JCI The Journal of Clinical Investigation

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J Clin Invest. 1966;45(5):714-723. https://doi.org/10.1172/JCI105386.

Research Article

Journal of Clinical Investigation
Vol. 45, No. 5, 1966

Variations among γ-Globulins at the Antigenic Site Revealed by Pepsin Digestion *

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The pepsin site, an antigenic determinant of human γ-globulin uncovered by proteolytic digestion of IgG [1] by pepsin, has previously been described and characterized by Osterland, Harboe, and Kunkel (3). By using anti-Rh antibodies digested with pepsin at pH 4.1, it was found that 20% of normal sera and 57% of sera from patients with rheumatoid arthritis contained anti-γ-globulin factors, primarily 7 S, which agglutinated cells coated with pepsin-digested human incomplete antibody. Of particular interest was the finding that these agglutination reactions could not be inhibited by whole γ-globulin, but only by pepsin or papain digests of human γ-globulin performed at pH 4.1. Under these conditions a 5 S pepsin fragment related to Fab-fragment remained, whereas Fc-fragment was digested to dialyzable peptides. Fab-fragment produced by papain at pH 7.4 did not harbor the pepsin site (3).

Since myeloma proteins represent products of individual monoclonal proliferations of cells manufacturing relatively homogeneous populations of IgG or IgA molecules, it seemed pertinent to examine them after pepsin digestion at pH 4.1 for their retention or relative loss of the pepsin site antigenic determinant. Moreover, a correlation between the retention of inhibitory capacity in the pepsin site agglutination system and the typing of individual myelomas for Gm(a) (4, 5), Gm(b) (6), and Gm(f) (7, 8) was sought, particularly since Gm(f) has been localized to the Fab-fragment (9). Also, it seemed possible that retention or loss of the pepsin site might be related to the H-chain subgroups recently described from several laboratories (1, 2). If such correlations could be found, another simple and convenient way of classifying isolated M-components could be added to Ouchterlony techniques already in use. Furthermore, it was the object of this study to learn more about the structural basis for complete expression of the pepsin site.

Methods

Digestion procedures. Human Fraction II,[2] individual myeloma proteins, and γ-globulins from normal human sera isolated by zone electrophoresis (10) were adjusted to pH 4.1 by dialysis at 4° C against acetate buffer of ionic strength 0.1. Pepsin digestion was carried out for 24 hours at 37° C by using an enzyme protein ratio of 1:100. Digestion was halted by dialysis in the cold at 4° C against several changes of phosphate-buffered saline, pH 7.3, ionic strength 0.1. Pepsin digestion of anti-Rh antibodies was performed by using γ-globulin isolated from serum by zone electrophoresis or by three precipitations with saturated ammonium sulfate. Preparations of γ-globulins and individual myeloma proteins were examined at several concentrations in the model E Spinco

* Submitted for publication November 8, 1965; accepted January 20, 1966.

Aided in part by grants AM 5461 and AM 07372 from the National Institute of Health and a grant from the Minnesota Arthritis Foundation.

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[1] Glossary of terms used: IgG = γG or 7 S gamma globulin; IgA = γA or β_{2A}-globulin. Fab-fragment and Fc-fragment refer to the two antigenically distinct 3.5 S fragments obtained after papain digestion of gamma globulin at pH 7.4; pepsin digestion of IgG at pH 4.1 produces a 5 S pepsin fragment antigenically related to papain Fab-fragment; Fd refers to the A piece or H-chain constituents of 5 S pepsin fragment. Gm(a), Gm(b), Gm(bw), and Gm(f) refer to genetically determined characteristics of γ-globulin molecules detected by inhibition of specific serologic agglutination systems. H-chain subclasses of γ-globulins include We or γ_{2b}, Vi or γ_{2c}, Ne or γ_{2a}, and Ge or γ_{2d} (1, 2); L-chain types are designated as either K or λ. M-components are isolated monoclonal myeloma proteins.

[2] American Red Cross, from E. R. Squibb and Sons, New York, N. Y., lot 20069.

analytical ultracentrifuge before and after pepsin digestion. All samples thus studied were equilibrated with pH 7.3, 0.1 M phosphate buffer and sedimentation coefficients calculated for S_{20}, w^{∞} at infinite dilution according to the methods described by Trautman (11). Loss of Fc-fragment antigenic determinants after pepsin digestion was confirmed by Ouchterlony analysis of all pepsin digests of γ-globulins and individual myeloma proteins by testing preparations before and after digestion with rabbit antisera specific for Fc antigenic determinants. Such antisera gave reactions with Fc-fragment, but they had been absorbed so as not to react with Fab or isolated L-chains. Absence of reaction with specific anti-Fc-fragment antiserum confirmed completeness of pepsin digestion at pH 4.1.

Serological test systems employed for assay of the pepsin site. Five human sera containing incomplete Rh antibodies of varying reactivity were used as the source of pepsin-digested human incomplete antibody. These antibodies included Rip, Matth, Garn, Buss, and Pet. Coats Rip and Buss in the whole state could be used for Gm(a) typing, Pet showed specificity for the Gm(b) (12) system, whereas Matth showed no Gm specificity but broad reactivity with many agglutinators. Garn coat in the whole state showed reactions only with occasional agglutinators. Twenty other high titer anti-Rh-antibody-containing sera were studied after pepsin digestion but gave weak or inconstant pepsin coats and were not studied further. Completeness of pepsin digestion of the γ-globulins from the anti-Rh sera was confirmed by loss of Gm specific agglutination of Gm(a) and Gm(b) coats (3, 13) and by absence of agglutination of pepsin-treated antibody-coated cells by specific rabbit anti-Fc antiserum (13).

Rh-positive cells (R_1R_1) coated with the five pepsin-digested human incomplete antibodies (3, 13) were tested against a panel of selected sera from patients with rheumatoid arthritis or subacute bacterial endocarditis, as well as against sera from normal donors (Table I).

A standard agglutinating dose consistently producing 2 plus agglutination was established for a smaller panel of nine agglutinating sera by repeated titration against the five pepsin-digested antibody coats; specificity of inhibition by pepsin-digested IgG, but not by whole IgG, was confirmed in all instances. Individual myeloma proteins digested at pH 4.1 by pepsin and adjusted to 1 mg per ml were tested in doubling dilutions for their ability to inhibit each agglutinating system. Inhibitory capacity of pepsin-digested M-components was remarkably constant from one set of tests to the next, varying no more than one doubling dilution. Inhibition was expressed as last $\log_2$ dilution of inhibitor producing complete inhibition of agglutination.

Isolated M-components were tested for Gm(a), (b), and (f) or (bw) as previously described (4, 7). Three different concordant Gm(b) typing systems were used.[3]

[3] Gm(f) and Gm(b) typing reagents were provided by Drs. L. Martensson, R. Grubb, A. G. Steinberg, and H. G. Kunkel.

TABLE I

Reactions between selected sera from patients with rheumatoid arthritis and subacute bacterial endocarditis and normal blood donors with five pepsin-digested human incomplete anti-Rh coats

Agglutinating serum	Pepsin-digested anti-Rh coats				
	Rip	Buss Gm (a)†	Pet Gm (b)†	Matth	Garn
Dri*	320	10	40	40	0
Dav	160	40	0	10	0
Schw	20	0	0	10	0
Rubl	40	10	5	20	0
Pa	10	40	10	10	0
Bigal	40	40	40	40	80
Ral	20	20	0	0	0
Dro	20	0	0	0	0
Mer	10	0	0	0	0
Fal	40	5	160	20	80
Bog	320	10	320	160	0
Turn	160	80	80	20	0
Pierr	320	80	160	40	0
Saund	40	160	160	80	0
7364	16	8	8	8	0
7239	16	4	0	4	0
7351	8	4	0	4	0
8702	32	8	4	0	0
8738	32	8	2	0	0
8705	16	0	0	0	0

* Serum agglutinators Dri to Mer are from patients with rheumatoid arthritis. Fal to Saund are agglutinators from subacute bacterial endocarditis. 7364 to 8705 represent normal blood donor sera.
† See footnote 1 of the text for definition.

Correlation of serological findings with precipitin reactions. Isolated IgG myeloma proteins were compared with Fraction II in quantitative precipitin curves by using rabbit antihuman γ-chain antisera with primary reactivity for Fc-fragment to establish their degree of antigenic deficiency (14). In addition, quantitative precipitin curves were performed using pepsin-digested myeloma proteins and rabbit antisera to pepsin digests of human IgG absorbed with L-chains (15).

Ouchterlony analysis of pepsin-digested M-components utilized rabbit antisera against pepsin-digested human IgG absorbed with Fab-fragment obtained after papain digestion at pH 7.4, or antipepsin-digested IgG antiserum absorbed with pooled L-chains.[4]

Reduction studies. In the case of pepsin digests of individual myeloma proteins reduced with various concentrations of mercaptoethanol, the extent of reduction expressed as free sulfhydryl groups produced was estimated by a modification of the method of Ellman (16). After addition of mercaptoethanol, the reaction was allowed to proceed for 1 hour. A portion was then added to 4 vol of cold 10% trichloroacetic acid. The resulting precipitate was washed four times with 10% trichloroacetic acid and taken up in 5 M guanidine HCl, pH 3.0. An equal

[4] Myeloma proteins typed for H-chain subgroups (1, 2) were generously supplied by Dr. Henry Kunkel in the case of proteins 1–15. Many other M-components H-chain groups were kindly typed by Drs. Edward Lichter and William Terry.

volume of guanidine HCl, buffered with Tris base to produce a final pH of 8.0, was added, and 0.1 mg of 5,5′ dithio (bis-nitrobenzoic acid) in 0.1 ml of ionic strength 0.1 phosphate buffer, pH 7.0, was immediately added; optical density at 412 mμ was determined.

Results

Basic inhibition studies. When 41 IgG pepsin-digested myeloma proteins were compared with pepsin digests of Fraction II for their inhibitory capacity in the pepsin site system, striking deficiency of inhibition was noted in ten (Table II). The remainder closely approximated pepsin-digested Fraction II in their inhibition potency. Among the first ten pepsin site deficient myelomas, nine showed no Gm(a), (b), or (f) character, but one was positive for Gm(b) (Uel). The four proteins Bjork, Dunk, Lou, and Metsa that showed $\log_2$ inhibition of 4 to 6 were of the Ge, or γ_{2a}, subclass of H-chains. Four (Brown, Hart, Murr, and Pik) were of the Ne, or γ_{2a}, H-chain subgroup. The deficiency of the myelomas tested bore no re-

TABLE II

Relative inhibiting capacity of pepsin-digested IgG myeloma proteins in a pepsin site agglutination system using nine different agglutinators and cells coated with pepsin-digested anti-CD Ripley

Pepsin-digested myeloma	L-chain	H-chain	Gm character	Agglutinators tested								
				Dri	Bog	Dav	Pierr	Turn	Saund	7239	7364	Schw
5 S pepsin fragment from pepsin digest of IgG	λ κ	Complete	Gm(a+b+f+)	12†	12	12	12	12	12	12	12	12
Nichol	κ	*	Gm(a−b−f−)	4	5	5	5	12	4	4	5	4
Brown	κ	Ne		4	4	4	4	3	4	5	5	4
Hart	λ	Ne		4	4	4	4	7	4	4	4	4
Murr	κ	Ne		7	7	8	7	6	8	7	8	7
Pik	κ	Ne		5	5	5	5	4	5	5	5	6
Bjork	λ	Ge		4	4	5	4	4	4	4	4	4
Dunk	κ	Ge		4	4	4	4	4	4	4	4	4
Lou	κ	Ge		4	5	5	5	4	5	5	4	5
Metsa	λ	Ge		6	6	6	6	10	6	6	6	6
Uel	κ	Vi	Gm(a−b+f−)	5	5	6	5	11	6	5	5	5
Bonap	κ	We	Gm(a−b−f−)	12	11	11	11	12	11	11	11	12
Stang	κ	We		12	12	12	12	12	12	12	12	12
Saxum	κ	We		11	11	12	12	11	12	12	11	12
Ander	κ	*		12	12	12	12	6	12	12	12	12
Wade	λ	Vi	Gm(a−b+f−)	11	11	11	11	7	11	11	11	11
Lang	κ	Vi		11	12	12	12	6	12	12	12	11
Nells	κ	We	Gm(a−b−f+)	11	11	12	11	12	11	12	12	11
Geisen	κ	We		12	12	12	12	12	12	12	12	12
Carl	κ	We		12	12	12	12	10	12	12	12	12
Clemen	λ	We		12	12	12	12	6	12	12	12	12
Sand	κ	We		12	12	12	12	6	12	12	12	12
Lavelle	κ	We		12	11	11	11	12	12	12	12	11
Ca	κ	We		11	11	11	11	10	11	11	11	11
Rand	λ	We		12	12	12	12	11	12	12	12	12
Swa	κ	We		12	12	12	12	3	12	12	12	12
Lauden	κ	We		12	12	12	12	10	12	12	12	12
Warnig	κ	We		12	12	12	12	12	12	12	12	12
Kane	κ	We		12	12	12	12	11	12	12	12	12
Goplan	κ	We		12	11	12	11	12	12	11	12	12
Swet	λ	We		12	12	11	12	6	12	12	12	12
Weberg	λ	We		12	12	11	12	11	12	12	12	12
Nixon	κ	We		12	12	12	12	11	12	12	12	12
Peder	κ	We	Gm(a+b−f−)	12	12	12	12	12	12	12	12	12
Fugu	κ	We		12	12	12	12	11	12	12	12	12
Smith	λ	We		12	12	12	12	5	12	12	12	12
White	κ	We		12	12	12	12	4	12	12	12	12
Lipsc	λ	We		12	12	12	12	9	12	12	12	12
Thomp	κ	We		12	12	12	12	11	12	12	12	12
Blix	λ	We		12	12	12	12	6	12	12	12	12
MacFar	λ	We		11	11	11	11	11	11	11	11	11
Triplett	λ	We		12	12	12	12	10	12	12	12	12

* Myelomas could not be typed as We, Vi, Ge, or Ne subgroups.
† Numbers represent $\log_2$ dilution of inhibitor effective in inhibition beginning at 1.0 mg per ml.

TABLE III

Inhibitory capacity of 15 additional pepsin-digested myelomas of known H-chain subclass in pepsin site system using pepsin-digested anti-CD Ripley incomplete antibody

Pepsin-digested myeloma	L-chain type	H-chain type	Agglutinators tested								
			Dri	Bog	Dav	Pierr	Turn	Saund	7239	7364	Schw
1	κ	Ne	2*	3	3	3	2	3	3	3	2
2	κ	Ne	4	5	5	4	4	4	5	4	5
3	κ	Ne	5	5	5	5	6	5	5	5	5
4	κ	Ge	5	4	4	4	3	4	4	4	4
5	κ	We	12	12	12	12	12	12	12	12	12
6	λ	Vi	11	11	11	11	11	11	11	11	11
7	κ	We	11	12	12	11	11	12	12	12	12
8	λ	Ne	2	3	3	2	4	3	2	2	3
9	κ	Ge	2	3	3	2	4	3	3	2	3
10	κ	We	11	12	11	11	9	12	12	11	12
11	κ	We	12	12	12	12	12	12	12	12	12
12	κ	We	12	11	12	12	10	12	12	12	12
13	κ	We	12	12	12	12	12	12	12	12	12
14	κ	Ne	5	4	5	5	6	5	5	5	4
15	κ	Ne	5	5	6	5	7	5	5	5	5

* Numbers represent $\log_2$ dilution of inhibitor effective in inhibition beginning at 1.0 mg per ml.

lationship to the kappa or lambda L-chain groups (17, 18). The majority of myelomas that showed pepsin site deficiency lacked all Gm characters. However, other myelomas that also lacked Gm character (Bonap, Stang, Saxum, Ander) showed no deficiency in pepsin site inhibition. The remainder of the pepsin-digested myelomas, including two proteins of the Vi (γ_{2c}) subgroup and 25 of the We (γ_{2b}) subgroup, showed no general deficiency with the panel of agglutinators listed in Table II. Inhibition patterns utilizing the other four pepsin-digested coats and the same panel of agglutinators produced essentially the same pattern as shown in Table II for pepsin Rip coat.

Of considerable interest were the inhibition patterns obtained with agglutinator Turn. In many systems a discrepancy between inhibition by a pepsin-digested M-component occurred between Turn and the other eight agglutinators tested. For Nichol, Uel, and Metsa pepsin-digested myelomas, Turn revealed no gross deficiency. On the contrary, in several instances of M-components not deficient with the other eight agglutinators (Smith, Swa, Swet, Clemen, and White), Turn revealed a deficiency in inhibition between $\log_2$ dilutions 3 and 6.

The initial group of ten myelomas deficient at the pepsin site appeared to contain no universal H-chain subgroup. Uel was positive for Gm(b) and typed as Vi H-chain subgroup; the remainder were Ge or Ne, one not falling into any major H-chain subclass. In an attempt to extend these relationships further, additional myelomas 1 to 15 [5] were tested for inhibition in the pepsin site system without prior knowledge of their H-chain subgroup or Gm type. Table III reveals that eight of these additional M-components were deficient—six were Ne, and two Ge, H-chain subgroup. Thus, all of the Ge or Ne pepsin-digested M-components tested for pepsin site inhibition showed marked deficiency. Of the 56 pepsin-digested IgG myelomas tested, four were Gm(b)+ and therefore of the Vi subgroup (8). Only one of these showed pepsin site deficiency. No myeloma positive for Gm(f) showed deficiency at the pepsin site, except with agglutinator Turn.

Studies of whole undigested myeloma proteins. Further experiments sought to answer two pertinent questions: had isolation or previous storage of M-components allowed spontaneous dissociation and thus variable exposure of the pepsin site, and did pepsin digestion produce the same 5 S fragment in all myelomas studied?

Freshly isolated myeloma proteins examined by immunoelectrophoresis against anti-whole gamma globulin antisera and against antisera to pepsin-digested IgG showed no gross dissociation or splitting. However, occasional isolated myelomas stored at 4° C for 2 to 3 weeks did appear to undergo some dissociation by immunoelectrophoresis. In all instances where any dissociation of whole isolated M-components was apparent by analytical

[5] Generously provided by Dr. H. Kunkel.

ultracentrifugation or immunoelectrophoresis, further digestion by pepsin was not carried out, and these myelomas were not studied further. Since inhibition of pepsin site agglutination by individual pepsin digests had proven a sensitive assay for the integrity or exposure of the pepsin site, the whole freshly-isolated M-components were tested for inhibition. In no instance did these fresh whole M-components show inhibition.

Physical studies. In the ten initial deficient myelomas, the majority of the whole M-components showed sedimentation rates of 6.9 S to 7.0 S proceeding to 4.9 S to 5.1 S after digestion. Exceptions to this included M-components Metsa and Hart, which proceeded to < 1.0 S and 3.9 and 5.7 S, respectively. In similar ultracentrifugal analyses, other isolated myelomas that showed full expression of the pepsin site in inhibition proceeded from values near 7.0 S in the whole state to 5.0 S after digestion (Table IV, Figure 1). Of interest in this regard were proteins Blix, Lavelle, and Smith which, after pepsin digestion, showed S_{20}, w^{∞} values of 1.4, 2.0, and 1.8 S, respectively, but which retained full inhibitory capacity for the pepsin site with the entire panel of agglutinators and pepsin-digested coats.

TABLE IV

Sedimentation coefficients of isolated myeloma proteins before and after pepsin digestion, as correlated with their inhibitory capacity for the pepsin site and precipitin reactions

Myeloma protein	L-chain group	H-chain group	S_{20}, w, whole protein	Reaction of whole with anti-Fc*	S_{20}, w, digest protein	Reaction with anti-Fc	Log_2 dilution of inhibitor
Nichol	κ	†	7.0	+	5.0	0	4–5
Brown	κ	Ne	7.0	+	5.0	0	4–5
Hart	λ	Ne	6.9	+	3.9, 5.7	0	4
Murr	κ	Ne	7.0	+	5.1	0	7–8
P.K.	κ	Ne	7.0	+	5.0	0	5–6
Bjork	λ	Ge	7.0	+	5.0	0	4–5
Dunk	κ	Ge	6.9	+	5.1	0	4
Lou	κ	Ge	7.0	+	5.0	0	4–5
Metsa	λ	Ge	7.0	+	<1.0	0	7
Uel	κ	Vi	6.9	+	5.0	0	5–6
Bonap	κ	We	7.0	+	5.0	0	11–12
Stang	κ	We	7.0	+	5.0	0	12
Saxum	κ	We	7.0	+	5.0	0	11–12
Ander	κ	†	6.9	+	5.0	0	12
Wade	λ	Vi	7.0	+	5.0	0	11
Lang	κ	Vi	7.0	+	5.0	0	11–12
Nelson	κ	We	6.9	+	5.0	0	11–12
Geisen	κ	We	7.0	+	5.0	0	12
Carl	κ	We	7.0	+	5.0	0	12
Clemen	λ	We	6.9	+	4.9	9	12
Sand	κ	We	7.0	+	5.1	0	12
Lavelle	κ	We	7.0	+	2.0	0	11–12
Ca	κ	We	7.0	+	5.0	0	11
Rand	λ	We	7.0	+	5.0	0	12
Swa	κ	We	7.0	+	5.0	0	12
Lauden	κ	We	6.9	+	5.1	0	12
Warnig	κ	We	6.9	+	5.0	0	12
Kane	κ	We	7.0	+	5.0	0	12
Goplan	κ	We	7.0	+	5.0	0	11–12
Swet	λ	We	7.0	+	5.0	0	12
Weberg	λ	We	7.0	+	5.0	0	12
Nixon	κ	We	7.0	+	5.0	0	12
Peder	κ	We	6.9	+	5.1	0	12
Fugu	κ	We	7.0	+	5.0	0	12
Smith	λ	We	6.9	+	1.8	0	12
White	κ	We	7.0	+	5.0	0	12
Lipsc	λ	We	7.0	+	5.1	0	12
Thomp	κ	We	7.0	+	5.0	0	12
Blix	λ	We	6.9	+	1.4	0	12
MacFar	λ	We	7.0	+	5.1	0	11
Triplett	λ	We	7.0	+	5.0	0	12

* See footnote 1 of the text for definition.
† Myelomas could not be typed for Ge, Ne, Vi, or We subgroups.

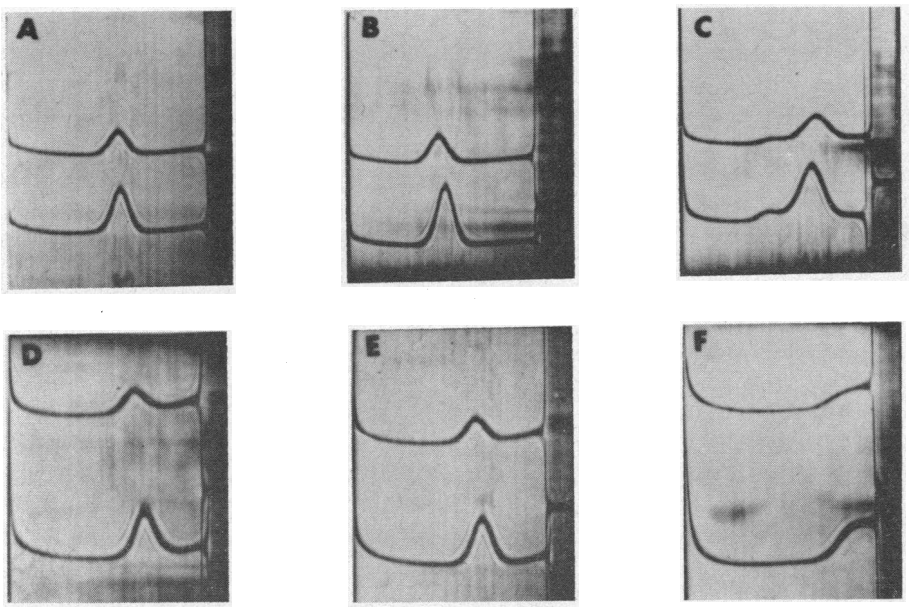

FIG. 1. ULTRACENTRIFUGAL STUDIES ON ISOLATED MYELOMA PROTEINS EXAMINED AT TWO CONCENTRATIONS IN THE WHOLE STATE AND AFTER PEPSIN DIGESTION. All photographs were taken after 64 minutes at 59,780, and all samples were examined in 0.1 M phosphate buffer, pH 7.3. Direction of sedimentation is from right to left. A and D represent M-component (isolated monoclonal myeloma protein) Lipsc before and after digestion; sedimentation rate in the whole state was 7.0 S_{20}, w^{∞} and 5.0 S_{20}, w^{∞} after digestion. B shows Brown M-component 7.0 S_{20}, w^{∞} before digestion, and 5.0 S_{20}, w^{∞} after pepsin digestion (E). Lipsc retains pepsin site inhibition, and Brown is deficient after digestion. F represents pepsin digest Lavelle, which has an S_{20}, w^{∞} value of 2.0 but retains pepsin site inhibitory capacity. C represents M-component Geist, which has dissociated into 3.5 S material on prolonged storage. A small 7 S shoulder of residual whole myeloma protein is noted. All M-components showing such dissociation after storage were not included further in the study.

Reduction studies. The role of disulfide linkages within pepsin-digested M-components as well as pepsin digests of γ-globulin was studied next with respect to the integrity of the pepsin site. Since relatively labile inter-H-chain disulfide bonds have been demonstrated in IgG digested by pepsin (19), it seemed pertinent to study the effects of progressive reduction on the inhibitory capacity of individual pepsin digests. Accordingly, 5 S pepsin fragment from human IgG was reduced in the presence of 0.001, 0.01, and 0.1 M β-mercaptoethanol in 0.5 M Tris buffer, pH 8.0. Acetylation was then carried out with excess of iodoacetamide, and preparations were subsequently dialyzed versus 0.15 M saline. Reduction of the 5 S pepsin fragment of IgG by 0.001 M mercaptoethanol resulted in some loss of inhibitory capacity for the pepsin site systems. Progressive loss of inhibitory power was noted after the reduction by 0.01 M and 0.1 M mercaptoethanol, the latter virtually destroying inhibitory capacity. When the 0.1 M reduced mercaptoethanol pepsin fragment was separated from the reducing agent by G25 Sephadex gel filtration and allowed to stand in an atmosphere saturated with oxygen for 12 hours, the reoxidized pepsin fragment regained full inhibitory capacity (Table V).

Reduction of pepsin-digested myelomas retaining full inhibition for the pepsin site was compared with reduction of pepsin fragments of myelomas deficient in the various agglutination systems. Amount of free or measurable sulfhydryl was determined by using Ellman's reagent after reduction at 0.001 M, 0.01 M, and 0.1 M mercaptoethanol. Reduction products were then tested in the nine pepsin site agglutination systems and $\log_2$ dilution of residual effective inhibition plotted against moles of SH per mole of pepsin-digested γ-globulin.

TABLE V

Loss of inhibitory capacity of 5 S pepsin fragment of IgG after reduction with β-mercaptoethanol and restoration after reoxidation in atmosphere saturated with oxygen

Inhibitors tested in pepsin site systems	Log_2 dilution effective as inhibitor of pepsin site agglutinating systems
Unaltered 5 S pepsin fragment of IgG	12
5 S pepsin fragment reduced with 0.001 M β-mercaptoethanol	8–9
5 S pepsin fragment reduced with 0.01 M β-mercaptoethanol	5–6
5 S pepsin fragment reduced with 0.1 M β-mercaptoethanol	0–1
5 S pepsin fragment reduced with 0.1 M β-mercaptoethanol and separated from reducing agent thereupon reoxidized in oxygen	11–12

Figure 2 indicates that myelomas retaining full inhibition as native pepsin digests showed (Δ) free SH changes quite similar to those of pepsin digests of Cohn Fraction II, and inhibition was progressively lost with increasing concentrations of mercaptoethanol used in reduction. Other deficient myeloma pepsin fragments, such as Brown and Dunkl, showed less inhibition with progressive reductions producing steeper plots. Of interest were studies with the pepsin digest of Lavelle, which was known to be 2.0 S, yet retained full inhibiting power. Very little free SH could be titrated after reduction, and no marked loss of inhibition was noted.

Since it was previously shown (3) that papain Fab-fragment produced at pH 7.4 in the presence of cysteine did not contain the pepsin site antigen, it seemed of interest to determine whether an interchain disulfide in Fc-fragment could contribute to the pepsin site antigen. Accordingly, Fc-fragment was isolated by zone electrophoresis after digestion by papain for 10 minutes in the presence of cysteine (20). Oxidation of Fc was carried out for 12 hours in the presence of an atmosphere of 100% oxygen. The Fc-fragment thus treated did not inhibit in the pepsin site agglutination systems.

Precipitin and Ouchterlony studies. As a parallel to the results of inhibition of pepsin site agglutination reactions, quantitative precipitin curves using anti-pepsin fragment antisera absorbed with L-chains and the isolated pepsin digests of M-components were constructed. The degree of deficiency of pepsin digests was compared to that of pepsin-digested IgG and to the degree of antigenic deficiency of the whole undigested M-component as determined by quantitative precipitin curves. The degree of deficiency with the absorbed anti-pepsin fragment antiserum correlated with the deficiency at the pepsin site as established by inhibition.

Rabbit antisera to 5 S pepsin fragment of IgG, after absorption with 3.5 S Fab produced by papain at pH 7.4, showed distinct residual precipitins with pepsin fragment of IgG. Such absorbed antisera showed weak or incomplete Ouchterlony reactions with pepsin-digested M-components deficient at the pepsin site (Figure 3) as did anti-pepsin fragment antisera absorbed with L-chains.

Inhibition studies with IgA myelomas and individual normal γ-globulins. Pepsin digestion of 11

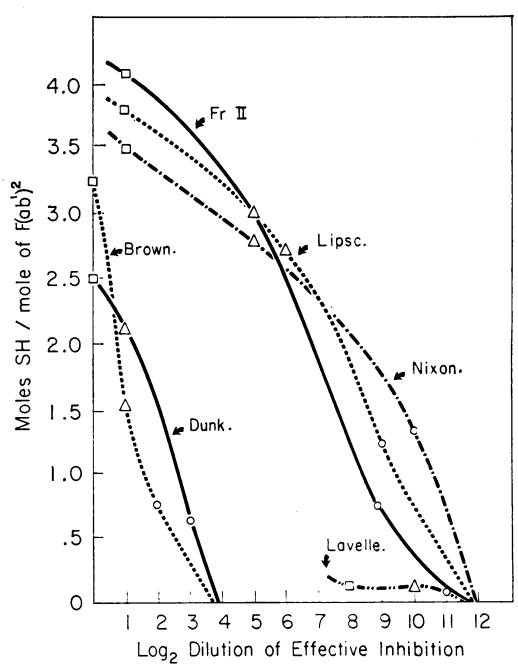

FIG. 2. PLOT OF MOLES OF FREE SH PER MOLE OF PEPSIN FRAGMENTS OF IgG AND VARIOUS MYELOMA PROTEINS [$F(ab')^2$] ACTUALLY MEASURED BY MODIFICATION OF ELLMAN'S METHOD (16) AGAINST RESIDUAL CAPACITY FOR INHIBITION IN PEPSIN SITE AGGLUTINATION SYSTEMS. Zero on the abscissa is the effective inhibition of the pepsin fragments before reduction. Points labeled –□– represent values obtained with 0.1 M mercaptoethanol, –△– with 0.01 M mercaptoethanol, and –○– with 0.001 M mercaptoethanol. Fr II = Cohn Fraction II.

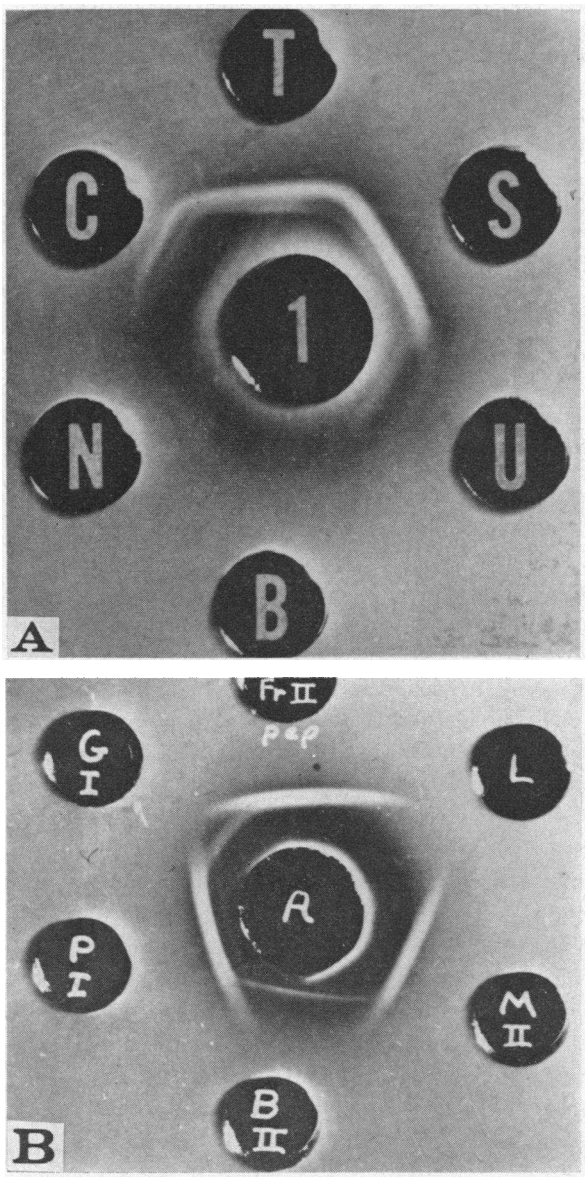

FIG. 3. A) OUCHTERLONY PLATE SHOWING REACTIONS OF THREE PEPSIN-DIGESTED M-COMPONENTS (C, T, AND S), WHICH RETAIN PEPSIN SITE INHIBITION IN AGGLUTINATION SYSTEMS AND WEAK OR INCOMPLETE REACTIONS WITH PEPSIN-DIGESTED M-COMPONENTS (N, B, AND U), WHICH ARE DEFICIENT IN INHIBITORY CAPACITY. B) REACTIONS OF VARIOUS PEPSIN-DIGESTED MYELOMA PROTEINS ADJUSTED TO 1 MG PER ML WITH ANTISERUM TO IgG DIGESTED WITH PEPSIN. In A, antiserum 1 is antiserum to pepsin-digested IgG absorbed with Fab-fragment produced by papain at pH 7.4. All digests are 1.0 mg per ml. Faint lines are present with deficient components N and U, and slight inhibition of precipitation of S line is noted where material from well U reaches it.

In B, the antiserum has been absorbed with pooled L-chains of IgG (A). Wells represented by P and M are pepsin-digested myeloma proteins showing no deficiency at pepsin site in inhibition. Wells G and B are pepsin-digested myeloma proteins that previously showed pepsin site inhibitory deficiency in agglutination reactions (Table II). L is light chains of IgG; FrII pep represents pepsin-digested γ-globulin. Roman numerals I or II indicate L-chain types of M-components tested.

IgA myeloma proteins isolated by zone electrophoresis produced products that were uniformly deficient in inhibition of the pepsin site antigen with all agglutinators tested. No inhibition was noted at 1.0 mg per ml with pepsin digests of all IgA myelomas tested.

No marked difference in inhibition patterns was noted when the 20 individual normal serum pepsin 5 S fragments were studied using the nine agglutinators in Table II and each of the five pepsin-digested incomplete antibody coats.

Discussion

Deficiency of IgG myeloma proteins for the pepsin site occurred in all M-components of the Ge or Ne H-chain subgroup; universal deficiency was not a feature of the Vi myelomas tested, since only one of four showed deficient pepsin fragments in the pepsin-digested human incomplete antibody systems. In similar fashion, all We myeloma proteins showing a Gm(a) or Gm(f) character were not deficient. Since previous work (1) has shown that part of the Vi subgroup antigen is localized to the 5 S pepsin fragments, the lack of full correlation of pepsin-site deficiency to the Vi subgroup indicates that a different antigen is involved. On the contrary, part of the antigenic determinants for the Ge subgroup appeared on the Fc fragment, showing unique behavior with rabbit antisera (1). In the pepsin site system, marked antigenic deficiency on the pepsin fragments of Ge proteins was apparent.

Of particular interest were the discrepant inhibition results obtained with agglutinator Turn. Deficiency in inhibition potency, though consistent within the other eight agglutinators, was not shared in some instances by Turn. Likewise, deficiency with Turn was present in some pepsin-digested myelomas not showing a deficiency with the remainder of the agglutinators. It seemed probable that in some instances agglutinating factors in serum Turn were selecting a pepsin site distinct from those shared by the remainder of the

agglutinating panel. When the 20 isolated normal γ-globulin pepsin digests were compared with Turn and Dri agglutinators and the pepsin coats, no marked differences, as had been present with the pepsin-digested myelomas, were apparent. The molecular size of the agglutinators was not a factor, as all nine were 7 S by sucrose gradient ultracentrifugation, confirming previous work (3).

The full integrity of the pepsin site did not bear a direct relationship to the sedimentation coefficients of the pepsin digests. Three digests showing sedimentation rates of 1.4 to 2.0 S retained full $\log_2$ dilution 12 inhibitory potency, whereas one of the less than 1.0 S (Metsa) showed distinct deficiency. In the case of the myelomas proceeding to small 1.0 to 2.0 S fragments after pepsin digestion, dialysis of the whole M-component against acetate buffer, pH 4.1, and examination in the ultracentrifuge in acid buffers revealed 7 S proteins of the same sedimentation rate as in neutral buffers of similar ionic strength.

That the pepsin site was a function of IgG and not shared by IgA molecules was apparent from the uniform lack of inhibiting potency of the 11 IgA pepsin-digested myelomas tested.

Since reduction by mercaptoethanol had the ability to virtually destroy the pepsin site in 5 S fragments of pepsin digests of both IgG and many myeloma proteins, it seemed likely that disulfide bonds were important in establishing the integrity of the pepsin site.

Insight into the area of the IgG molecule involved with the pepsin site antigen was gained by the Ouchterlony analysis of pepsin-digested myelomas using anti-5 S pepsin fragment antisera absorbed with 3.5 S papain Fab. Pepsin-digested myelomas deficient in the inhibition systems employed showed uniform weak or incomplete reactions with such absorbed antisera. This would suggest that the area of the IgG molecule participating in the pepsin site is on the Fd-fragment, comprising antigenic structures on the H-chain left by pepsin and not shared by H-chain determinants of 3.5 S Fab. Since pepsin-digested IgG contains an inter-H-chain disulfide bond, and since mercaptoethanol has been shown to virtually destroy the antigenicity of the pepsin-digested IgG, as well as that of individual myelomas digested with pepsin, it would appear that the configuration of the pepsin site may depend on this inter-H-chain disulfide bond. Clear evidence that the Fab-fragment produced by pepsin and that produced by papain are structurally different or result from cleavage at different sites on the H-chain has not been obtained in previous studies (21). Furthermore, studies by Frangione and Franklin (22) have emphasized the heterogeneity of Fd-fragments of myeloma proteins. This area of the IgG molecule is responsible for the pepsin site antigens.

Summary

The buried antigenic determinant uncovered by pepsin proteolytic digestion of 7 S γ-globulin at pH 4.1 was studied in 56 isolated IgG myeloma proteins. Eighteen of the pepsin-digested M-components (isolated monoclonal myeloma proteins) were deficient in inhibition of agglutination systems comprised by 7 S human agglutinators and cells coated with pepsin-digested incomplete antibody. All pepsin-digested myeloma proteins that showed deficiency were of the Ge or Ne H-chain subgroups, with the exception of one that was typed as Vi and one that did not fall into any of the major H-chain subgroups. Pepsin-digested IgA myelomas lacked the ability to inhibit in the pepsin site system. Relationship of the pepsin site antigen to disulfide bonds was shown by loss of inhibitory capacity of both 5 S pepsin-digested γ-globulin or pepsin fragments of individual myelomas by progressive reduction with mercaptoethanol. Antigenic structures present on the 5 S pepsin fragment but not shared by the 3.5 S Fab-fragment obtained with papain digestion were detected by antisera to pepsin-digested human γ-globulin absorbed with Fab-fragment produced by papain. Such antisera showed weak or incomplete precipitin reactions with pepsin-digested myelomas previously shown to be deficient in inhibition reactions. The pepsin site antigenic structure thus appears to reside on the Fd portion of γ-globulin.

Acknowledgments

We are indebted to Dr. Henry G. Kunkel for encouragement and many helpful suggestions during this study.

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