Autologous Agglutinators to Erythrocytes Sensitized with the Ripley Anti-Rh Antibody Digested with 14 Different Proteolytic Enzymes*†

MARION WALLER

Department of Medicine, Medical College of Virginia, Richmond

Isologous antigenic determinants of γ-globulin revealed by proteolytic digestion of the globulin molecules were discovered by Osterland, Harboe, and Kunkel (1963) in human IgG and by Mandy, Fudenberg, and Lewis (1965) in rabbit γ G. These determinants are detected by autologous and isologous antibodies found in most human sera and in all rabbit sera, respectively. These antibodies were named pepsin agglutinators (Natvig, 1966a and b) for pepsin digested human globulins and homoreactant (Mandy, et al., 1965) for digested rabbit globulins.

Subsequent studies with human globulins have shown that these serum agglutinators are primarily 7S; are usually specific for the enzymatic modification of the globulin molecule (Waller, in press); are distinct from other anti-globulin antibodies (Harboe et al., 1965); are found in most human sera albeit in low titer (Waller and Blaylock, 1966); and the titers of the pepsin agglutinators are not readily influenced by other immunologic events occurring in the individual.

Most investigators have confined their studies to pepsin and papain digestion of the γ-globulin molecules. However, Lawrence and Williams (1966) found that rheumatoid factors revealed a variety of antigenic determinants in gamma chains following digestion of these chains by trypsin, chymotrypsin, and bacterial protease. These authors did not distinguish between 7S serum agglutinators and 19S rheumatoid factors in their inhibition studies.

This study was undertaken to determine the effects of 14 different proteolytic enzymes on the IgG globulins and specifically the anti-Rh antibodies of a single serum, Ripley. As IgG is known to be heterogeneous (Dray, 1960; Rowe, 1962; Edelman et al., 1960) in chemical composition and on electrophoretic and chromatographic analyses and is subject to enzymatic attack at positions which may vary from molecule to molecule (Heimer et al., 1967), these parameters were limited as much as possible by confining the study to a single human serum.

None of the enzymes studied under the conditions as reported had the ability to so modify or degrade the antibody combining site of the molecule that this anti-Rh (Ripley) Fab was unable to bind to the Rh positive cells. However, titers of serum agglutinators to some of the modified IgG globulins were noted to always be lower when the digest preparations were allowed to proceed for a full 24 hours as opposed to four hours of digestion. Heimer et al. (1967) found that prolonged exposure to pepsin resulted in changes in the chemical nature of the component with the antibody combining site. This change was characterized by a fall in sedimentation coefficient.

The enzymes could be divided into three groups, only one of which invariably separated or degraded the Fc portion of the molecule so that the 19S rheumatoid factors were unable to bind to the digested globulin on the sensitized cells.

Absorption of heterologous anti-Fc and anti-IgG antisera by the Fab sensitized cells revealed differences between the papain, pepsin, and subtilisin enzymatic degradation of the antibody molecule, resulting in different Fab particles on the sensitized cells.

Materials and Methods

The Ripley Anti-Rh Antibody

This single serum was used in the preparation of the enzyme digested fragments of the IgG globulins and also for autologous agglutinator activity (antibody) to his own enzymatically degraded IgG anti-Rh antibodies.

Sera from normal individuals or from patients with rheumatoid

* Supported in part by the Charles Thomas Arthritis Fund, NIH Grant AM 04549, and NIH Training Grant T1 AM 5056. This is publication #10 from the Thomas Fund.

† Studies were carried out with the technical assistance of Nellie Curry and Jean Mallory.

arthritis were used as indicated in the text.

Preparation of the Digested Globulins

The IgG fraction of the serum studied was obtained by precipitation with $(NH_4)_2 SO_4$ followed by dialysis in 0.02 M phosphate buffer pH 8.0 and applied to a column of DEAE cellulose (Carl Schleicher & Schuell Co.) which was equilibrated with the same buffer. The first peaks obtained with starting buffer were isolated and dialyzed against the specific buffer used in the digestion.

Proteolytic Enzymes Used in the Study

Fourteen proteolytic enzymes were selected to degrade the IgG molecules. All enzymes were used at 1% of the weight of the protein. The following enzymes—pepsin, ficin, papain, trypsin, chymotrypsin, urease, collagenase, elastase, carboxypeptidase A and carboxypeptidase B—were obtained from Worthington Biochemicals Corp. Bromelin, rennin, subtilisin, and proteinase were obtained from Nutritional Biochemical Corp., Cleveland, Ohio.

Digestion Procedures

All digestions were carried out in the presence of 0.01 M cysteine and 0.002 M ethylenediaminetetraacetate (EDTA) and digested at 37°C for 18 hours. Modifications of the time of digestion are mentioned in the instances to which they apply.

Pepsin digestion was at pH 4.1 with 0.10 M acetate buffer; papain, proteinase, chymotrypsin, trypsin, and rennin at pH 7.4 with 0.10 M phosphate buffer; ficin at pH 7.0 with 0.10 M phosphate buffer; bromelin at pH 6.5 with 0.10 M phosphate buffer; urease at pH 7.0 with .75 M phosphate buffer; elastase at pH 8.8 with .2 M tris buffer; carboxypeptidase A at pH 7.5 with 0.025 M tris buffer containing .5 M NaCl; carboxypeptidase B and subtilisin at pH 7.5 with a 0.025 M tris buffer containing 1 M NaCl; and collagenase at pH 7.4 with .067 M phosphate buffer containing .45% NaCl.

To stop the digestions, the pH was raised to 8.0 followed by dialysis against saline for pepsin. For the other digests, the reaction was stopped by adding 1 volume of 1.25 gms% N-ethylmaleimide to 9 volumes of the protein solution, followed by dialysis against saline.

Sensitizations. One ml (containing 5 mg of digested globulin) was added to 0.1 ml human O DCe/DCe washed packed cells and sensitized at 37°C for one hour. The same cells from a selected donor were used throughout. The sensitized cells were then washed three times with saline and reconstituted in saline to a 2% suspension. The cells were tested with goat anti-Fc and anti-Fab (Hyland Laboratories) antisera. For erythrocytes sensitized with undigested anti-Rh antibodies, the anti-Rh sera were prepared by the same method, i.e., precipitation and column chromatography and the cells were sensitized with 5 mg/ml of globulins.

Agglutinations. Agglutination tests were performed in tubes by adding 0.1 ml aliquots of the sensitized or unsensitized cells to an equal volume of undiluted or serial two-fold dilutions of sera to be tested. The cells were then allowed to stand at room temperature for five to 10 min then spun for one min at 1000 rpm and read with the naked eye. The results were scored as 0 to 4+.

Inhibition Tests. Serum agglutinator activity can be removed either by inhibition with the digested globulins or by absorption of the activity with erythrocytes sensitized with enzyme digested anti-RH antibodies. In inhibition tests, saline was replaced by the material to be tested for inhibiting capacity. In absorption tests, unsensitized cells served as a control with the erythrocytes sensitized with the digested anti-RH antibodies.

Trypsin Modified Rh Positive Cells. The method used is but slightly modified from that of Morton and Pickles (1947).

Tests for Rheumatoid Factor. Methods for the slide latex (Hyland), sensitized human cell (SHC), and sensitized sheep cell (SSC) have been previously described (Waller et al., 1961).

Results

1. *Enzymes divided into three groups*

Certain proteolytic enzymes such as papain, pepsin, bromelin, ficin, and subtilisin rapidly degrade the Ripley globulin molecules. In the presence of the reducing agent, the anti-Rh antibody becomes univalent, and the Fc portion of the molecule is separated from the Fab portion of the molecule.

Erythrocytes sensitized with the anti-Rh antibodies, digested with other proteolytic enzymes, such as trypsin, chymotrypsin, elastase, rennin, collagenase, carboxypeptidase A and carboxypeptidase B, urease, and proteinase, always give positive results with anti-Fc serum diluted >5000. Some of these so-called Fc positive sensitized cell preparations are capable of binding both serum agglutinators and rheumatoid factor. Serological reactivity with these sensitized cell preparations in the 19S peak following DEAE chromatography has been demonstrable only in those sera giving positive tests for rheumatoid factor. This agglutinating activity was removed by absorption of these macroglobulin peaks with cells sensitized with undigested anti-Rh antibody. The macroglobulin peaks from sera giving negative tests for rheumatoid factor always gave negative agglutination reactions with these digest sensitized cells. The agglutinators in the 7S peak following DEAE chromatography, are not absorbed by cells sensitized with undigested antibody. Some of the proteolytic enzymes, such as collagenase, proteinase, urease, carboxypeptidase

A and carboxypeptidase B, bring about such a minor modification of the γ-globulin molecule that sera from individuals giving negative tests for rheumatoid factor always give negative reactions with cells sensitized with the digested anti-Rh antibodies. Rheumatoid sera show the characteristic reactions of the rheumatoid factor and this agglutination can be removed by absorbing the sera with cells sensitized with undigested antibody. Thus, we can divide these enzymes into three groups in regard to the modifications they make in the γ-globulin molecules as expressed by serological reactivity in human sera.

Group 1. Destruction or separation of all or most of the Fc portion of the γ-globulin molecule. Most human sera show serological reactivity with erythrocytes sensitized with these Fab portions of the anti-Rh antibody Heterologous anti-Fc sera diluted 1/200 give negative reactions with the sensitized cells. Rheumatoid factors will not bind to these sensitized cells. Agglutinators are found in the 7S peak after DEAE chromatography of whole sera. Representative enzymes are pepsin, papain, ficin, bromelin, and subtilisin.

Group 2. Anti-Fc sera >5000 give positive reactions with the sensitized cells. Both rheumatoid factors (19S) and serum agglutinators (7S) will bind to the sensitized cells. Percentage of normal sera reactive with these sensitized cells is usually less than with group 1. Representative enzymes are trypsin, chymotrypsin, elastase, and rennin.

Group 3. All sensitized cells are Fc positive >5000. Normal sera are not reactive with these sensitized cells. Rheumatoid sera give reactions characteristic of rheumatoid factor. Modification of the globulin molecule is slight or not at all. Representative enzymes are collagenase, urease, proteinase, carboxypeptidase A and B.

Table 1 shows the agglutinator titers of the Ripley serum with Rh positive erythrocytes sensitized with his own digested and undigested anti-Rh antibodies. These agglutinator titers are rather typical of normal sera. Usually the bromelin agglutinator shows the highest titer, and titers of other agglutinators for the enzyme digested anti-Rh antibodies in group 1 and group 2 vary between reactions only in undiluted sera to titers of 1:320. Sera giving negative reactions for rheumatoid factor will not agglutinate erythrocytes sensitized with undigested antibodies or antibodies digested with the group 3 enzymes. A rheumatoid serum usually gives positive agglutination reactions with erythrocytes sensitized with all three groups of enzyme modified anti-Rh antibodies. However, the enzymatic modification of the group 3 enzymes is minimal and the agglutinations observed with erythrocytes sensitized with antibodies "modified" by these enzymes as well as the agglutinations observed with undigested antibodies, merely represent reactions of rheumatoid factor. Rheumatoid sera show the same titer of factor with the group 3 enzyme modified antibodies as with undigested antibody.

TABLE 1
Titers of Serum Agglutinators in Serum Ripley with Erythrocytes Sensitized by Donor's Own Anti-Rh Antibodies Digested with a Variety of Enzymes at 37° for 18 Hours

Erythrocytes sensitized with digested anti-Rh antibody Ripley 5 mg/ml	Anti-Fc titers	Neat	Ripley serum titrated in saline					
			5	10	20	40	80	160
GROUP 1								
1. Pepsin	80	4	2	2	1	0	0	0
2. Papain	40	4	3	2	1	0	0	0
3. Ficin	40	4	2	1	0	0	0	0
4. Bromelin	160	4	4	4	4	3	2	1
5. Subtilisin	160	4	3	2	2	1	0	0
GROUP 2								
6. Trypsin	>5000	4	1	0	0	0	0	0
7. Chymotrypsin	>5000	4	3	3	2	1	0	0
8. Elastase	>5000	4	4	4	3	2	1	0
9. Rennin	>5000	2	0	0	0	0	0	0
GROUP 3								
10. Collagenase	>5000	0	0	0	0	0	0	0
11. Proteinase	>5000	0	0	0	0	0	0	0
12. Urease	>5000	0	0	0	0	0	0	0
13. Carboxypeptidase A	>5000	0	0	0	0	0	0	0
14. Carboxypeptidase B	>5000	0	0	0	0	0	0	0
15. Undigested globulins	>5000	0	0	0	0	0	0	0

There is no relationship between the anti-Fc titers of the sensitized cells and the serum agglutinator titers.

2. *Enzyme digested anti-Rh antibodies and reactions with anti-Fc serum.*

Table 2 shows the titers of anti-Fc (goat) and anti-Fab (goat) with erythrocytes sensitized with anti-Rh antibodies modified by the group 1 enzymes, erythrocytes sensitized with anti-Rh antibodies modified by the group 2 enzyme elastase, and with undigested anti-

TABLE 2
Titers of Anti-Fc and Anti-Fab with Erythrocytes Sensitized with Anti-Rh Antibody Ri Digested with Six Different Enzymes at 37 °C for 18 Hours

Erythrocytes sensitized with digest 5 mg/ml	Anti-Fc titrated in saline									
	1/10	1/20	1/40	1/80	1/160	1/320	1/640	1/1280	1/2560	1/5120
1. Pepsin	4	3	3	1	—	—	—	—	—	—
2. Papain	4	3	2	—	—	—	—	—	—	—
3. Ficin	3	3	1	—	—	—	—	—	—	—
4. Bromelin	4	4	2	1	1	—	—	—	—	—
5. Subtilisin	3	2	2	1	1	—	—	—	—	—
6. Elastase	4	4	4	4	4	4	4	3	2	1
7. Undigested antibody	4	4	4	4	4	4	4	4	2	2
	Anti-Fab titrated in saline									
		1/50	1/100	1/200	1/400	1/800	1/1600	1/3200	1/6400	
1. Pepsin		4	4	4	3	3	3	2	1	
2. Papain		4	4	4	4	4	4	3	2	
3. Ficin		4	4	4	4	4	3	2	1	
4. Bromelin		4	4	4	4	4	4	2	2	
5. Subtilisin		4	4	4	4	4	3	2	1	
6. Elastase		4	4	4	4	4	3	2	1	
7. Undigested antibody		4	4	4	4	4	3	2	1	

body. Note that at a 1/10 dilution of the anti-Fc antiserum all sensitized erythrocytes give strongly positive reactions. However, there is a marked difference in titer between the so-called Fc positive (>5000) and the Fc negative sensitized erythrocytes (<160). The anti-Fab titers are comparable for all the sensitized cells.

Table 3 shows the results of absortption of anti-Fc antiserum (goat) with erythrocytes sensitized with five different enzyme modified anti-Rh preparations. Williams and Lawrence (1966) found that rabbit antisera to 5S pepsin fragment of IgG after absorption with 3.5S Fab produced by papain at pH 7.4 showed distinct residual precipitins with the pepsin fragment of IgG.

The notation Fab and Fab′ (Cohen, 1965) will not be used because the enzyme subtilisin produces an Fab fragment distinct from these two. The notation Fab (pepsin, papain, bromelin, ficin, or subtilisin) will be used. Results of absorption of anti-Fc antisera diluted 1:10 with Fab (papain) is in accord with the previous studies of Williams and Lawrence (1966). Absorption of the anti-Fc antisera with the Fab (pepsin) left no residual antibody activity for erythrocytes sensitized with Fab (papain, ficin, bromelin, or pepsin) but did leave strong reactivity with erythrocytes sensitized with Fab (subtilisin). Thus, digestion with subtilisin results in a fragment which contains antigenic determinants not demonstrable in the fragments digested by pepsin, papain, ficin, or bromelin.

These absorptions with erythrocytes sensitized with enzyme modified anti-Rh antibodies (Ripley) were performed with an additional anti-Fc serum (goat) and with two additional anti-IgG antisera (rabbit) and comparable results were obtained. It was observed that heterologous antibody activity (anti-Fc) for subtilisin modified anti-Rh antibodies was the most difficult to absorb, requiring many more absorptions than the other sensitized erythrocytes.

3. *Serological evaluation of the proteolytic digestions of the Ripley IgG globulins.*

Use of whole digests rather than sensitized cells in inhibition experiments presents certain problems. Absorption tests with sensitized cells utilize only that portion of the anti-Rh antibody which is bound to the Rh positive cell. The size of the antibody or Fab fragment will depend upon the proteolytic enzyme used for digestion. On the other hand, the whole digest contains not only the Fab portions

of γ-globulin molecules, but the Fc portions and other antibody fragments (Heimer et al., 1967). Furthermore, all antibody molecules are available in the whole digest, not merely the anti-Rh antibodies.

Digests prepared from Ripley globulins may show the agglutinator activity of his whole serum (table 1). Therefore, in inhibition experiments the digest preparations will sometimes agglutinate the sensitized cells without the addition of the agglutinator. Only the digests prepared with the enzymes in group 1 consistently fail to show agglutinator activity. With this group of enzymes, the Fab portions of the globulin molecules are rapidly separated from each other and there is no bridge to bind the sensitized cells together.

When the whole digest preparations are used to inhibit the reaction between agglutinators and sensitized cells, as shown previously (Waller, in press) the inhibition may be quite specific. Pepsin digested globulins specifically inhibit the agglutinator activity to cells sensitized with pepsin digested anti-Rh antibodies. Pepsin digested globulins will not inhibit the agglutinator activity to papain digested Ripley sensitized cells. However, if the digest is prepared with an enzyme from group 2 or 3, the Fab portions of the globulin molecules may not be separated and any agglutinator activity present in the whole serum may be demonstrable in the digest preparations. The agglutinator with the highest titer (in all human sera) is to the bromelin digested antibodies and this bromelin agglutinator activity in the digest may be used as an indicator of the presence of bivalent IgG antibodies in the digest.

The reducion of incomplete anti-Rh antibodies to univalent fragments is also associated with the loss of ability to agglutinate enzyme modified Rh positive cells (Fudenberg et al., 1964). Thus, these digest preparations may be evaluated for antibody valence by two methods: (1) the bromelin agglutinator, and (2) agglutination of trypsinized Rh positive cells.

Table 4 presents a detailed study of two enzyme preparations. The Ripley globulins were digested by two different enzymes, bromelin (group 1) and elastase (group 2). The digestions were stopped at 30 min, one hour, four hours, seven hours, and 24 hours. Section (*a*) compares the anti-Fc titers with erythrocytes sensitized with the bromelin digested antibodies and the undigested antibodies and anti-Fc titers with erythrocytes sensitized with the elastase digested antibodies as compared with the undigested antibodies. The anti-Fc titers with elastase digested antibodies are >5000 with all sensitized preparations. However, the titers with the bromelin digested antibodies progressively declines to 1:40 at 24 hours. Section (*b*) shows the titrations of a single normal serum A. J. with erythrocytes sensitized with the bromelin and the elastase digested antibodies. With bromelin digested antibodies, the titer of agglutinator tends to progressively decline with increase in time of digestion. In other words, the agglutinator titer is highest after 30 min to one hour of digestion at a time when the anti-Fc serum with the same cells is showing very high titers. Erythrocytes sensitized with undigested antibody give negative results. Unlike bromelin, elastase digestions of the anti-Rh antibodies for varying lengths of time are not reflected in significant changes in elastase agglutinator titers.

Section (*c*) shows the titers of the whole digests serially doubly diluted in saline starting with 5 mg/ml with erythrocytes sensitized with bromelin digested anti-Rh antibody. Digestion with the group 1 enzyme destroys the bromelin agglutinator activity present in the undigested globulins, very rapidly, despite the fact that the same bromelin sensitized cells give good titers with the anti-Fc serum after 30 min to one hour of digestion. The elastase digests show bromelin agglutinator activity even after seven hours of digestion, albeit in lesser amount than the undigested material. Of course, these differences in titers are only comparable within the individual test situation, i.e., the digested versus the undigested globulins.

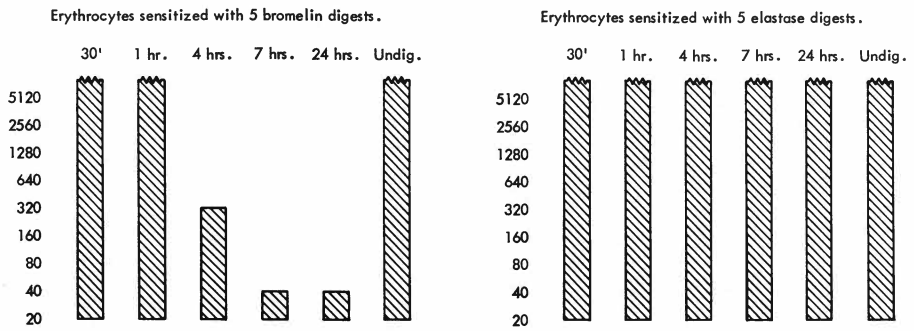
TABLE 3
Absorption of Anti-Fc Antiserum with Erythrocytes Sensitized with Anti-Rh Antibodies (Ripley) Digested with Five Different Proteolytic Enzymes for 18 Hours at 37°C

Sensitized erythrocytes 5 mg/ml	Titer unabsorbed	Anti-Fc antiserum (diluted 1/10) absorbed with sensitized cells					
		Pepsin	Papain	Ficin	Bromelin	Subtilisin	Undigested
1. Pepsin	1:80	0	<1:40	1:40	<1:40	0	0
2. Papain	1:40	0	0	0	0	0	0
3. Ficin	1:40	0	0	0	0	0	0
4. Bromelin	1:160	0	0	0	0	0	0
5. Subtilisin	1:160	>1:80	>1:80	>1:80	>1:80	0	0
6. Undigested	>5000	>2560	>2560	>2560	>2560	>2560	160*

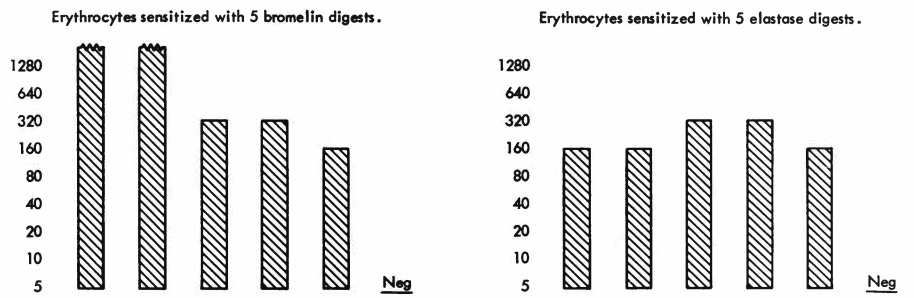
* Absorptions of anti-Fc were not carried beyond x5 because of the wastage of valuable preparations.

TABLE 4 SEROLOGICAL EVALUATION OF THE RIPLEY GLOBULINS DIGESTED WITH BROMELIN AND ELASTASE FOR VARYING LENGTHS OF TIME

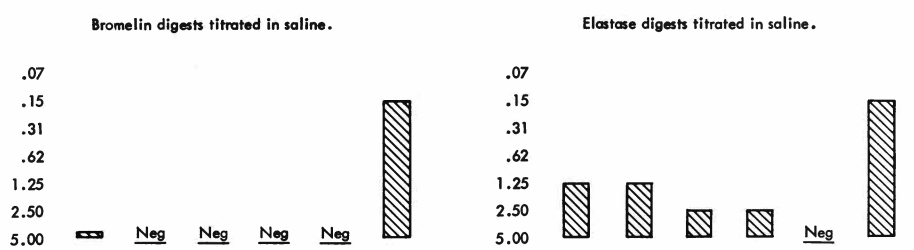
(a) Anti-Fc Titers



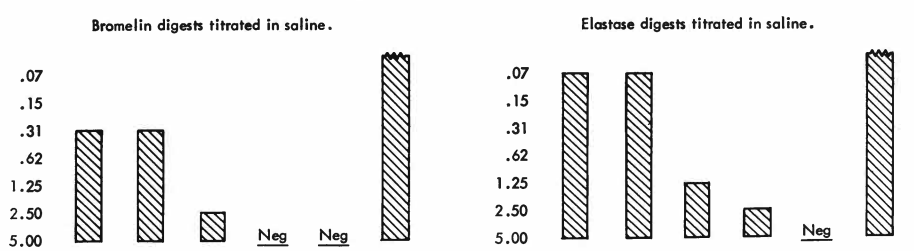
(b) Titers of Normal Serum A.J.



(c) Erythrocytes Sensitized with Bromelin Digested Antibodies 37° for 18 hours



(d) Trypsinized Rh Positive Cells



The effect of reducing the time of digestion on titers of (a) anti-Fc antiserum; (b) serum agglutinators; (c) bivalent Ripley agglutinator activity in the digests; and (d) ability of digested anti-Rh antibodies to agglutinate trypsinized Rh positive erythrocytes.

Section (*d*) shows these same digests in another system (Fudenberg et al., 1964), utilizing the well-known agglutination reactions of incomplete anti-Rh antibodies and trypsin modified Rh positive erythrocytes. After four hours of digestion, the bromelin digested incomplete anti-Rh antibodies are univalent and are unable to agglutinate the trypsin treated Rh positive cells. The elastase digested anti-Rh antibodies after 24 hours of digestion are also unable to agglutinate the trypsinized cells. This finding is of interest since there has not been a fall in titer of the anti-Fc serum with erythrocytes sensitized with the 24 hour elastase digest preparation.

Additional studies on 24 hour digest preparations with the group 3 enzymes showed no reduction in titer with trypsinized Rh positive cells as compared with undigested globulins, all standardized at 5 mg/ml. Among the group 2 enzymes, after 24 hours of digestion only elastase and trypsin digestions showed no ability to agglutinate the trypsinized cells; the chymotrypsin and rennin preparations showed as high a titer as the undigested globulins.

4. *Rheumatoid sera and enzyme digested Ripley anti-Rh antibodies.*

Table 5 shows the serologic reactions of the isolated IgG and IgM fractions of a rheumatoid serum (J. J.) with erythrocytes sensitized with the Ripley anti-Rh antibodies digested with bromelin for varying lengths of time. The IgG fraction lacks rheumatoid factor and the erythrocytes sensitized with the undigested antibodies are not agglutinated. However, this fraction is rich in bromelin agglutinator. The serologic activity with the bromelin digested anti-Rh antibodies is mercaptoethanol resistant and is not absorbable with erythrocytes sensitized with undigested antibody.

TABLE 5
IgG and IgM Fractions* of Rheumatoid Serum J. J. Tested with Erythrocytes Sensitized with Undigested Ripley Anti-Rh Antibodies and with Antibodies Digested with Bromelin for Varying Lengths of Time.

Cells sensitized with 5 mg/ml	IgG Fraction Titrated in Saline								
	2	4	8	16	32	64	128	256	512
Undigested bromelin digest	—	—	—	—	—	—	—	—	—
30 minutes	4	4	3	3	3	3	2	2	1
1 hour	4	3	3	3	3	2	1	—	—
4 hours	4	3	3	3	2	1	—	—	—
7 hours	4	3	3	3	2	1	—	—	—
24 hours	4	3	3	2	1	—	—	—	—
Cells sensitized with 5 mg/ml	IgM Fraction Titrated in Saline								
	2	4	8	16	32	64	128	256	512
Undigested bromelin digest	4	4	4	4	3	2	1	±	—
30 minutes	4	4	4	4	3	3	3	2	1
1 hour	3	3	3	2	2	2	2	1	—
4 hours	1	1	—	—	—	—	—	—	—
7 hours	1	1	—	—	—	—	—	—	—
24 hours	—	—	—	—	—	—	—	—	—

* Isolated by DEAE chromatography.

This activity can be inhibited with bromelin digested globulins and can be absorbed with erythrocytes sensitized with bromelin digested anti-Rh antibodies. Note that the bromelin agglutinator titer is highest with erythrocytes sensitized with antibodies digested for only 30 min. Additional time of digestion leads to a fall in titer of bromelin agglutinator. This was noted previously (Waller, in press). The anti-Fab titers of the bromelin digested anti-Rh antibodies showed very slight reduction after 24 hours of digestion as compared with 30 min of digestion.

The IgM fraction of the serum is rich in rheumatoid factor. Erythrocytes sensitized with the undigested antibodies are agglutinated. Also, the antibodies digested for 30 min to one hour are also agglutinated. Table 4 shows the anti-Fc titers to be >5000 with these preparations. After four hours of digestion, the anti-Fc titer is down to about 300 and rheumatoid factor agglutinates the cells very poorly.

The serologic activity in this fraction is mercaptoethanol sensitive and is completely absorbed with erythrocytes sensitized with undigested antibody.

The group 2 enzymes, trypsin, chymotrypsin, elastase, and rennin, characteristically do not destroy or remove the Fc portion of the antibody molecule. Serological activity with erythrocytes sensitized with antibodies digested with these enzymes may be found in both the IgG and IgM fractions of rheumatoid sera. However, the activity in the IgM fraction is absorbable with undigested antibodies and is believed to be rheumatoid factor that has bound to the partially digested antibodies. We have never found serum agglutinator activity in the 19S fraction of any serum.

Discussion

The Ripley serum does not differ from other normal (non-rheumatoid) sera in titer or specificity of agglutinator activity. In keeping with other normal sera, no agglutinator activity was detected in the macroglobulin fraction of the donor's serum. His IgG agglutinators are apparently unrelated to his anti-Rh antibodies. In these studies, no differences were noted between autologous and isologous agglutinators. The agglutinator activity demonstrable in the macroglobulin fractions of rheumatoid sera is absorbable with undigested Ripley antibody, is mercaptoethanol sensitive, and thus is technically rheumatoid factor.

It is of interest that no agglutinator activity is demonstrable in human sera for anti-Rh antibodies digested by those enzymes eliciting little or no alteration in the globulin molecules (group 3). In accordance with this, erythrocytes sensitized with anti-Rh antibodies "modified" by these enzymes are agglutinated by the rheumatoid factors.

Since we are studying the digested and undigested anti-Rh antibodies of a single human serum, it is difficult to imagine that the serum agglutinators and rheumatoid factors are completely unrelated. It appears that almost all humans have some of the agglutinators to digested gamma globulins but that the patient with rheumatoid factor has gone one step further and also has antibody to undigested γ-globulin.

Heterologous (goat) anti-Fc serum is standardized by Ouchterlony precipitin reactions which are much less sensitive than agglutination reactions. The anti-Fc serum actually gives positive agglutination reactions with erythrocytes sensitized with all digest preparations and presumably this represents contamination with some anti-Fab. Nevertheless, the serum can be made monospecific by absorbing it with appropriately sensitized cells.

Nomenclature (Cohen, 1965) for Fab portions of the globulin molecule is probably a bit premature, since subtilisin produces an Fab quite different from pepsin or papain and it is possible that other enzymes may cleave the globulin molecules at sites different from papain, pepsin, or subtilisin.

The ability of incomplete anti-Rh antibodies to agglutinate trypsin modified Rh positive cells is a reflection of the bivalency of the antibody or antibody fragment. Likewise, the ability of the digest preparations to show bromelin serum agglutinator activity is a reflection of the bivalency of the IgG globulins. These serologic reactions are valuable tools in detecting bivalent antibody molecules following digestions, reduction, or other types of molecular degradation.

Studies with the group 2 enzymes may prove to be of great interest in elucidating the reactions of the rheumatoid factors. Elastase is the example studied in detail here. After 24 hours of digestion with this enzyme, we find that erythrocytes sensitized with the digested antibodies give an anti-Fc titer of >5000. The elastase agglutinator titer has not significantly altered using preparations digested for varying lengths of time and we can presume that the digestive process was achieved early and that continued slow deterioration of the molecule is not proceeding as is the case with the bromelin digestions. However, we find that the ability of the digest preparations to agglutinate trypsinized cells is deteriorating (table 4, *d*) and actually the reaction is negative in the 24 hour digest. That the bivalent IgG globulins are slowly becoming univalent is also reflected in the loss of agglutinator activity (table 4, *c*). These reactions are probably due to the reduction of disulphide bonds (Nisonoff et al., 1960; Nisonoff et al., 1961).

Elastase digested anti-Rh antibodies will bind rheumatoid factors of some sera but not others. Elastase agglutinators are found in both normal sera and rheumatoid sera

and they are always IgG globulins. Additional studies with this enzyme may reveal information about the site on the Fc portion of the molecule where rheumatoid factor is bound.

Summary

Studies with 14 different enzyme digested globulin preparations of a single anti-Rh serum Ripley has revealed an astonishing array of autoagglutinators to his enzymatically degraded anti-Rh antibodies. These serum agglutinators did not differ from isologous agglutinators found in most human sera. They were found to be IgG globulins and were not demonstrable in the macroglobulin fraction of non-rheumatoid sera. The IgM agglutinating activity in rheumatoid sera is indistinguishable from rheumatoid factor.

Heterologous anti-Fc antisera absorbed with Fab fragments prepared from five different enzymes has revealed differences between the fragments produced by papain, pepsin, and subtilisin.

References

COHEN, S. Nomenclature of human immunoglobulins. *Immunology* 8: 1–5, 1965.

DRAY, S. Three γ-globulins in normal human serum revealed by monkey precipitins. *Science* 132: 1313–1314, 1960.

EDELMAN, G. M., J. F. HEREMANS, M. T. HEREMANS, AND H. G. KUNKEL. Immunological studies of human γ-globulin. *J. Exp. Med.* 112: 203–223, 1960.

FUDENBERG, H., W. J. MANDY, AND A. NISONOFF. Are incomplete antibodies univalent? Proc. 9th Congr. Int. Soc. Blood Transf., Mexico 1962. S. Karger Basel. Separatum. 562–568, 1964.

HARBOE, M., B. RAU, AND K. AHO. Properties of various anti-γ-globulin factors in human sera. *J. Exp. Med.* 121: 503–519, 1965.

HEIMER, R., S. S. SCHNOLL, AND A. PRIMACK. Products of the peptic digestion of human IgG immunoglobulin. *Biochemistry* 6: 127–133, 1967.

LAWRENCE, T. G., AND R. C. WILLIAMS. Reactions of human anti-γ-globulin factors with digested and urea-reduced γ-chains. *J. Immunol.* 97: 319–330, 1966.

MANDY, W. J., H. H. FUDENBERG, AND F. B. LEWIS. A new serum factor in normal rabbits. I. Identification and characterization. *J. Immunol.* 95: 501–509, 1965.

MORTON, J. A., AND M. M. PICKLES. Use of trypsin in the detection of incomplete anti-Rh antibodies. *Nature* 159: 779–780, 1947.

NATVIG, J. B. Reactions of human anti-antibodies with incomplete anti-D and its sub-units. *Acta Path. Microbiol. Scand.* 66: 383–395, 1966a.

NATVIG, J. B. Heterogeneity of anti-γ-globulin factors detected by pepsin digested human γ G-globulin. *Acta Path. Microbiol. Scand.* 66: 369–382, 1966b.

NISONOFF, A., F. C. WISSLER, L. M. LIPMAN, AND D. L. WOERNLEY. Separation of univalent fragments from the bivalent rabbit antibody molecule by reduction of disulfide bonds. *Arch. Biochem. and Biophy.* 89: 230–244, 1960.

NISONOFF, A., G. MARKUS, AND F. C. WISSLER. Separation of univalent fragments of rabbit antibody by reduction of a single, labile disulphide bond. *Nature* 189: 293–295, 1961.

OSTERLAND, C. K., M. HARBOE, AND H. G. KUNKEL. Anti-γ-globulin factors in human sera revealed by enzymatic splitting of anti-Rh antibodies. *Vox Sang.* 8: 133–152, 1963.

ROWE, D. S. Antigenic characteristics of γ-globulins. *Immunology* 5: 533–548, 1962.

WALLER, M., B. DECKER, E. C. TOONE, AND R. IRBY. Evaluation of rheumatoid factor tests. *Arthritis Rheumat.* 4: 579–591, 1961.

WALLER, M., AND K. BLAYLOCK. Further studies on the anti-globulin factors in human serum to the pepsin digested fragment of the Ri anti-Rh antibody. *J. Immunol.* 97: 438–443, 1966.

WALLER, M. Serum agglutinators of enzyme digested anti-Rh antibodies. *Vox Sang.* (In press).

WILLIAMS, R. C., AND T. G. LAWRENCE. Variations among pepsin-digested gamma globulins. *J. Clin. Invest.* 45: 714–723, 1966.