buffer, pH 6.7. Usually the fractions collected between 42-54 ml were combined and reduced in vol to about 4 ml in an Amicon ultrafiltration cell.

Treatment of protease-reacted IgG with glutathione was carried out in a buffer of composition 0.02 M Tris, 0.015 M K phosphate, 1 mM EDTA, pH 7.5. They contained 2-3 mg of IgG in vol of 1 ml. Some incubated samples were applied directly to a Sepharose 6B column (0.9 x 30 cm) for analysis. They were passed through the column with 0.02 M K phosphate buffer, pH 6.7, at a rate of 10 ml per hr. If the sample was to be analyzed by electrophoresis, it was first carbamylated. The sample buffer was changed to Peacock buffer (6) by concentration and reconstitution in an Amicon ultrafiltration cell, the sample incubated with an equal vol of 2 M KNCO for 30 min at 45°C and the excess KNCO removed by concentration and reconstitution as before. Electrophoresis was carried out using 4% acrylamide slab gels (E-C apparatus, E-C Corporation, St. Petersburg, FL) for 2 hr at 75 ma in Peacock buffer. Protein bands were visualized by staining them with Commassie blue.

For immunization 5 lb New Zealand white male rabbits were injected intravenously at 3-4 day intervals 4 times with the IgG sample. The total protein injected per rabbit was about 15 mg. The animals were bled 2 wks after the last injection and serum prepared from the blood. Testing of sera for rheumatoid factor was done using human IgG coated latex particles supplied by ICN Scientific, Fountain Valley, CA per their directions.

## Results and Discussion

As judged by molecular sieving in Sepharose 6B incubation of IgG with 1 mM GSH did not alter its structure (Fig. 1). However, if the IgG was first treated with leukocyte protease before incubating it with GSH, changes were clearly seen (Fig. 2). The protease-treated IgG samples used in these experiments did not serve as a substrate for glutathione insulin transhydrogenase indicating that only limited proteolysis of the protein had occurred (Table

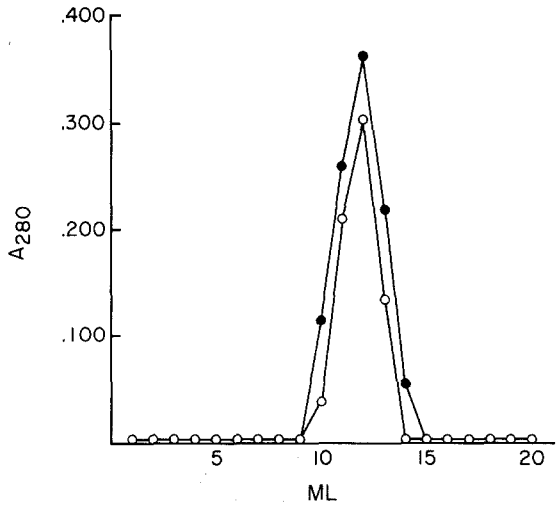


Figure 1. Sepharose 6B analysis of native IgG. 0---0, no additions; ●---●, 1 mM GSH. Samples were incubated 60 min at 37°C.

I). IgG treated with relatively large amounts of either chymotrypsin or papain did serve as a substrate for the enzyme (Table I). If the protease-treated IgG was incubated with a mixture of GSH/GSSG (10:1) rather than GSH alone, different products were obtained (Fig. 2). Electrophoresis of this sample confirms that the IgG has undergone changes in structure when compared to protease-reacted IgG (Fig. 3). Protease-reacted IgG incubated with 1 mM

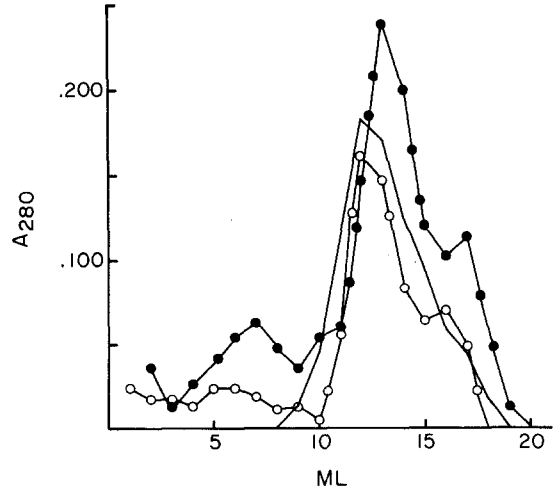


Figure 2. Sepharose 6B analysis of protease-reacted IgG. —— , no additions; 0---0, 1 mM GSH/0.1 mM GSSG; ●---●, 1 mM GSH. Samples were incubated 60 min at 37°C.

Table I

IgG as a Substrate for Glutathione Insulin Transhydrogenase*

| Sample | NADPH Oxidized/9min, 30°C |
|---|---|
| | μmoles |
| native IgG | 0.02 |
| leukocyte protease-reacted IgG | 0.02 |
| chymotrypsin-reacted IgG | 0.09 |
| papain-reacted IgG | 0.06 |
| insulin | 0.15 |

*Reaction mixtures contained in 3 ml of 0.07 M Tris-HCl, 5 mM EDTA buffer, pH 7.5, 0.1 mM NADPH, 1 mM GSH, 0.5 unit of yeast glutathione reductase, liver glutathione insulin transhydrogenase, and 0.5 mg of the different proteins. Preparation of leukocyte protease-reacted IgG was described in Materials; for the other two protease-reacted samples 0.5 mg of IgG was incubated with 0.5 mg of either chymotrypsin or papain at pH 7.5 for 60 min at 37°C.

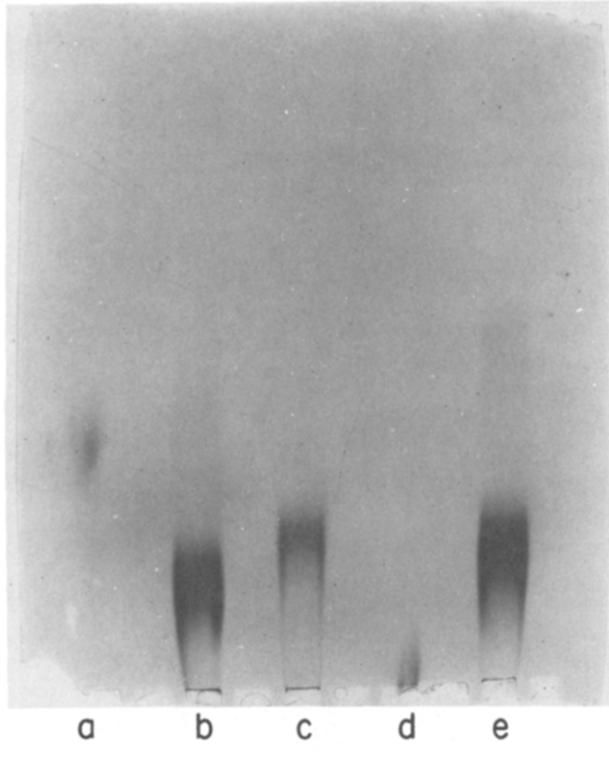


Figure 3. Electrophoresis of rabbit IgG treated in different ways. a) serum albumin b) carbamylated protease-reacted-IgG c) carbamylated IgG d) IgG e) protease-reacted IgG incubated with 1 mM GSH/0.1 mM GSSG for 60 min at 37°C and carbamylated.

Table II

Assay for Rheumatoid Factor in Sera From Rabbits Injected with Different Rabbit IgG Samples

| IgG Sample | Rheumatoid Factor scale 0-(+4) |
|---|---|
| protease-reacted | 0, 2+ |
| protease-reacted and incubated with 1 mM GSH | 1+, 1+ |
| protease-reacted and incubated with 1 mM GSH/0.1 mM GSSG | 2+, 1+ |
| native | 0, 0 |

GSH alone upon electrophoresis move slightly behind the GSH/GSSG sample (sample not shown). Other evidence that the treated IgG samples are structural different from native IgG is that they are antigenic in the autologous host (Table II). Previously, IgG fragments produced by proteases have been shown to be antigenic in the autologous host (7). Gauged by the behavior of the IgG products in Sepharose 6B, especially the products obtained by reaction with GSH/GSSG (Fig. 2), they are not simply fragments but have retained their large molecular size.

If reduction of protein disulfide bonds is the only reaction promoted by glutathione in protease-reacted IgG, then the same products would be expected in every case. This was not found. The findings are compatible with sulfhydryl-disulfide interchange reactions taking place in the protease reacted IgG forming unique protein species. Because information for forming "correct" disulfide bonds is lacking in the protease-reacted IgG, mismatched bonds formed by sulfhydryl-disulfide interchange reactions can give such products. That the products of IgG catabolism are different depending on the GSH/GSSG is particularly relevant to the leukocyte, because of the oxidative stress it undergoes in the inflammatory lesion, which would be expected to perturb the GSH/GSSG. The occurrence of such products in the intact cell, where proteolysis of IgG and its reaction with glutathione take place concurrently, is now being investigated.

Table III

Recovery of Lactate Dehydrogenase, Neutral Protease, and Glutathione Insulin Transhydrogenase after Different Treatments of Leukocytes*

| Enzyme | Homogenization | Triton X-100 | Freeze-Thawing |
|---|---|---|---|
| | % | % | % |
| Lactate dehydrogenase | 85 | 100 | -- |
| Neutral protease | 20 | 100 | -- |
| Glutathione insulin transhydrogenase | 100 | -- | 100 |

*About 200 million cells per ml of 0.015 M Tris-HCl, 0.14 M NaCl, 5 mM EDTA, pH 7.5 were homogenized using 5 strokes of a motor-driven Teflon pestle in a glass homogenizer. The homogenate was centrifuged at 30,000 xg for 30 min and the supernate assayed for enzyme activities. Triton X-100, final concentration 1%, was added to other homogenates or they were frozen and thawed 5 times and the enzyme assays of supernate repeated. These values were taken as 100% recovery of enzyme. Glutathione insulin transhydrogenase was assayed using [$^{125}$I] insulin (8) and neutral protease using azo-casein (10). Lactate dehydrogenase was assayed as described by Bergmeyer et al. (11).

Glutathione insulin transhydrogenase, an enzyme that catalyzes reduction of protein disulfide bonds by GSH, has been described in human leukocytes (8). It was of considerable interest to ascertain if it were granule-associated where it could participate in IgG degradation. Similar enzymes in other tissues are found associated with endoplasmic reticulum (9). As can be seen in Table III, leukocyte glutathione insulin transhydrogenase is recovered with lactate dehydrogenase, a cytoplasmic enzyme, and not with neutral protease, a granule-associated activity. Because of its cellular location, it is unlikely that it would participate in degradation of ingested IgG.

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