

Effect of Papain on the Interaction Between Human Monocytes, Erythrocytes, and IgG

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The mechanism by which papain detaches IgG-sensitized erythrocytes from the monocyte surface has been explored in an *in vitro* assay for the monocyte IgG receptor using red cells quantitatively sensitized with IgG anti-Rh D immunoglobulin. Papain treatment of IgG-sensitized erythrocytes diminished the ability of these cells to bind to the monocyte surface; however, treatment of erythrocytes with papain prior to sensitization with IgG did not inhibit binding,

and at papain concentrations ≥ 38 $\mu\text{g/ml}$ binding was enhanced. IgG receptor activity was not diminished by prior treatment of monolayer cells with papain and was enhanced with high concentrations of papain. These studies suggest that papain detaches erythrocytes from the monocyte surface by virtue of its proteolytic effect on IgG and not by an effect of papain on the D antigen of red cells or the IgG receptor of monocytes.

HUMAN PERIPHERAL BLOOD monocytes possess a receptor for the IgG class of human immunoglobulin¹⁻³ capable of binding IgG-sensitized erythrocytes to the monocyte surface.²⁻⁴ This interaction, which may serve as a model for the *in vivo* clearance of IgG-sensitized erythrocytes in man, has been studied in considerable detail by several investigators.²⁻⁵ The proteolytic enzyme papain has been observed to cause detachment of bound erythrocytes from the monocyte surface.² Papain cleaves IgG into at least two antigenic fragments, destroying the antibody combining site.^{6,7} Additionally, papain has a direct effect on cell membranes and may digest and solubilize cell membrane antigens.⁸ The capacity of papain to remove IgG-sensitized erythrocytes from the monocyte IgG receptor attachment site might therefore relate to an effect of papain on the erythrocyte antigen, IgG antibody, or the monocyte IgG receptor. We have examined the site of action of papain as part of an attempt to define further the nature of the monocyte receptor for IgG.

MATERIALS AND METHODS

Isotonic veronal-buffered saline, pH 7.4, containing 0.1% gelatin and 0.0015 *M* CaCl₂ and 0.0005 *M* MgCl₂ (VBS) and veronal-buffered saline containing 0.1% gelatin and 0.01 *M* ethylenedinitrilotetracetic acid (EDTA buffer) were prepared as previously described.^{9,10} Rabbit antiserum to human IgM and rabbit antiserum to human IgG (Behring Diagnostics, Somerville, N.J.), human anti-Rh D antiserum (Sera-Tec Biologicals, New Brunswick, N.J.), human CI (Cordis Lab., Miami, Fla.), Hanks' Balanced Salt Solution (HBSS) (Gibco, Grand Island, N.Y.), crystalline papain (Sigma Chemical, St. Louis, Mo.), and cysteine (Fisher Scientific, King of Prussia, Pa.) were obtained as indicated. Peroxidase stain was performed according to Kaplow.¹¹

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Isolation of Rabbit IgG and Complement Components

Three milliliters of rabbit anti-IgG antiserum were applied to a 5 × 100-cm Sephadex G-200 column equilibrated with 0.0035 M phosphate buffer, pH 7.4, containing 0.15 M NaCl and fractionated by upward flow at 15 ml/hr. The IgG fraction was pooled, concentrated to the starting volume, and utilized in the quantitative IgG studies below.

Fresh-frozen guinea pig serum was obtained from Rockland (Gilbertsville, Pa.). Partially purified guinea pig C2 and human C1 were prepared by modification of established methods,^{9,12,13} as were cells coated with hemolytic antibody and the fourth component of complement (EAC4).^{10,14}

Preparation of Human Erythrocytes Sensitized With Human Anti-D Antisera

Whole blood was obtained from Rh D-positive donors and either diluted three fold with Alsever's solution⁹ and held at 4°C or diluted with sodium heparin (Riker Labs, Northridge, Calif.; 10 U of heparin per 10 ml whole blood) and used immediately. The diluted whole blood was washed twice with EDTA buffer with removal of the buffy coat, resuspended to 1×10^8 RBC per ml¹⁰ with EDTA buffer, sensitized with an equal volume of anti-D antiserum diluted in EDTA buffer, washed twice with 0.15 M NaCl, and resuspended with HBSS to a final concentration of 5×10^7 sensitized erythrocytes (EA) per ml. In all experiments utilizing EA, control erythrocytes (E) were prepared by exposing washed erythrocytes to EDTA buffer in the absence of antiserum.

Hemagglutination titers were performed using a microtiter technique. 2.5×10^6 washed, sensitized human erythrocytes in a volume of 25 μ l were added to 50 μ l of serial three fold falling dilutions of monospecific rabbit antihuman IgG or antihuman IgM antiserum diluted in EDTA buffer. The suspensions were mixed and incubated at 37°C for 30 min and at room temperature for an additional 30 min and examined for agglutination. In all experiments there was no detectable agglutination of sensitized erythrocytes incubated with EDTA buffer alone or with anti-IgM antisera, while marked agglutination was seen with greater than 1 : 1000 dilution of anti-IgG antisera.

Quantitation of the Number of IgG Molecules Per Erythrocyte

The C1 fixation and transfer method was used to determine the average number of IgG molecules per erythrocyte in the sensitized erythrocyte population.^{10,15} Rh D-positive erythrocytes suspended in EDTA buffer were sensitized with twofold decreasing dilutions of human anti-D antiserum diluted in EDTA buffer as indicated in the preparation of EA. The average number of C1-fixing sites generated was determined following sensitization with rabbit IgG antihuman IgG, and typical dose-response curves with a slope of 1.0 were obtained, directly relating the number of C1-fixing sites to the number of IgG molecules per erythrocyte.^{10,16} Similar results were obtained using three different preparations of anti-IgG. In all experiments erythrocytes were sensitized with approximately 900–1000 IgG molecules per red blood cell.

Preparation of Monocyte Monolayer

Whole blood was obtained from normal human donors, anticoagulated with sodium heparin (10 U per ml of whole blood), and applied to a Ficoll-Hypaque density gradient.¹⁷ The mononuclear cell layer was harvested, diluted with HBSS to 1×10^6 mononuclear cells per ml and 1 ml was placed in replicate 35-mm plastic tissue culture dishes (Falcon Plastics, Oxnard, Canada) for 45 min at room temperature; nonadherent cells were decanted, and the adherent cells were washed five times with HBSS. In all experiments, monolayer plates contained greater than 97% mononuclear cells, of which approximately 80% were peroxidase positive. Morphologically, approximately 90% of cells appeared to be monocytes when stained with Wright-Giemsa and examined by light microscopy.

EA-monocyte rosette formation was achieved by incubating 1 ml (5×10^7 erythrocytes per ml) of E or EA with each monolayer for 2 hr at 37°C and washing five times with HBSS; the monolayers were then air-dried and stained with Wright-Giemsa. One hundred consecutive mononuclear cells per plate were counted, and the percentage of mononuclear cells having at

least three erythrocytes bound to their surface was determined. This figure was designated the percentage of rosette-forming cells. In all experiments, less than 1% of the mononuclear cells formed rosettes when incubated with E.

Statistics

The results are expressed as mean \pm standard error of the mean. Comparisons were evaluated using Student's *t* test for unpaired samples.

RESULTS

Incubation of EA-monocyte Rosettes With Papain

Mononuclear cell monolayers previously incubated with E or EA were incubated for 15 min at 37°C with 1.0 ml of VBS containing 0.02 *M* cysteine (VBS-cysteine) or VBS-cysteine containing increasing concentrations of papain. Each plate was washed three times with HBSS and the percentage residual rosette-forming cells determined. Prior to the addition of papain or buffer, 49% of the mononuclear cells formed rosettes. As little as 19 $\mu\text{g}/\text{ml}$ of papain resulted in a marked decrease in the percentage of mononuclear cells forming rosettes (Fig. 1), while there was $\leq 10\%$ decrease in the percentage of rosette-forming cells treated with buffer alone.

Incubation of EA and E With Papain

In order to evaluate the mechanism by which papain treatment of EA-monocyte rosettes abolished rosette formation, the site of action of papain was investigated. The effect of papain treatment of E and EA on the ability of these unsensitized and sensitized erythrocytes to subsequently bind to mononuclear cells (to form rosettes) was first examined. 5×10^7 E and EA were suspended in

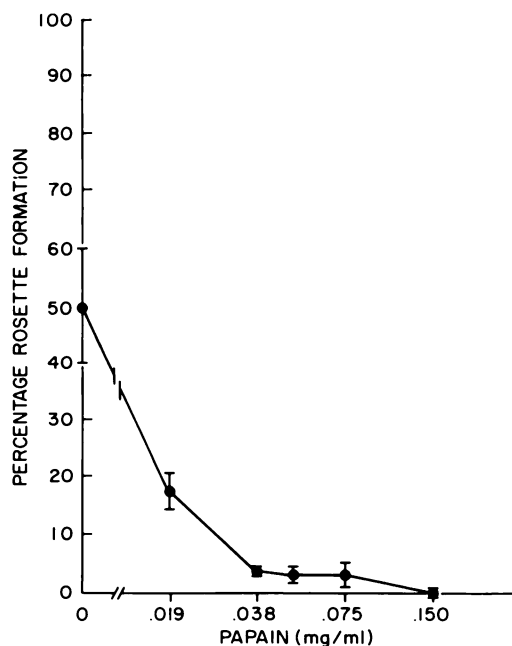


Fig. 1. Removal of sensitized (IgG) erythrocytes from the monocyte surface with papain. Bars indicating the mean \pm standard error of the mean are shown for each concentration of papain in this and subsequent figures.

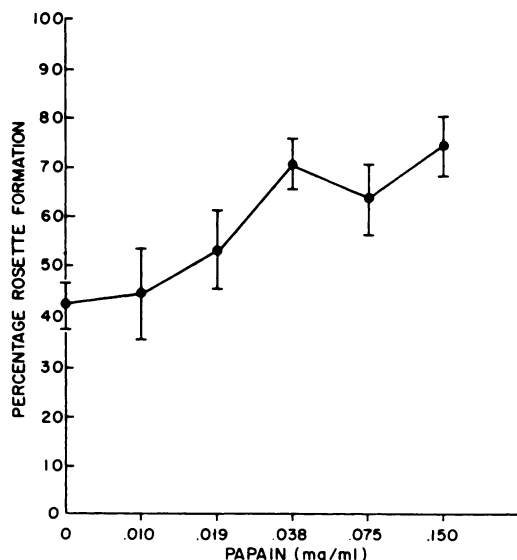


Fig. 2. Papain treatment of erythrocytes prior to sensitization with IgG anti-D antisera: effect on monocyte rosette formation. Each concentration of papain represents the mean of at least four separate experiments when compared to no papain. $p < 0.01$ for papain concentrations ≥ 0.038 mg/ml.

1 ml VBS-cysteine containing increasing concentrations of papain for 15 min at 37°C. The mixtures were sedimented, washed once with VBS and once with HBSS, resuspended to a concentration of 5×10^7 /ml in HBSS, and placed on the monolayer to assess rosette formation. As little as 19 μ g/ml of papain abolished the ability of EA to form rosettes, while EA incubated with VBS-cysteine without papain, as previously, elicited rosette formation in approximately 50% of the mononuclear cells. Unsensitized erythrocytes (E) incubated with papain resulted in less than 1% rosette formation.

Human erythrocytes were also incubated with papain prior to sensitization with anti-D antiserum, and the ability of these cells to form rosettes when subsequently sensitized was assessed. 5×10^7 erythrocytes were first incubated in 1 ml VBS-cysteine containing increasing concentrations of papain for 15 min at 37°C, sedimented, washed once with VBS, once with EDTA buffer, resuspended to a concentration of 1×10^8 /ml in EDTA buffer, and then sensitized with anti-D antiserum and placed on a monolayer to assess rosette formation. Sensitized erythrocytes which were not previously exposed to papain contained approximately 900–1000 IgG molecules per cell, while erythrocytes exposed to papain and then sensitized contained the same number of IgG molecules. Incubation of human erythrocytes with papain prior to sensitization with anti-D antiserum did not diminish the ability of the sensitized erythrocytes to form rosettes at all concentrations of papain examined (Fig. 2). In fact, preincubation of erythrocytes with high concentrations of papain (≥ 38 μ g/ml) resulted in an increase in the percentage rosette formation when they were subsequently sensitized with anti-D antiserum. At these higher concentrations of papain, agglutination of erythrocytes could be detected following sensitization with anti-D antiserum using a microtiter hemagglutination method. Erythrocytes preincubated with papain and incubated with EDTA buffer in the absence of anti-D antiserum elicited less than 1% rosette formation.

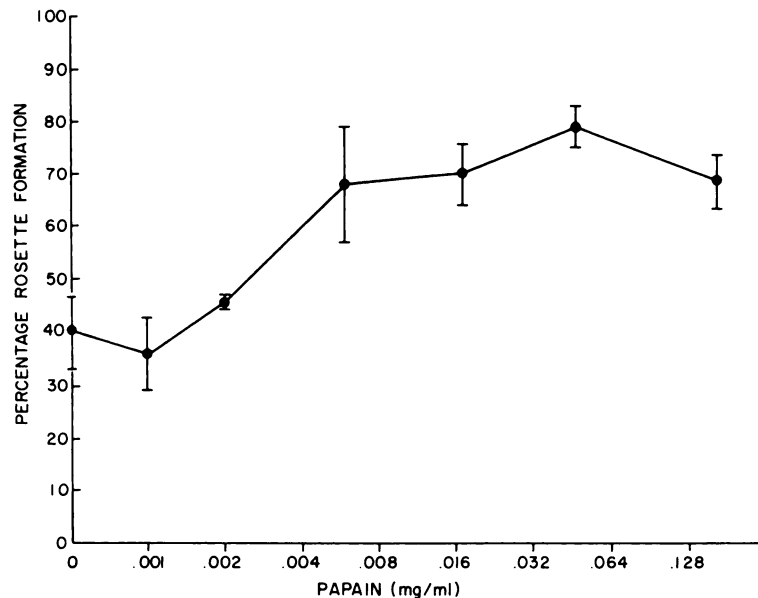


Fig. 3. Papain treatment of monocytes: effect on monocyte rosette formation when compared to no papain. $p < 0.01$ for papain concentrations ≥ 0.050 mg/ml.

Incubation of Mononuclear Monolayer Cells With Papain

In order to explore further the site(s) of papain action, monolayers were incubated with papain prior to the introduction of EA. One milliliter of VBS-cysteine containing increasing concentrations of papain was applied to each monocyte monolayer for 15 min at 37°C. The monolayers were washed twice with HBSS, and E or EA was applied to assess rosette formation. Pretreatment of the monolayer with papain did not diminish the ability of EA to form rosettes (Fig. 3). With concentrations of papain $> 2 \mu\text{g/ml}$, rosette formation appeared to increase, but statistical significance was noted only with papain concentrations $\geq 5 \mu\text{g/ml}$. Unsensitized erythrocytes (E) elicited less than 1% rosette formation. There was no apparent change in monocyte morphology by light-microscopy examination with all concentrations of papain employed.

Thus, it appeared that the papain effect on the erythrocyte membrane was insufficient to alter sensitization and that papain did not destroy the active site of the mononuclear monolayer cell IgG receptor. Rather, papain appeared to act upon the IgG antibody molecule bound to the erythrocyte and the IgG molecule which bound the erythrocyte to the mononuclear monolayer cell membrane. The integrity of the mononuclear monolayer cell IgG receptor following removal of bound IgG-sensitized erythrocytes by papain was investigated by examining the ability of these cells to reform rosettes. After removal of bound sensitized erythrocytes with papain, the monolayer was washed three times with HBSS, 5×10^7 EA or E in 1 ml VBS applied for 1 hr at 37°C and the plates washed three times with HBSS, air dried, stained with Wright-Giemsa, and assessed for rosette formation. Although papain treatment of the mono-

Table 1

Papain (mg/ml)	Percentage Rosette Formation		
	Before Papain (%)	Following Papain (%)	Following Addition of EA (%)
0.15	42	0	74*
0.05†	(40-43)		(71-78)
0.05†	43	2	70‡
	(40-49)	(0-8)	(40-85)
0.019†	44	17	50
	(40-49)	(10-24)	(4-82)
0§	43	33	35
	(40-49)	(21-48)	(11-58)

* $p < 0.01$.

†Mean of at least three experiments.

‡ $p = 0.05$.

§Mean of at least four experiments.

Parentheses indicate the range of experimental values.

cyte rosettes removed the sensitized erythrocytes from the monolayer in a dose-response fashion, the majority of monocytes on the monolayer were still capable of forming rosettes with new EA (Table 1). The capacity to form new rosettes appeared related to the extent of rosette removal with papain. Unsensitized erythrocytes did not form rosettes with monocytes either prior to or following treatment with papain. Thus, the binding of IgG-sensitized erythrocytes to the monocyte IgG receptor did not appear to increase the sensitivity of the IgG receptor to the proteolytic activity of papain. Moreover, IgG fragments formed during papain removal of IgG-sensitized erythrocytes from the mononuclear monolayer surface did not appear to diminish IgG receptor function.

DISCUSSION

Human peripheral blood monocytes have a receptor capable of binding human erythrocytes specifically sensitized with IgG immunoglobulin.¹⁻⁴ LoBuglio and his co-workers observed that IgG-coated erythrocytes which were bound to the monocyte could be removed by treatment with papain.² Incubation of IgG with papain results in loss of the antibody-combining site⁶ and the appearance of IgG digestion products of varying molecular weight.⁷ Additionally, incubation of intact cells with papain has resulted in removal of transplantation antigens¹⁸ and solubilization of antigens from the surface of various leukemic cells.⁸ Thus, papain's site(s) of action in removing IgG-coated erythrocytes from the monocyte IgG receptor might involve an effect of papain on IgG, the red cell, or the monocyte receptor. We have confirmed the ability of papain to remove IgG-sensitized erythrocytes from the monocyte surface and have explored the site of action of papain in this interaction.

These studies were performed in an in vitro assay for the monocyte IgG receptor using red cells quantitatively sensitized with IgG anti-Rh D immunoglobulin. There was no detectable IgM on the sensitized erythrocyte surface which might have interfered with the assay, and the presence of 0.01 M EDTA in the sensitizing buffer prevented participation of the complement system in

the reaction sequence. Several lines of evidence suggested that the cell monolayer contained predominantly monocytes. Approximately 90% of the monolayer cells appeared morphologically as monocytes by light microscopy, and 80% contained peroxidase-positive granules. Additionally, the IgG receptor could be detected on approximately 75% of the monolayer cells using red cells sensitized with high concentrations of IgG (unpublished observation).

Papain treatment of IgG-coated erythrocytes markedly diminished the capacity of these cells to bind to the monocyte IgG receptor. This action of papain could be attributed to an effect on IgG, since papain pretreatment did not prevent the binding of erythrocytes subsequently sensitized with IgG (Fig. 2). The increased capacity of papain ($\geq 30 \mu\text{g/ml}$)-treated erythrocytes to form rosettes following sensitization was not due to an increase in the number of IgG molecules per erythrocyte. Whether or not papain altered the erythrocyte so as to permit more efficient binding of IgG-sensitized erythrocytes to the monocyte IgG receptor remains to be determined.

The activity of the monocyte IgG receptor was not diminished by exposure to papain (Fig. 3). The apparent increase in the percentage of mononuclear cells forming rosettes after incubation with high concentrations of papain suggests that papain either exposed new receptor sites or increased the affinity of receptors for EA. Similar results were obtained in antibody-mediated and antibody-independent lymphocyte cytotoxicity studies when lymphocytes were pretreated with papain.¹⁹ Papain pretreatment of human monocytes⁴ and guinea pig macrophages²⁰ had previously been observed not to diminish IgG receptor activity.

When IgG-sensitized red cells were bound to the monocyte and then removed by papain, monocytes were still capable of binding IgG-sensitized erythrocytes (Table 1), and the extent of binding related to the concentration of papain in a dose-response fashion. Thus, rosette formation did not appear to alter the susceptibility of the monocyte receptor to papain. In fact, with papain concentrations $\geq 0.05 \text{ mg/ml}$, rosette formation was increased. This result may be attributed to the effect of papain on the monocyte, per se, as seen in Fig. 3.

Papain digestion of IgG produces two major antigenic fragments, Fab and Fc. The antibody-combining site resides in the Fab portion of the molecule,⁶ and the monocyte IgG receptor⁵ and the macrophage IgG receptor in animal systems^{21,22} are specific for the Fc segment of the IgG molecule. Our studies are consistent with the concept that the capacity of papain to remove IgG-sensitized erythrocytes from the monocyte receptor relates to the proteolytic effect of papain on IgG. Thus, IgG antibody may serve as a "bridge" between the erythrocyte and monocyte which is effectively cleaved by papain, separating the IgG antibody-combining site from the IgG Fc segment associated with the monocyte receptor. The ability of these monocytes subsequently to bind IgG-sensitized cells may reflect the resistance of the monocyte IgG receptor to papain or may relate to the exposure of new monocyte receptor sites by papain. Alternatively, the ability of these mononuclear monolayer cells to reform rosettes may involve the participation of IgG receptor sites not initially involved in rosette formation or point to an inability of the fragments of papain digestion (e.g., Fc fragments) to block the IgG receptor active site. Inability of

such Fc fragments to block IgG receptor activity may relate to the relatively small number of Fc fragments generated compared to the available number of IgG receptor sites per monocyte.

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