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Research Article

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A Comparative Analysis of the $C\bar{1}$-Binding Ability of Fragments Derived from Complement-Fixing and Noncomplement-Fixing IgM Proteins

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ABSTRACT The purpose of this study was to examine the molecular parameters necessary for initiation of complement fixation by IgM proteins. To determine why some IgM molecules are capable of complement fixation while others are not, several different Waldenström IgM proteins were examined for their ability to fix total hemolytic complement in the CH_{50} assay. Subsequently, the $C\bar{1}$ fixing ability of a 56-residue fragment derived from the $C\mu4$ domain of each of these IgM molecules was studied with the $C\bar{1}$ fixation assay.

One of the three Waldenström IgM proteins (Gr) used in the present study was found unable to consume complement in a CH_{50} assay when tested at the same concentration as the two complement-consuming IgM molecules (Dau and Bus). However, when the 56-residue C_H4 fragment from the $C\mu4$ domain of each IgM molecule was tested for $C\bar{1}$-fixing ability, all three were found to bind $C\bar{1}$.

On the basis of these observations, it is proposed that a $C\bar{1}$ binding site exists within the $C\mu4$ domain of both complement-fixing and noncomplement-fixing IgM molecules. Presumably, the latter molecules are unable to interact in their native state with $C\bar{1}$ in the manner required for initiation of the classical complement pathway, possibly due to the configurational inaccessibility of the entire $C\bar{1}$ binding site.

INTRODUCTION

There have been several reports in the literature suggesting the existence of two IgM subclasses, one capable of fixing complement and the second consisting of noncomplement-fixing IgM antibodies (1–4). In one such study Onoue and Ikeda (3) reported the identification of three noncomplement-fixing Waldenström IgM proteins out of a group of six. In a more recent publication, Tanimoto, et al. (4) reported that whereas a group of three monoclonal IgM-rheumatoid factor molecules were found to lack complement-fixing activity in a direct hemolytic assay system, three polyclonal rheumatoid factors were found capable of fixing and activating complement via the classical pathway. These authors suggested that IgM-rheumatoid factor may be composed of a mixture of complement-fixing and noncomplement-fixing antibodies, the noncomplement-fixing IgM either lacking the $C\bar{1}$ binding site or possessing configurations that interfere with $C\bar{1}$ binding.

We reported previously that a 6,800 mol wt fragment, designated C_H4 fragment, derived from the terminal $C\mu4$ domain of the complement-fixing Waldenström IgM, Dau, was capable of binding the activated first component of complement ($C\bar{1}$) (5). Due to mounting evidence that a considerable degree of homology in primary structure exists within the Fc region of all human pathological μ heavy chains (6), it seemed logical that the isolated C_H4 fragment from either a complement-fixing or noncomplement-fixing IgM should be able to fix $C\bar{1}$. We therefore compared the $C\bar{1}$ fixing ability of the C_H4 fragment isolated from the noncomplement-fixing IgM molecule, Gr, with that of the same fragment derived from the complement-fixing IgM molecules, Dau and Bus. The C_H4 fragment from each of these three molecules was found to bind $C\bar{1}$, lending support to the hypothesis that noncomplement-fixing IgM proteins do indeed contain $C\bar{1}$ binding sites, these sites being either inaccessible to $C\bar{1}$ in the native state, or only partially accessible so that even though $C\bar{1}$ does attach, it is unable to interact with the other early complement components.

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METHODS

Preparation of IgM and its $(Fc)_5\mu$ and C_H4 fragments. Human Waldenström IgM from patients Dau, Bus, and Gr was obtained as described by Hester et al. (7). $(Fc)_5\mu$ and Fabμ fragments were prepared by trypsin digestion (trypsin-TPCK, Worthington Biochemical Corp., Freehold, N. J.) at 60°C for 20 min (7, 8). After separation of $(Fc)_5\mu$ and Fabμ on Sephadex G-200 (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) in 1% NH_4HCO_3, pH 8, the $(Fc)_5\mu$ was further purified on Bio-Gel A-15 (Bio-Rad Laboratories, Richmond, Calif.). $(Fc)_5\mu$ fragments were incubated with 0.05 M cysteine (Fisher Scientific Co., Pittsburgh, Pa.) for 8 min at 25°C in 0.05 M Tris (Sigma Chemical Co., St. Louis, Mo.) buffer (pH 8.6), containing 0.1 M sodium chloride to produce monomeric Fcμ fragments. After alkylation with a 10% excess of iodoacetamide (Sigma Chemical Co.), homogeneous Fcμ fragments were isolated by gel chromatography on Sephadex G-200 (7). Limited tryptic cleavage of Fcμ to produce the C_H4 fragment was performed by the method of Hester et al. (7). Trypsin-TPCK was added at an enzyme-to-substrate ratio of 1:100 to a 5 mg/ml preparation of Fcμ fragments in 0.05 M Tris buffer (pH 8.0) containing 0.1 M sodium chloride and 0.01 M $CaCl_2$. After incubation at 37°C for 16 min, digestion was terminated by the addition of an amount of soybean trypsin inhibitor (Worthington Biochemical Corp.) equivalent in weight to the quantity of trypsin used. Solid guanidine-HCl (Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, N. Y.) was then added to a final concentration of 5 M and the sample applied to a Sephadex G-75 column. The C_H4 fragment was further purified by passage through a column of Bio-Gel P-10 in 5 M guanidine-HCl.

A detailed account of these methodologies and characterization of the fragments obtained from Gr IgM has been published by Hester et al. (7). The fragments isolated from Dau and Bus IgM molecules behaved identically to the Gr fragments during both fractionation and characterization.

After centrifugation for 1 h at 100,000 *g*, protein concentrations were determined spectrophotometrically at 280 nm with $E_{1\,cm}^{1\%}$ of 12.0 for IgM and for $(Fc)_5\mu$. The C_H4 fragments were desalted in 1% NH_4HCO_3, lyophilized, and weighed directly. A measured aliquot of the parent stock solution of each C_H4 fragment was removed for quantitative amino acid analysis.

Amino acid analyses. Samples were hydrolyzed in constantly boiling HCl in vacuum-sealed tubes for 20 h. After evaporation, the samples were analyzed on a Durrum model D-200 automatic amino acid analyzer with high-speed analysis and the single-column technique (Durrum Instrument Corp., Palo Alto, Calif.).

Gel electrophoresis. Disc electrophoresis was performed with 5% polyacrylamide, as described by Maizel (9). The gels were run in 0.1 M sodium phosphate buffer (pH 7.2) containing 0.1% sodium dodecyl sulfate and 0.5 M urea, stained for 15–30 min with 1% amido black (Gallard-Schlesinger Chemical Mfg. Co., Carle Place, N. Y.) in 7% acetic acid, and destained in 40% absolute methanol and 10% acetic acid.

Buffers used in complement assays. Gelatin-veronal buffer is barbital-buffered saline (VBS)[1] containing 0.1% gelatin, 0.15 mM $CaCl_2$, and 1 mM $MgCl_2$, with a relative salt concentration of 0.15. The relative salt concentration of each buffer was monitored by relating the electrical resistance of the buffer at 0°C to a standard curve obtained by graphing the resistance of NaCl solutions vs. the NaCl molarities (10). Dextrose-gelatin VBS^{++} is VBS diluted with 5% dextrose to a relative salt concentration of 0.075, containing standard amounts of gelatin, Ca^{++}, and Mg^{++} (11). Dextrose VBS^{++}, the buffer used for dilution of immunoglobulin and its fragments, refers to dextrose-gelatin VBS minus the gelatin. VBS containing 0.02 M EDTA and 0.1% gelatin (EDTA-gelatin-VBS) was also used as a buffer in the complement assays (11).

Complement components and intermediates. Partially purified human $C\bar{1}$ and guinea pig C2 were prepared as described by Nelson et al. (12). $EAC4^{hu}$ (sheep erythrocytes sensitized with rabbit antibody to E, to which complement components have been added) were made by a slight modification of the method described by Borsos and Rapp (13), using guinea pig (gp) C1 (500 effective molecules/cell). $EAC1^{gp}4^{hu}$ were prepared by adding 500 effective molecules of C1 per EAC4 cell. As a source of the later-reacting complement components (C3–9), guinea pig serum was used at a dilution of 1:40 in 20 mM EDTA buffer.

CH_{50} fixation assay. Consumption of human hemolytic complement was demonstrated by quantitative assay of residual hemolytic activity in fresh normal human serum after incubation with immunoglobulin fragments, or with buffer as a control, for 1 h at 37°C. In this procedure 0.25-ml aliquots of fresh normal human serum were mixed with 1.125 ml of immunoglobulin fragment dissolved in gelatin VBS^{++}. After incubation for 1 h at 37°C, the residual hemolytic complement activity was measured as described by Mayer (11).

$C\bar{1}$ binding assay. The $C\bar{1}$ fixing ability of the IgM molecules and their respective fragments was examined with a modified $C\bar{1}$ fixation assay (5). The buffer utilized throughout the assay was dextrose-gelatin VBS^{++} of relative salt concentration 0.065, containing optimum amounts of Ca^{++} and Mg^{++} (10). An amount of functionally pure human $C\bar{1}$ (14) sufficient to yield 63% lysis was incubated with serial dilutions of the test fragments or with buffer as a control for 10 min at 30°C. EAC4 (1.5×10^8/ml) were then added and incubation was allowed to proceed for an additional 10 min. The resulting $EAC\bar{1}4$ cells were washed twice to remove unbound $C\bar{1}$ and resuspended in the same buffer. An excess of functionally pure C2 (12) was then added, followed by an excess of C3–9, and the degree of lysis was determined spectrophotometrically at 412 nm.

The controls used for this assay consisted of the following: (*a*) a cell blank (cells plus buffer), (*b*) a complete lysis control (cells plus water to obtain 100% lysis), (*c*) a cell blank plus complement (buffer substituted for test serum dilutions to insure that no extraneous C1 was in any of the reagents), and (*d*) a complement color (buffer plus guinea pig serum-EDTA) (10).

RESULTS

After passage of the 60°C tryptic digests of each IgM protein through a Sephadex G-200 column, and subsequent purification of the $(Fc)_5\mu$ fragments on Bio-Gel A-15, an $(Fc)_5\mu$ fraction was obtained that gave a single immunoelectrophoretic band with anti-IgM, and did not react with anti-Bence Jones κ or anti-Bence Jones λ antisera (Meloy Laboratories Inc., Springfield, Va.). Dau $(Fc)_5\mu$ has been characterized by Zikán and Bennett (15), and Hester et al. (7) have published a detailed analysis of Gr $(Fc)_5\mu$.

Pentameric Fc was reduced with 50 mM cysteine and the 34,000-mol wt monomer isolated on a Sephadex

[1] *Abbreviation used in this paper:* VBS, veronal-buffered saline.

G-200 column in pH 7.5 phosphate buffer (7). Disc electrophoresis in 5% polyacrylamide with a pH 7.2 sodium phosphate-0.1% sodium dodecyl sulfate-50 mM urea buffer revealed a single band whose molecular weight was not lowered after reduction and alkylation in 5 M guanidine-HCl (7). Furthermore, Hester et al. (7) found that total reduction of Gr monomeric Fcμ released four SH groups per fragment, indicating that the two intrachain disulfide loops had remained intact.

The Fcμ fragment was treated with trypsin for 16 min at 37°C and the digest passed through a Sephadex G-75 column in 5 M guanidine-HCl (7). The peak containing the C_H4 fragments was pooled, desalted on Sephadex G-25 in 1% NH_4HCO_3, lyophilized, and applied to a Bio-Gel P-10 column in 5 M guanidine-HCl. After elution from the P-10 column, the C_H4 fragments from each of the IgM proteins gave a single band on 5% sodium dodecyl sulfate acrylamide gels. The gel filtration patterns and fragment analyses obtained during the fragmentation of Gr IgM have been published by Hester et al. (7). Similar patterns and products resulted from the fragmentation of Dau and Bus IgM molecules. Partial sequence analysis and a comparison of the amino acid composition of the Gr C_H4 fragment and of its respective isolated peptide chains with that published for the IgM, Ou (6), enabled Hester et al. (7) to pinpoint its location within the μ chain. The C_H4 fragment was found to be composed of the Cμ4 domain of the Fcμ minus two tryptic peptides in the center of the loop (residues 468–491 disulfide bonded to residues 515–546, according to the residue numbering of Ou[6]). Amino acid compositional analysis of the Dau and Bus C_H4 fragments (Table I) and a spot check via double sequencing in our automated sequencer of the intact Dau C_H4 fragment (16) revealed no differences as compared to the Gr C_H4 fragment (7).

TABLE I

Amino Acid Composition of the C_H4 Fragment from Dau and Bus IgM

	Dau C_H4 Fragment*	Bus C_H4 Fragment*	Theor.‡
His	1.9	1.8	2
Arg	2.3	2.2	2
S-CMC	ND§	ND	
Asp	3.0	3.0	3
Thr	6.3	6.2	7
Ser	3.7	3.5	4
Glu	7.9	7.7	8
Pro	2.0	2.0	2
Gly	2.0	2.0	2
Ala	4.7	4.7	5
Val	5.7	5.6	6
Met	1.2	1.1	1
Ile	2.0	2.0	2
Leu	3.0	3.0	3
Tyr	1.7	1.7	2
Phe	2.7	2.8	3
Trp	ND	ND	2
Lys	0	0	0

* Residues per three residues of leucine.

‡ Residues 468–491 and 515–546 of the IgM, Ou, from Florent et al. (6).

§ ND, not determined.

TABLE II

Consumption of Human Complement by Dau IgM C_H4 and $(Fc)_5\mu$ Fragments

Test sample	Total complement CH_{50}	Total complement Molar ratio*
	$nmol^{-1}$	
C_H4	2.2	1.0
$(Fc)_5\mu$	17.0	7.7
IgM	23.0	10.4

* For each series of titrations the molar ratios refer back to the C_H4 fragment as 1.

Initially, the CH_{50} assay was used to test the overall complement-consuming ability of the Waldenström IgM, Dau, and its $(Fc)_5\mu$ and C_H4 fragments. As seen in Table II, the Dau C_H4 fragment consumed 2.2 CH_{50} U/nmol, while the Dau $(Fc)_5\mu$ consumed 17 CH_{50} U/nmol. $(Fc)_5\mu$ was therefore 7.7 times more efficient than the C_H4 fragment at consuming total complement. Furthermore, on a molar basis, intact Dau IgM appeared to be approximately 10 times more efficient in consuming total complement than the monomeric C_H4 fragment. This latter observation provides an impressive quantitative correlation, since there are 10 C_H4 fragments per IgM molecule.

Subsequently, the ability of two other Waldenström IgM proteins (Bus and Gr) to fix total complement was examined with the CH_{50} assay. As depicted in Table III, Bus IgM was found to consume total complement, whereas an equivalent amount of Gr IgM did not.

For the final set of experiments, the C_H4 fragment was isolated from Dau, Bus, and Gr IgM proteins and tested in the $C\bar{1}$ fixation assay (Table III). It was found that the C_H4 fragment from each of these IgM molecules was able to fix $C\bar{1}$, with 8 μg of Dau and Bus C_H4 fragments and 24 μg of Gr C_H4 fragment required to fix one-half of the available $C\bar{1}$. Fig. 1 is a graph of these results. It should be noted that although the Gr C_H4 fragment appears to be slightly less efficient than the Dau and Bus C_H4 fragments in binding $C\bar{1}$, the molar ratios for the three fragments are of the same order of magnitude.

TABLE III

Complement-Binding Properties of Three Different Waldenström IgM Molecules and of Their Respective C_H4 Fragments

Source of IgM	Total C′ fixation by IgM		$C\bar{1}$ fixation by C_H4 fragments	
	CH_{50}	Molar ratio*	Amount‡	Molar ratio§
	$nmol^{-1}$		μg	
Dau	23.0	1.0	8	1
Bus	4.8	4.8	8	1
Gr	0	>100	24	3

* For these titrations the molar ratios refer back to Dau IgM as 1.

‡ Amount required to bind 50% of the available $C\bar{1}$.

§ For this titration the molar ratios refer back to the Dau C_H4 fragment as 1.

DISCUSSION

It has been suggested by Edelman et al. (17) that certain repeating regions of immunoglobulin polypeptide chains are folded into compact globular structures called domains, each evolved to perform a particular biologic function. According to this concept, it should be possible to associate specific biological effector functions, such as complement fixation, with a specific submolecular domain in certain immunoglobulin molecules. Evidence to support this hypothesis has been obtained from the data of Connell and Porter (18) and of Ellerson et al. (19) demonstrating that the Cγ2 domain of IgG contains a $C\bar{1}$ binding site (Fig. 2) and may therefore play a role in complement activation by this molecule.

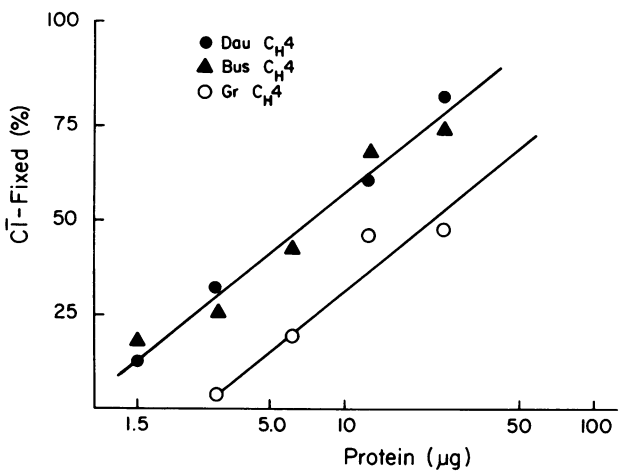

FIGURE 1 Fixation of human $C\bar{1}$ by the C_H4 fragments isolated from three different Waldenström IgM proteins, Dau, Bus, and Gr. Enough $C\bar{1}$ to produce 63% lysis of the cells in absence of test material was used. After incubation, the amount of unbound $C\bar{1}$ was determined and expressed as a percentage of that found when no test material was added during the incubation.

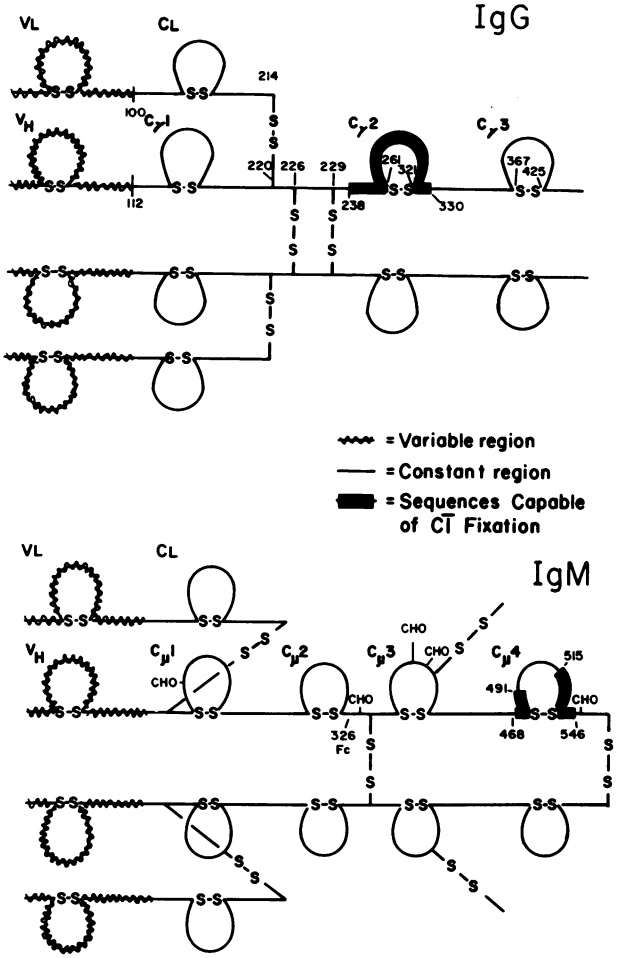

FIGURE 2 Diagrammatic representation of an IgG 1 myeloma protein and of one of the five subunits of a Waldenström IgM protein. The four homology regions of the IgG heavy chain (V_H and Cγ1–Cγ3) and the five homology regions of the IgM heavy chain (V_H and Cμ1–Cμ4) are marked. The locations of disulfide bonds, carbohydrate groups, and amino acid residues were taken from Edelman et al. (17) for IgG and from Florent et al. (6) for IgM. The sequences found to be capable of $C\bar{1}$ fixation are indicated with broad lines.

Few attempts have been made to delineate the structural basis for complement binding to IgM. However, two meaningful observations relative to this question have been published. First, the 7S subunit of IgM was found to bind $C\bar{1}$ as effectively as nonaggregated, monomeric IgG in a $C\bar{1}$ fixation test (20), and second, the $(Fc)_5\mu$ and Fcμ fragments of IgM were found to have similar complement-fixing capacities in a CH_{50} assay, each being 31 times as effective as intact IgM by weight and 19 times as effective on a molar basis (21).

In a more recent study from this laboratory (5), it was reported that a 56-residue fragment (designated C_H4 fragment), derived from a Waldenström IgM protein and consisting of 24 residues of the amino-terminal portion of the $C\mu4$ domain disulfide bonded to 32 residues of the carboxy-terminal region of the loop, possessed $C\bar{1}$ fixing ability in a $C\bar{1}$ fixation assay. On the basis of these data, and the observation that upon incubation with normal human serum the C_H4 fragment consumes C4 (16), it is proposed that a classical complement fixation function, i.e., C1 binding and activation, can be localized within the $C\mu4$ domain of the IgM molecule (Fig. 2).

The sequence data presented by Florent et al. (6) indicate that only minor differences exist in the primary structure of the Fc region of both pathological and normal IgM molecules. This being true, it follows that if primary structure relates directly to function, the C_H4 fragment from complement-fixing and from noncomplement-fixing molecules should possess $C\bar{1}$ fixing ability. Whether or not each respective IgM could fix $C\bar{1}$ so that it could subsequently interact with the other early complement components would therefore depend upon the relative exposure of the $C\mu4$ domain in the intact molecule. This was indeed borne out by the fact that the isolated C_H4 fragment from the noncomplement-fixing IgM, Gr, was able to fix $C\bar{1}$, and did so nearly as efficiently as the C_H4 fragments from the complement-fixing IgM molecules, Dau and Bus. The threefold difference in $C\bar{1}$-fixing ability observed between the C_H4 fragments from complement-fixing and noncomplement-fixing molecules was not felt to be significant.

That the C_H4 fragments from two complement-fixing IgM molecules (Dau and Bus) and from an apparent noncomplement-fixing IgM protein (Gr) were each capable of $C\bar{1}$ fixation raises interesting possibilities. In this regard it should be pointed out that Isenman et al. (22) found that while noncomplement-fixing IgG4 does not bind $C\bar{1}$, Fc fragments derived from this subclass do fix $C\bar{1}$, and do so with an affinity comparable to the corresponding fragment from IgG1. In an earlier publication these authors reported that the $C\bar{1}$ fixing site of β_2-microglobulin remains active after reduction and alkylation (23). Furthermore, they obtained spectral data indicating that in the absence of an intact intrachain disulfide bond, β_2-microglobulin does not fold into the conformation characteristic of the native state. On the basis of these data Isenman and coworkers postulated that little secondary or tertiary folding is required for the existence of an active C1 binding site, and that the chemical features of the linear amino acid sequence required for binding of $C\bar{1}$ may be commonplace but may be prevented from reacting because of local folding patterns or interaction with one-dimensionally distant regions of the molecule (22). Such an interpretation is consistent with our observation that fragments derived from the $C\mu4$ domain of both complement-fixing and noncomplement-fixing IgM molecules retain the ability to fix $C\bar{1}$.

Considering our data on IgM relative to that of Isenman and colleagues on IgG4 and β_2-microglobin, we suggest that a $C\bar{1}$ fixing site may exist in all IgG and IgM proteins, the noncomplement-fixing molecules possessing configurations that interfere with correct $C\bar{1}$ binding.

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