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Inhibition of the Classical Complement Pathway by Synthetic Peptides from the Second Constant Domain of the Heavy Chain of Human Immunoglobulin G†

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ABSTRACT: The $C_\gamma2$ domain of immunoglobulin G (IgG) is reported to have a tryptophan residue and cationic residues at or near its C1q-binding site. The present study has used synthetic IgG peptides to explore the involvement of the 275–290 region of the $C_\gamma2$ domain of the heavy chain of human IgG1 in binding to C1. This region (Phe-Asn-Trp-Tyr-Val-Asp-Gly-Val-Gln-Val-His-Asn-Ala-Lys-Thr-Lys) contains Trp-277 and the cationic residues His-285, Lys-288, and Lys-290. The following peptides were synthesized by the solid-phase method and purified to homogeneity by using reverse-phase high-pressure liquid chromatography: the hexadecapeptide 275–290 and its N^t-formyl derivative 275–290F containing both Trp-277 and the cationic residues; the N^α-acetylpentapeptide 275–279A comprising the hydrophobic region around Trp-277; and the cationic decapeptide 281–290. When examined in the Augener assay for inhibition of C1-mediated immune hemolysis, peptides 275–290F and 281–290 were about half as active as monomeric 7S human IgG on a molar basis and essentially as active on a site basis. Since both peptides containing residues 281–290 inhibited hemolysis in a manner similar to the $C_\gamma2$ domain, the cationic 281–290 region containing His-285, Lys-288, and Lys-290 may be a part of the C1q-binding site of $C_\gamma2$. These results are consistent with the tertiary structure of the Fc fragment of IgG, in which the 275–279 region is part of the hydrophobic core of the $C_\gamma2$ domain and the 281–290 region is exposed on the surface.

When immunoglobulin G (IgG)[1] binds to a bacterium or virus, the resulting immune complex binds to C1q, which leads to the activation of the classical complement pathway and the destruction and removal of the invading organism (Müller-Eberhard, 1975). During the past two decades, many studies have explored the location and chemical nature of the C1q-binding site of IgG. Several studies of IgG fragments have suggested that this site resides in the $C_\gamma2$ domain. Progressive proteolysis of the Fc region of IgG, which is responsible for complement activation (Ishizaka et al., 1962), abolished complement binding when the amino-terminal portion was degraded (Utsumi, 1969). Kehoe & Fougereau (1969) found that a 62-residue fragment (residues 253–314) of the $C_\gamma2$ domain of murine IgG2a inhibited complement fixation when bound to polystyrene latex beads. This observation has been supported by the observations that a $C_\gamma2$-containing fragment from intact IgG (Colomb & Porter, 1975), the isolated $C_\gamma2$ domain (Yasmeen et al., 1976), and a 54-residue fragment (residues 253–306) of human IgG1 (Lee & Painter, 1980) each inhibit C1-mediated hemolysis. But the direct binding of the $C_\gamma2$ domain or an IgG fragment to C1q has not been demonstrated.

Two chemical features of the C1q-binding site of IgG have been reported. IgG aggregates bearing modified tryptophan residues fail to activate complement, which suggests that a tryptophan residue is at or near the C1q-binding site (Allan & Isliker, 1974a,b). In addition, a peptide corresponding to residues 277–281 of IgG and containing Trp-277 was reported

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[1] Abbreviations used: Ac, acetyl; Boc, *tert*-butyloxycarbonyl; C1q, C1r, C1s, C2, C3, and C9, protein components of the serum complement cascade; $C_\gamma2$, second constant domain of the γ (heavy) chain of IgG; For, formyl; HPLC, high-pressure liquid chromatography; IgG, immunoglobulin G; I_{50}, 50% inhibition of C1-mediated immune hemolysis; TLC, thin-layer chromatography; TLE, thin-layer electrophoresis; DMF, dimethylformamide.

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Table I: Amino Acid Sequences of Synthetic Peptides from the $C_\gamma 2$ Domain of Human IgG1

peptide	amino acid sequence
	275 277 279 281 285 288 290
	For
275-290F	Phe-Asn-Trp-Tyr-Val-Asp-Gly-Val-Gln-Val-His-Asn-Ala-Lys-Thr-Lys
275-290	Phe-Asn-Trp-Tyr-Val-Asp-Gly-Val-Gln-Val-His-Asn-Ala-Lys-Thr-Lys
281-290	Gly-Val-Gln-Val-His-Asn-Ala-Lys-Thr-Lys
275-279A	Ac-Phe-Asn-Trp-Tyr-Val

to inhibit hemolysis (Boackle et al., 1979). Both aliphatic and aromatic diamines inhibit C1-mediated hemolysis, the more hydrophobic diamines being better inhibitors (Wirtz & Becker, 1961; Sledge & Bing, 1973). Taken together, these studies suggest that the C1q-binding site contains or is near a hydrophobic region and a cationic region.

X-ray crystallographic studies of the three-dimensional structure of the Fc fragment of human IgG indicate that the $C_\gamma 2$ domain bears an exposed cationic region (residues 285–292) containing four positively charged residues (His-285, Lys-288, Lys-290, and Arg-292) and an adjacent buried hydrophobic region (residues 275–279) containing Trp-277 (Deisenhofer et al., 1976; Deisenhofer, 1981).

On the basis of these biochemical and crystallographic studies, we initiated a synthetic study of the 275–290 region of the $C_\gamma 2$ domain as the most plausible region for interaction with C1q (Prystowsky et al., 1977). More recently, two other groups have also predicted specific regions of the $C_\gamma 2$ domain as possible C1q-binding sites. Brunhouse & Cebra (1979) speculated that the C1q-binding site of human IgG1 is determined by the extended chain formed by residues 290–295. In contrast, Burton et al. (1980) proposed that the C1q-binding site involves residues 317–340, which comprise the two COOH-terminal β-sheet strands of $C_\gamma 2$. The present study suggests that residues 285–290 may be a part of the C1q-binding site of $C_\gamma 2$.

Experimental Procedures

Amino Acids. Amino acids were protected at the α-amino position with the *tert*-butyloxycarbonyl (Boc) group. The side-chain-protected amino acids were the benzyl esters of aspartic acid and glutamic acid, benzyl ether of threonine, 2,6-dichlorobenzyl ether of tyrosine, N^{ϵ}-(3-chlorobenzyloxycarbonyl)lysine, N^{im}-(2,4-dinitrophenyl)histidine, and N^{i}-formyltryptophan.

N^{ϵ}-(3-Chlorobenzyloxycarbonyl)-L-lysine. This intermediate was prepared by the method of Erickson & Merrifield (1973). After crystallization from 70% aqueous ethanol, it was homogeneous by thin-layer chromatography (TLC) in 17:2:1 (v/v/v) chloroform–methanol–acetic acid (R_f 0.06) and in 5:5:1:3 (v/v/v/v) 1-butanol–pyridine–acetic acid–water (R_f 0.77). Anal. Calcd for $C_{14}H_{19}ClN_2O_4$: C, 53.41; H, 6.09; Cl, 11.28; N, 8.90. Found: C, 53.51; H, 6.07; Cl, 10.96; N, 8.93.

N^{α}-tert-Butyloxycarbonyl-N^{ϵ}-(3-chlorobenzyloxycarbonyl)-L-lysine. This derivative was prepared by a modification of the procedure of Schnabel (1967). N^{ϵ}-(3-Chlorobenzyloxycarbonyl)-L-lysine (19.3 mmol) was dissolved in 2:1 (v/v) dioxane–water (150 mL), and 1.8 molar equiv of MgO and 4.5 molar equiv of Boc azide were added. The reaction was conducted at 50 °C for 72 h and stopped by addition of 5 molar equiv of hydrazine hydrate. After acidification and extraction into ethyl acetate, the solvent was evaporated to afford the Boc derivative as a colorless oil. It was homogeneous by TLC in 17:2:1 (v/v/v) chloroform–methanol–acetic acid (R_f 0.74) and 5:5:1:3 (v/v/v/v) 1-butanol–pyridine–acetic acid–water (R_f 0.85). Anal. Calcd for $C_{19}H_{27}ClH_2O_6$: C, 54.99; H, 6.56; Cl, 8.56; N, 6.75. Found: C, 55.03; H, 6.82; Cl, 8.40; N, 6.57.

Table II: Protocol for One Synthetic Cycle

step	reagent or solvent	time (min)
A, deprotection	dichloromethane	0.5
	50% trifluoroacetic acid	2.0
	50% trifluoroacetic acid	30.0
	dichloromethane, 5 times	0.5
	2-propanol, 2 times	0.5
	dichloromethane, 5 times	0.5
B, neutralization	diisopropylethylamine, 3 times	2.0
	dichloromethane, 5 times	0.5
C, coupling	Boc-amino acid	2.0
	N,N'-dicyclohexylcarbodiimide	30.0
	dichloromethane, 5 times	0.5
	2-propanol, 2 times	0.5
	dichloromethane, 2 times	0.5
	2-propanol, 2 times	0.5
	dichloromethane, 5 times	
D, neutralization	repeat step B	
E, coupling	repeat step C	

Boc-Amino Acid-Resin. Chloromethylated copoly(styrene–1% divinylbenzene) beads (0.7 mequiv of Cl/g, 200–400 mesh; Bio-Rad Laboratories, Richmond, CA) were used for all syntheses. The first amino acid was esterified to the resin by the method of Gisin (1973). The cesium salt of the Boc-amino acid in 1.8–2.0 molar excess over the chloromethyl groups was dissolved in *N*-methyl-2-pyrrolidinone. The resin was swelled in a minimal amount of this solvent, the cesium salt of the Boc-amino acid was added, and the slurry was stirred at 50 °C for 4–6 days. The amount of Boc-amino acid present per gram of resin, which was measured by the picrate monitoring method of Gisin (1972), was 0.48 mmol/g for Boc-Lys(3-ClZ)-resin and 0.60 mmol/g for Boc-Val-resin.

Solid-Phase Synthesis. Peptides were synthesized by the solid-phase method (Merrifield, 1963; Erickson & Merrifield, 1976) using a Schwarz/Mann automatic peptide synthesizer. The primary structures of the synthetic peptides are shown in Table I, and a detailed protocol for one synthetic cycle is given in Table II. The Boc-amino acid-resin was first deprotected with 50% trifluoroacetic acid in CH_2Cl_2 for 30 min and then thoroughly washed (step A). The free α-amino groups were neutralized with 5% diisopropylethylamine in CH_2Cl_2, and the resin was washed again (step B). Then 2–3 molar equiv of the next amino acid in CH_2Cl_2 and an equimolar amount of *N,N'*-dicyclohexylcarbodiimide in CH_2Cl_2 were added to the resin, the reaction mixture was shaken at room temperature for 30 min, and the resin was thoroughly washed again (step C). Finally, neutralization step B and coupling step C were repeated. After both coupling steps with the same amino acid had been performed, the next synthetic cycle was started. During the synthesis of peptide 275–279, the peptide-resin was monitored with picric acid (Gisin, 1972) after the second coupling reaction to detect unreacted amino

groups. If the monitoring value was unacceptable (<95% coupled), a third coupling cycle was performed with equimolar amounts of Boc-amino acid, 1-hydroxybenzotriazole, and dicyclohexylcarbodiimide.

Alanine-287 was incoporated as [^{3}H]alanine. Asparagine and glutamine were coupled either with dicyclohexylcarbodiimide alone or in the presence of 1-hydroxybenzotriazole (Hruby et al., 1973). Acetylation of peptide 275–279 was performed on the peptide-resin at room temperature by using a limiting amount of [^{3}H]acetic anhydride (400 mCi/mmol) in pyridine for 24 h followed by an excess of a 1:2 (v/v) solution of nonradiolabeled acetic anhydride and pyridine for 3 h. Peptides were cleaved from the resin by using 9:1 (v/v) anhydrous HF in anisole (Sakakibara & Shimonishi, 1965). Prior to cleavage with HF, the N^{im}-dinitrophenyl protecting group of histidine was removed by treatment overnight at room temperature with 1000 molar equiv of thiophenol in DMF (Lin et al., 1972). Peptides 275–279F and 281–290 were treated with HF for 1 h at 0 °C. Peptide 275–290F was treated with HF for 30 min at −25 °C and for 30 min at 0 °C to minimize formation of β-aspartyl residues (Baba et al., 1973). After treatment with HF, the resins were washed with diethyl ether to remove organic byproducts formed during HF cleavage. The crude peptides were eluted from the resin with 10% aqueous acetic acid, glacial acetic acid, or mixtures of aqueous acetic acid and methanol, depending upon the solubility of the synthetic peptide.

Purification. Peptides were purified by using a modular high-pressure liquid chromatography (HPLC) system (Waters Associates, Milford, MA) consisting of two Model 6000A pumps, a Model 660 solvent programmer, and a U6K manual sample injector and were monitored by using a variable wavelength UV detector. The major mode for purification was reverse-phase chromatography using a column containing μBondapak C18 (3.9 mm × 30 cm; Waters), μBondapak phenyl (3.9 mm × 30 cm; Waters), or Nucleosil C18 (10 mm × 25 cm; Rainin Instrument, Brighton, MA). The mobile phase consisted of varying proportions of methanol and aqueous acetic acid. The column eluate was monitored by UV absorbance, TLC, and liquid scintillation counting of the radioactive peptides.

Characterization. The homogeneity of each synthetic peptide was shown in three different solvent systems by using thin-layer chromatography (TLC) on Woelm silica gel (250 μm thick; Analtech, Newark, DE). The R_f values for the synthetic peptides ranged between 0.2 and 0.8. Peptides were visualized on the thin-layer plates by spraying with ninhydrin and heating and by exposure to a Cl_2 atmosphere followed by spraying with *o*-toluidine. Thin-layer electrophoresis (TLE) was performed on the peptides soluble in aqueous systems by using 25:1:225 (v/v/v) pyridine–acetic acid–water at pH 6.5 for 30 min (2000 V, about 27 mA) on hard-layer silica plates with arginine, glutamic acid, and alanine as standards. Electrophoretic mobilities are expressed as the relative distance of migration from Ala toward Arg (R_{Arg}) or Glu (R_{Glu}).

The ultraviolet spectrum (200–400 nm) was recorded for peptides containing Trp or Trp(For) (Ohno et al., 1972). Reverse-phase HPLC using methanol–aqueous acetic acid solvent systems was also employed analytically. A peptide was considered to be homogeneous if it eluted as one sharp peak with a k' value between 1.5 and 12, where $k' = (V_p - V_0)/V_0$; V_p is the volume of the peptide peak, and V_0 is the breakthrough volume as measured by an unretained solute.

Synthetic peptides were hydrolyzed in preparation for automated amino acid analysis by one or more of the following methods: 6 N HCl containing 0.3% (w/v) phenol for 24 and 48 h at 110 °C (Moore & Stein, 1963); 4 N NaOH containing thiodiglycol for 24 and 48 h at 110 °C (Hugli & Moore, 1972) as modified;[2] and enzymatically (Salnikow et al., 1973) with aminopeptidase M in 0.1 M ammonium carbonate (pH 7.5) for 20 h at room temperature or carboxypeptidase Y in 0.1 M ammonium acetate (pH 5.4) for 3 h at 37 °C (1 μg of enzyme per nmol of peptide).

C1 Inhibition Assay. The C1 inhibition assay used in this study, first described by Borsos & Rapp (1963) and modified by Augener et al. (1971) and Painter et al. (1974), was designed to measure the ability of a compound to inhibit activation of the classical complement pathway through binding to C1. Three IgG proteins were used as standards. The IgG1 myeloma protein Pen was chromatographed twice on Sephadex DEAE A-50 and gel filtered once on Sephadex G-200 and was found by analytical ultracentrifugation at a concentration of 4.5×10^{-6} M to consist of heterogeneous IgG aggregates with an average $s_{20,w}$ value of 12.5 S. The IgG1 myeloma protein Bah was purified by Pevikon block electrophoresis and used as the monomeric IgG standard. The Fc fragment of IgG was prepared by papain digestion of the slowly migrating portion of the FII fraction obtained from Pevikon block electrophoresis of pooled human serum (Edelman et al., 1960).

Each peptide and protein was assayed at a series of concentrations produced by successive 2-fold dilution. Since each peptide contained a known specific activity of radiolabel, its dilution was monitored by liquid scintillation counting. The amounts of peptide in the solutions of highest and lowest dilution were calculated by determining the radioactivity of these solutions in duplicate. The average dilution factor was calculated as $(A_H/A_L)^{1/N}$, where A_H and A_L were the average radioactivity in counts per minute for the highest and lowest dilutions, respectively, and N was the number of 2-fold dilutions. The mean and standard deviation of the population of dilution factors was 2.01 ± 0.11.

Human complement components were purchased from Cordis Laboratories (Miami, FL). The EAC4 cells were sheep erythrocytes coated with IgG and human C4. The buffer used in this C1-inhibition assay was a sucrose–gelatin–veronal buffer (SGV) (Rapp & Borsos, 1970) containing 1 mM $MgCl_2$, 0.15 mM $CaCl_2$, 0.06 M NaCl, 0.17 M sucrose, 0.1% (w/v) gelatin, and 5 mM sodium 5,5-diethylbarbiturate (pH 7.3–7.4). This low ionic strength buffer (μ = 0.065) increases the association of C1 with IgG and immune complexes (Borsos & Rapp, 1963). C–EDTA solution containing complement components C3–C9 was prepared by diluting 1 part of normal human serum with 49 parts of a buffer consisting of 4.5 mM sodium 5,5-diethylbarbiturate, 0.135 M NaCl, 0.1% (w/v) gelatin, and 0.01 M EDTA (pH 7.3–7.4). Titration of C1 was performed before each assay to ensure that only about 61% of 8.5×10^6 cells would be lysed. The amount of C1 used in each assay was approximately 25 fmol or approximately 500 C1 molecules per erythrocyte.

All assays were performed in triplicate. SGV buffer, IgG, or peptide (0.10 mL) was incubated with C1 (0.10 mL) for 10 min at 30 °C. The C1 that was not complexed with IgG or peptide was available to react with activated erythrocytes and was measured in the following test system. A solution (1.0 mL) containing 8.5×10^6 EAC4 cells was added, and the mixture was incubated at 30 °C for 10 min. The resulting $EAC\overline{14}$ cells were washed once with SGV buffer at 4 °C. Then C2 (10 CH_{50} units in 0.10 mL) was added and the

[2] P. Blackburn and S. Moore, personal communication.

Table III: Analytical Thin-Layer Chromatography of Purified Peptides

solvent system[a]	volume ratio	R_f values for peptides 281–290	275–290F	275–279A	275–279AF
BuOH:EtOAc:AcOH:H_2O	4:1:1:4	0.08	0.37		
EtOAc:Pyr:AcOH:H_2O	5:5:1:3	0.18	0.41		
BuOH:Pyr:AcOH:H_2O	3:1:1:3	0.42	0.59		
BuOH:AcOH:H_2O	1:1:2	0.28			
BuOH:Pyr:AcOH:H_2O	1:1:1:3	0.77			
$CHCl_3$:CH_3OH:AcOH	15:3:1			0.33	0.43
C_6H_6:CH_3OH:AcOH	10:2:1			0.38	0.45
C_6H_6:CH_3OH:AcOH	7:2:2			0.48	0.55

[a] Abbreviations used: AcOH, acetic acid; BuOH, 1-butanol; EtOAc, ethyl acetate; Pyr, pyridine.

mixture incubated at 30 °C for 10 min. The temperature was raised to 37 °C, and the reaction mixture was incubated with the C-EDTA solution (1.0 mL) for 1 h. After the unlysed erythrocytes were pelleted by centrifugation, the amount of lysis was determined spectrophotometrically by measuring the absorbance at 412 nm of the hemoglobin released into the supernatant. Complete lysis of 6.7×10^6 erythrocytes in 1 mL gave an absorbance of 0.660.

Absorbance values for three controls were observed for each experiment. Complement-mediated hemolysis (H_1) was measured from a buffer control lacking peptide or IgG but containing C1, EAC4, C2, and C-EDTA. Background hemolysis (H_2) was determined from a control lacking peptide, IgG, and C1 but including EAC4, C2, and C-EDTA. Maximum possible hemolysis (H_3) was measured after hypotonically lysing the washed EAC4 cells with distilled water. Since C-EDTA was not present in this control, the complement color value, which is the absorbance at 412 nm of the dilute C-EDTA solution, was added to H_3.

The amount, Z, of free C1 capable of initiating hemolysis (Borsos & Rapp, 1963) is defined (Painter et al., 1974) as

$$Z = -\ln \{1 - [(H_{exptl} - H_2)/(H_3 - H_2)]\}$$

where H_{exptl} is the observed absorbance for a given amount of IgG or peptide. The percentage of free C1 remaining after complex formation with IgG or peptide was calculated as $100(Z/Z_1)$, where Z_1, the total amount of complement lysis found in the first control, was calculated from H_1. The percentage of C1 remaining was plotted against the log of the concentration of the IgG or peptide inhibitor. The peptide concentration at which 50% of the initial C1 was inhibited from initiating hemolysis (I_{50}) is a convenient measure of the ability of the peptides to bind to C1 and inhibit the classical complement pathway.

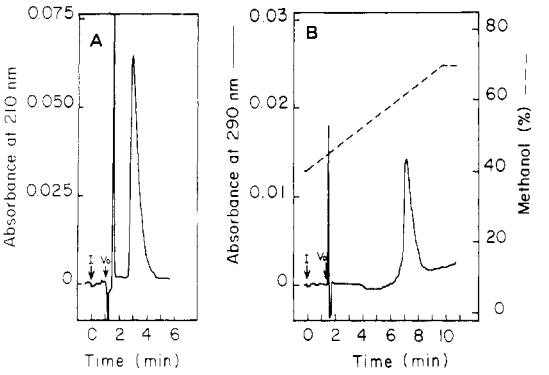

FIGURE 1: Analytical reverse-phase HPLC of purified peptides 281–290 and 275–290F. (A) Isocratic elution of 281–290 ($k' = 2.0$) from a μBondapak C18 column using 2% methanol in 1% aqueous acetic acid. (B) Elution of 275–290F ($k' = 4.1$) from a μBondapak phenyl column using a linear gradient (2 mL/min, 10 min) from 40 to 70% methanol in 1% aqueous acetic acid. I, injection; V_0, void volume.

Results

IgG1 Eu γ-Chain-(281–290)-decapeptide. The crude decapeptide 281–290 was chromatographed on superfine Sephadex G-25 and then on a μBondpak C18 column, and the fractions were analyzed by TLC and monitored for radioactivity. Pure 281–290 chromatographed as one peak by reverse-phase HPLC (Figure 1), showed one spot in five TLC systems (Table III), exhibited one spot ($R_{Arg} = 0.59$) on TLE at pH 6.5, and gave satisfactory amino acid ratios after acid or enzyme hydrolysis (Table IV). Aminopeptidase M digestion revealed only Asn and Gln without a trace of Asp or Glu above a background control containing only enzyme. The specific activity was 210 cpm/nmol.

N^i-Formyl-IgG1 Eu γ-Chain-(275–290)-hexadecapeptide. Crude synthetic peptide 275–290F was chromatographed on Bio-Gel P-2 and then on a Nucleosil C18 column to obtain pure peptide 275–290F. It showed a single peak on reserve-phase HPLC (Figure 1), one spot in three different TLC systems (Table III), and one spot ($R_{Glu} = 0.05$) on TLE at pH 6.5 and gave satisfactory amino acid ratios after acid hydrolysis (Table IV). The ultraviolet spectrum exhibited the 300-nm band characteristic of the N^i-formyltryptophan residue. The presence of the amide residues was confirmed by aminopeptidase M digestion followed by amino acid analysis (data not shown). The specific activity was 170 cpm/nmol.

IgG1 Eu γ-Chain-(275–290)-hexadecapeptide. The N^i-formyl protecting group was removed from 70% of the tryp-

Table IV: Amino Acid Composition of Purified Synthetic Peptides Based on the $C_\gamma 2$ Domain of Human IgG1[a]

amino acid	281–290 acid	281–290 ApM	275–290F acid	275–279AF acid	275–279AF base	275–279AF CpY	275–279A acid	275–279A base
Asp	1.04 (1)		2.67 (1)	0.65 (1)	[1]		[1]	[1]
Asn						0.62 (1)		
Thr	0.98 (1)	2.50[b] (3)	1.04 (1)					
Glu	1.05 (1)		1.10 (1)					
Gly	1.00 (1)	1.02 (1)	0.97 (1)					
Ala	[1]	[1]	[1]					
Val	1.99 (2)	1.99 (2)	2.78 (3)	0.91 (1)	0.96 (1)	0.89 (1)	1.01 (1)	0.72 (1)
Tyr			1.01[c] (1)	0.99 (1)	0.97 (1)	1.11 (1)	0.97 (1)	0.74 (1)
Phe			0.80 (1)	[1]	1.01 (1)		1.10 (1)	1.01 (1)
His	0.99 (1)	1.00 (1)	0.95 (1)					
Lys	2.04 (2)	2.01 (2)	2.07 (2)					
Trp			(1)	0.39 (1)	0.88 (1)	1.00[d] (1)	(1)	0.90 (1)

[a] Amino acids were obtained by hydrolysis with acid, base, aminopeptidase M (ApM), or carboxypeptidase Y (CpY). The theoretical values are shown in parentheses. [b] Includes Asn, Gln, and Thr. [c] Corrected for 5% destruction per 24 h. [d] N^i-Formyltryptophan.

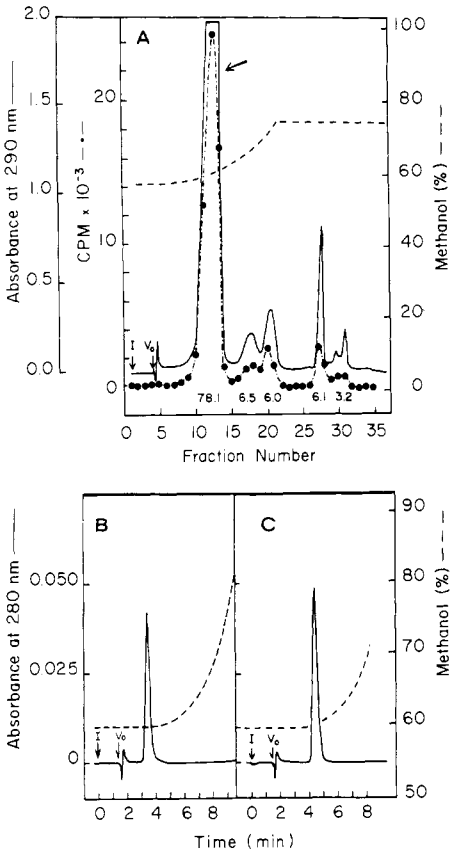

FIGURE 2: Reverse-phase HPLC of peptides 275–279AF and 275–279A on a μBondapak phenyl column. (A) Semipreparative elution of the part of crude peptide 275–279AF soluble in 1:1 (v/v) methanol–1% aqueous acetic acid using a concave gradient (no. 8, 2 mL/min, 10 min) from 60 to 75% methanol in 1% aqueous acetic acid. The number under each peak is the percentage of tritium in that peak; 275–279AF is indicated by the arrow. (B) Analytical elution of purified 275–279A (k' = 1.5) using a concave gradient (no. 10, 2 mL/min, 10 min) from 60 to 90% methanol in 5% aqueous acetic acid. (C) Purified 275–279AF (k' = 2.2) under the conditions given in (B). I, injection; V_0, void volume.

tophan residues of peptide 275–290F by treatment at room temperature for 77 h with 0.01 M aqueous ammonium carbonate adjusted to pH 9.0 with ammonium hydroxide (Yamashiro & Li, 1973). Peptide 275–290 (k' = 2.5) was separated from the starting peptide 275–290F (k' = 5.9) on a μBondapak C18 column by isocratic elution (3 mL/min) using 35% methanol in 1% aqueous acetic acid.

N^{α}-Acetyl-N^{i}-formyl-IgG1 Eu γ-Chain-(275–279)-pentapeptide. When a solution of crude pentapeptide 275–279AF in 10% aqueous acetic acid was stored at 4 °C, a white precipitate slowly formed. When redissolved in 1:1 (v/v) methanol–1% aqueous acetic acid and chromatographed on a μBondapak phenyl column, the solid was found to be mainly the desired peptide 275–279F plus four minor components (Figure 2). Semipreparative chromatography on this column provided pure peptide 275–279AF. It gave single spots in three TLC systems (Table III), one peak on reverse-phase HPLC (Figure 2), satisfactory amino acid ratios after hydrolysis with acid, base, or carboxypeptidase Y (Table IV), and ultraviolet maxima at 290 and 300 nm characteristic of N^{i}-formyltryptophan. The specific activity was 2040 cpm/nmol.

N^{α}-Acetyl-IgG1 Eu γ-Chain-(275–279)-pentapeptide. Peptide 275–279AF was deformylated by treatment for 49 h at room temperature with 1:1 (v/v) methanol–0.01 M aqueous ammonium carbonate adjusted to pH 9.0 with sodium hydroxide. The reaction mixture was chromatographed on a μBondapak phenyl column to afford pure peptide 275–279A.

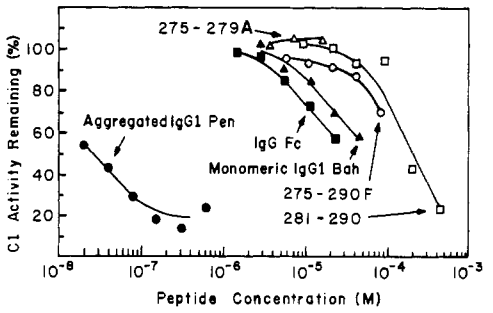

FIGURE 3: Inhibition of C1-mediated immune hemolysis. The percentage of C1-mediated hemolytic activity remaining is plotted vs. the molar concentration of the synthetic peptide or protein standard present in the initial C1 reaction mixture.

Table V: Inhibition of C1-Mediated Hemolysis[a]

inhibitor	I_{50} (μM)	relative molar activity
aggregated IgG	0.025	3000
Fc fragment of IgG	47	1.6
monomeric IgG	74	[1.0]
peptide 275–290F	150	0.5
peptide 281–290	180	0.4
peptide 275–290	>20	
peptide 275–279A	>16	

[a] Peptides 275–290 and 275–279A were inactive at the concentrations indicated.

It showed one peak on reverse-phase HPLC (Figure 2), one spot in three TLC systems (Table III), ultraviolet maxima at 280 and 290 nm characteristic of unformylated Trp, and satisfactory amino acid ratios after acid and base hydrolysis (Table IV). The Tyr and Val values after base hydrolysis were low due to incomplete cleavage of the Tyr-Val dipeptide. The specific activity was 2040 cpm/nmol.

C1 Inhibition Assay. Four synthetic peptides were compared with three protein controls (aggregated IgG, monomeric IgG, and the IgG fragment Fc) for their ability to inhibit the classical complement pathway by binding to C1. The dose–response curves are shown graphically in Figure 3, and the I_{50} values are given in Table V. Aggregated IgG was the most active material tested, producing 50% inhibition at 0.025 μM. It was approximately 3000 times more active than monomeric IgG and about 2000 times more active than Fc. The cationic decapeptide 281–290 was about 40% as active as monomeric IgG. Peptide 275–290F showed 13% inhibition at 40 μM and 35% inhibition at 80 μM, which yields an extrapolated value for 50% inhibition of about 150 μM. Thus, this formylated hexadecapeptide 275–290F, which contains both the hydrophobic region and the cationic region, was essentially as active as the decapeptide. The deformylated hexadecapeptide 275–290 showed no inhibition over the concentration range tested (2–20 μM); insufficient material was available to examine the effect of higher concentrations. Acetylated peptide 275–279, a hydrophobic peptide containing tryptophan-277, showed no inhibition at a saturating concentration (16 μM).

Discussion

The 275–290 region of the $C_{\gamma}2$ domain of human immunoglobulin G was selected as a potential site for interaction with the C1q subcomponent of serum complement because it includes the hydrophobic 275–279 region around the invariant tryptophan-277 and contains the cationic 281–290 region that resembles a diamine. Four $C_{\gamma}2$ peptides (Table I) were synthesized by the solid-phase method and purified by high-pressure liquid chromatography: the hexadecapeptide 275–290

and its N^i-formyl derivative 275–290F, which contain both the cationic and hydrophobic regions; decapeptide 281–290 containing the cationic region but lacking the hydrophobic region; and N^{α}-acetylated pentapeptide 275–279A containing the hydrophobic region but lacking the cationic region.

The peptides were synthesized by the solid-phase method. The amino acid sequences of these synthetic peptides suggested several potential side reactions, so strategies were adopted to circumvent them. Racemization of the His residue during the coupling step was avoided by using a strongly electron-withdrawing group (2,4-dinitrophenyl) to reduce the basicity of the imidazole ring (Shaltiel, 1967). Formation of β-Asp residues was minimized by avoiding the Asp-Gly sequence through omission of Asp-280 from peptide 281–290 and by conducting the HF cleavage of peptide 275–290F at low temperatures (Baba et al., 1973). Branched peptides having extra residues attached to the side-chain amino group of Lys were avoided by using a more acid-stable protecting group (3-chlorobenzyloxycarbonyl) for Lys (Erickson & Merrifield, 1973). Dehydration of Asp and Gln residues (Katsoyannis et al., 1958; Paul & Kende, 1964) during the coupling reaction to form β-cyanoalanine and γ-cyano-α-aminobutyric acid, respectively, was not a serious problem because infrared spectroscopy of crude peptide 275–290F after HF cleavage revealed that less than 4% of these cyano derivatives was present.

All of the synthetic peptides prepared in this study were purified by reverse-phase HPLC, which routinely allowed high-resolution analysis and preparative separation of the desired peptide in a minimal amount of time. As illustrated by peptide 275–279AF, the use of reverse-phase HPLC alone rapidly produced a large quantity of the homogeneous peptide. Even when dealing with a complex mixture of peptides, rechromatography of carefully pooled fractions allowed the separation of the desired product from the byproducts. The homogeneity and composition of the synthetic peptides were demonstrated by TLC, TLE, HPLC, amino acid analysis, and UV spectroscopy.

The C1 inhibition assay used in this study involves the binding of uncomplexed, activated human C1 to sheep erythrocytes bearing rabbit anti-sheep erythrocyte IgG and human C4. Sequential addition of human complement components C2 and C3–C9 results in the hemolysis of the activated erythrocytes. Compared to a standard complement fixation assay, in which any step from C1 activation to formation of the C5b–9 complex might be inhibited, the present assay is relatively specific for C1. The assay was designed initially to quantitate the amount of C1 indirectly by measuring hemolysis (Borsos & Rapp, 1963). It was used later to measure the ability of large polypeptides, such as IgG and its Fc fragment, to inhibit C1-mediated immune hemolysis (Augener et al., 1971). Monomeric IgG and Fc cause inhibition not by activating and consuming C1 but by binding to C1q, which blocks C1 from binding to erythrocyte-bound IgG. A small peptide with undefined binding specificity, however, might inhibit the interaction between erythrocyte-bound IgG and C1 by binding to either C1q or IgG. Alternatively, it might inhibit hemolysis by dissociating the C1 complex or by binding to C4 and inhibiting its interaction with C2. Finally, it might inhibit by binding to the active site of $C1\bar{s}$ and thus inhibit C2 activation. Since the human C1 used in the assay was predominantly in the enzymatically active form, inhibition of C1s activation by $C1\bar{r}$ is not relevant.

Yasmeen et al. (1976) used this C1 inhibition assay to show that the $C_\gamma2$ domain of IgG, which bears a carbohydrate chain, inhibits C1-mediated hemolysis before but not after reduction and alkylation. The $C_\gamma3$ domain of IgG and β_2 microglobulin lack carbohydrate, but each inhibits C1-mediated immune hemolysis after reduction and alkylation (Isenman et al., 1975; Yasmeen et al., 1976). Thus, a linear polypeptide region of β_2 microglobulin or $C_\gamma3$ may be responsible for the inhibition of C1-mediated hemolysis. After reduction and alkylation of the $C_\gamma2$ domain, however, the carbohydrate moiety linked to $C_\gamma2$ might induce this domain to adopt a different set of conformations than those assumed by β_2 microglobulin and $C_\gamma3$, which might distort or block the C1-binding site of $C_\gamma2$ and thus prevent inhibition of C1-mediated hemolysis. In analogy with denatured β_2 microglobulin and $C_\gamma3$, either denatured $C_\gamma2$ lacking carbohydrate or a synthetic linear polypeptide region of $C_\gamma2$ might also inhibit C1-mediated hemolysis.

The concentrations of protein standard or synthetic peptides at which 50% of the C1-mediated immune hemolysis was inhibited (I_{50}) are shown in Table V. The aggregated IgG standard had an average $s_{20,w}$ value of 12.5 S, which corresponds to a size range consistent with efficient complement activation (Hyslop et al., 1970). As expected from previous studies (Augener et al., 1971; Yasmeen et al., 1976), aggregated IgG was much more active than monomeric IgG, and monomeric IgG and Fc were essentially equally active in inhibiting the classical complement pathway.

Peptides 275–290F and 281–290 display their range of inhibitory activities (inactive to highly active) over a 3–4-fold concentration range, similar to the ranges observed for $C_\gamma2$ and a $C_\gamma2$ dimer (Yasmeen et al., 1976), whereas the IgG proteins exhibit this range of activities over a 10–20-fold concentration range (Figure 3). This difference might reflect the involvement of a cooperative phenomenon, such as aggregation or micelle formation. When peptide 281–290 was examined by sedimentation velocity and sedimentation equilibrium methods at concentrations that would have resulted in greater than 80% inhibition of C1-mediated hemolysis, however, it sedimented as a homogeneous compound with an apparent molecular weight of 1007. Since the monomeric form of peptide 281–290 has a molecular weight of 1082, peptide 281–290 is clearly monomeric at concentrations sufficient to inhibit C1-mediated hemolysis. Since the peptides are active over the same 3–4-fold concentration range observed for the isolated $C_\gamma2$ domain (Yasmeen et al., 1976), the difference in the range of activities between proteins and peptides may reflect the difference between a site within a large protein and a more isolated binding site. Alternatively, since immune complexes or IgG aggregates are responsible in nature for binding to and activation of C1, inhibition of C1 by part or all of the $C_\gamma2$ domain might be a separate phenomenon.

On a molar basis, the synthetic peptides 275–290F and 281–290 were each as active as one $C_\gamma2$ domain. Intact monomeric IgG contains two $C_\gamma2$ domains and thus potentially two equivalent C1-binding sites. Each molecule of peptide, however, could mimic only one of these sites. If the two C1-binding sites in the $C_\gamma2$ domain function independently and noncooperatively, the I_{50} per C1-binding site of IgG is 150 μM. Thus, peptides 275–290F and 281–290 are just as inhibitory as one of the two C1-binding sites of the native IgG molecule. Furthermore, these peptides probably do not inhibit the classical complement pathway by binding to erythrocyte-bound IgG. When separately tested by equilibrium dialysis for binding to pooled human IgG, none of these peptides bound under conditions where 5% binding would have indicated a dissociation constant of 100 μM.

Monomeric IgG binds to C1q with a dissociation constant of 47 μM (Schumaker et al., 1976). Since peptides 275–290F and 281–290 exhibit I_{50} values twice that of monomeric IgG (Table V), they should bind to C1q with a dissociation constant about twice that of monomeric IgG, or about 100 μM. Preliminary results (Prystowsky, 1978) for binding of these peptides to purified human C1q indicated that no binding occurred under conditions where the minimum detectable binding would have corresponded to a dissociation constant of 100 μM. Efforts to increase the sensitivity of the assay by increasing the concentration of C1q did not succeed because of the limited solubility of C1q in physiological buffers. Although this attempt to demonstrate direct binding of IgG peptides 275–290F and 281–290 to human C1q was not successful, it was not unexpected because the assay was not sensitive enought to detect weak binding. Also, direct binding of an IgG fragment or the $C_\gamma2$ domain to C1q has not yet been reported. The possibility that the cationic decapeptide 281–290 is a part of the C1q-binding site of $C_\gamma2$ cannot be ruled out by these binding studies. The weak interaction of these IgG peptides with C1q would have to be relatively specific because it would involve the binding of a cationic peptide with the cationic C1q protein, which has an isoelectric point of 10.0–10.6 (Lin & Fletcher, 1978).

The possibility that the decapeptide contains at least a part of the C1-binding site of $C_\gamma2$ is consistent with the X-ray crystallographic structure (Deisenhofer et al., 1978; Deisenhofer, 1981) of the complex between Fc and fragment B, one of four structurally similar regions of staphyloccal protein A. Examination of this structure suggests that the presence of the entire protein A molecule would sterically hinder the C1q molecule from binding to the cationic 281–290 region of $C_\gamma2$. Indeed, protein A does inhibit the binding of C1q to IgG1 in vitro (Stalenheim et al., 1973). In addition, it is consistent with the observation that human IgG1 proteins with a 15-residue deletion in the hinge region are unable to bind to C1q (Klein et al., 1981). This result was ascribed to decreased segmental flexibility and increased steric hindrance of the Fc segment by the Fab segments. These same factors probably account for the inability of human IgG4 immunoglobulins to bind C1 and activate the classical complement pathway.

IgG1 peptide 275–290F contains most of the structural features of both hydrophobic peptide 275–279A and cationic peptide 281–290 but shows essentially the same activity as peptide 281–290 in inhibiting C1-mediated immune hemolysis. Thus, peptide 275–290F probably acts through its cationic 281–290 region rather than through the hydrophobic 275–279 region containing N^i-formyltryptophan-277. These findings are consistent with the X-ray crystallographic result (Deisenhofer et al., 1976; Deisenhofer, 1981) that Trp-277 is part of the hydrophilic core of $C_\gamma2$ and thus is not readily available for C1q binding (Figure 4). Inhibition of complement activation by alkylation of Trp-277 (Allan & Isliker, 1974a,b) probably results from distortion of the secondary structure of the 275–290 region.

The human IgG γ-chain pentapeptide 277–281 (Trp-Tyr-Val-Asp-Gly) is reported to inhibit immune hemolysis by 50% at a concentration of 2.67 μg/mL, which is equivalent to 4.1 μM (Boackle et al., 1979). The assay used (Boackle et al., 1974) differs from the C1 inhibition assay of the present study (Borsos & Rapp, 1963; Painter et al., 1974) by not including decantation and washing of the $\overline{EAC14}$ cells, which removed soluble peptide and C1, before the addition of C2 and C3–C9. Thus, peptide 277–281 may have inhibited immune hemolysis not just at the initial step involving the binding of C1 to EAC4 cells but at any later step in the classical complement cascade. In the present study, negligible inhibition (<10%) of the first step of immune hemolysis was observed over the concentration range of 2–20 μM for the unprotected peptide 275–290 containing the entire 277–281 region and over the range of 1.6–16 μM for the acetylated pentapeptide 275–279A, which shares the segment Trp-Tyr-Val with peptide 277–281. Peptides 275–290 and 275–279A are thus completely inactive in the more specific assay at concentrations 4–5 times higher than the I_{50} value for peptide 277–281 in the less specific assay.

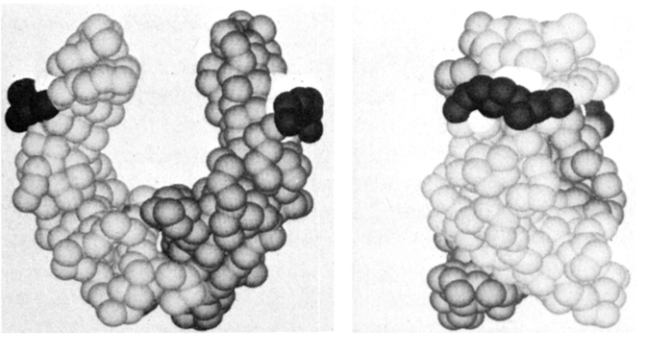

FIGURE 4: Computer simulation of the α carbon connectivity map of the Fc region of human IgG. One heavy chain is shown in charcoal gray and the other in light gray. The exposed cationic region (residues 281–290) corresponding to peptide 281–290 is shown in black, and the buried hydrophobic region (275–279) corresponding to peptide 275–279A is shown in white. (Left panel) Side view of both domains showing the exposed cationic region 281–290. The central cavity between the chains is normally filled with carbohydrate (not shown). (Right panel) View looking directly at the exposed cationic region 281–290. These representations are based on the revised X-ray coordinates of Deisenhofer (1981) and were generated by Richard J. Feldmann, Division of Computer Research and Technology, National Institutes of Health.

Two alternative explanations of these results are possible. First, peptide 277–281 may inhibit not the initial binding of C1 to IgG but some later step in the classical complement cascade. Second, the pentapeptide 277–281 bearing terminal ammonium and carboxylate groups may block the binding of C1 to IgG and yet be at least 10 times less active when these charged groups are not present due to incorporation of residues 277–281 into a larger peptide, such as 275–290. In either case, the activity reported for peptide 277–281 is about 20 times greater on a site basis than that observed for each C1q-binding site of human IgG. Thus, the relevance of peptide 277–281 to the location of the C1q-binding sites of IgG is not clear.

Since the hydrophobic 275–279 region of IgG is mostly buried in the interior of the $C_\gamma2$ domain (Deisenhofer et al., 1976; Deisenhofer, 1981), one would not expect this region to be a major part of the C1q-binding site of IgG. Acetylated peptide 275–279A is completely inactive at a concentration of 16 μM in inhibiting C1-mediated immune hemolysis. Because of its limited solubility, this was the highest concentration at which it could be tested. The unprotected 16-residue peptide 275–290 is completely inactive in this relatively specific assay at a concentration of 20 μM. Examination of its activity at higher concentrations was precluded by the limited amount of synthetic peptide available. The protected 16-residue peptide 275–290F, which bears a formyl group on the indole nitrogen atom of Trp-277, is only about 20% more active on a molar basis than the decapeptide 281–290. Even though the formylated indole ring is only two atoms (C=O) larger than the free indole ring, the formyl group might still prevent an interaction of Trp-277 with C1q. Thus, the present results provide no experimental evidence for a contribution of the hydrophobic 275–279 region to the C1q-binding site of human

IgG. On the other hand, they do not directly exclude such a contribution.

The major goal of the present study was to explore the location and structural features of the C1q-binding site of IgG. Two homogeneous synthetic peptides related to the 275–290 region of the $C_\gamma 2$ domain of human IgG1 were found to inhibit C1-mediated immune hemolysis. Since decapeptide 281–290 is as active as the entire $C_\gamma 2$ domain in inhibiting C1-mediated hemolysis, the 281–290 region containing the positively charged residues His-285, Lys-288, and Lys-290 may be part of the C1q-binding site of $C_\gamma 2$.

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