DEFICIENCY OF C1r IN HUMAN SERUM

EFFECTS ON THE STRUCTURE AND FUNCTION OF MACROMOLECULAR C1*

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Lepow et al. (1) and Naff et al. (2) showed that the first component of human complement is a macromolecule composed of three "subcomponents" which they called C1q, C1r, and C1s. C1q was shown to be the same protein as the previously described 11S component (3), C1r was introduced as a new component, and C1s was identified as the proesterase or precursor of the esterase activity associated with C1. All of these subcomponents were found to be required for C1 to be hemolytically active. In serum, hemolytic C1 was found to have a molecular size of approximately 18S and could be rendered hemolytically inactive by dissociating it into its "subunits" or components by a calcium chelating agent (1, 2). This suggested that in normal serum the subunits of C1 existed in a macromolecular complex, the structural and functional integrity of which seemed dependent on the presence of Ca^{++}.

To date, information concerning the relationship of the subcomponents of C1 to the whole molecule and to each other has been derived from in vitro experiments dependent on the use of calcium chelating agents to dissociate the subcomponents. It is the purpose of this report to describe studies of C1q, C1r, and C1s in a human serum found to be extremely deficient in hemolytic C1 activity. These studies show that the deficiency is predominantly related to a severe deficiency or absence of C1r and that the other subunits of C1, though present in normal or moderately reduced amounts, do not react to form a complex (C1q-C1s) when C1r is deficient.

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The information derived from the study of this serum suggests that the synthesis and assembly of the subcomponents of C1 are the functions of more than one cell type or perhaps are under separate genetic control.

Materials and Methods

Salts of Ethylenediaminetetraacetic Acid (EDTA)[1].—Reagent grade Na_2H_2EDTA was titrated to pH 7.4 at a stock concentration of 0.15 M resulting in a solution largely in the form Na_3HEDTA. $Na_2MgEDTA$ (Geigy Chemical Corp., Ardsley, N. Y.) was also titrated to pH 7.4 at a stock concentration of 0.15 M.

Buffers.—Triethanolamine-buffered saline (TBS)(1) at pH 7.4, ionic strength 0.15, containing 10^{-3} M Ca^{+} and 0.05% gelatin (Knox Gelatine, Inc., Camden, N. J.) was used in all experiments unless stated otherwise. Veronal-buffered saline at pH 7.4 containing Ca^{++}, Mg^{+}, and gelatin were prepared according to Mayer (4).

Human Serum.—Human blood was allowed to clot at room temperature for 1 hr, stored overnight at 5°C, and the serum removed after centrifugation at 0°C. Pools from several donors were frozen in portions and stored at −70°C.

Human C1 Subcomponents.—C1q, C1r, and C1s were prepared by diethylaminoethyl (DEAE)-cellulose chromatography of euglobulin preparations in the presence of Na_3HEDTA as described previously (1). Pools of these three preparations were dialyzed against TBS buffer at 1°C for 16 hr to reduce the EDTA concentration to 10^{-8} M; for purposes of expressing dilutions, these pools were considered undiluted.

Resolution of a euglobulin preparation from the C1-deficient serum was carried out on DEAE-cellulose by this same method (1).

Guinea Pig C2.—Partially purified C2 was prepared from guinea pig serum (Texas Biological Laboratory, Inc., Fort Worth, Texas) according to the method described by Nelson (5).

Sensitized Sheep Erythrocytes (EA).—Sheep blood was collected and processed as previously described (1).

EAC1.—Cell intermediates carrying guinea pig C1 were prepared according to methods previously described (6).

EAC1, 4 and EAC4.—Cell intermediates carrying these components were prepared from human serum according to the method described by Borsos and Rapp (7).

Assay of C1 Subcomponents.—C1q, C1r, and C1s were measured as previously described (1, 2). Briefly, buttons of EA containing 5×10^8 cells were suspended in 0.5 ml of the sample to be tested for a given subcomponent. Portions of 0.5 ml of 1/100 dilutions (in TBS) of the other two subcomponents were then added. After incubation for 10 min at 37°C, the resulting EAC1 were lysed by adding 2.5 ml of human serum diluted (1/62.5) in TBS containing $Na_2MgEDTA$ (final concentration of 16×10^{-3} M) and allowing an additional 60 min incubation at 37°C.

An alternative method was employed on some occasions with identical results. A button of EAC4 (5×10^7 cells) was suspended in 0.5 ml of the test material. The appropriate two of the three subcomponents were added and, after 10 min at 37°C, 0.5 ml of functionally purified guinea pig C2 containing 100 (50%) hemolytic units was added. This was followed by incubation for a period of 15 min at 30°C. The resulting EAC142 were lysed by adding 0.5 ml of 1/12.5 dilution of guinea pig complement (GPC) in 0.04 M EDTA and allowing a further incubation for 60 min at 37°C. After this incubation, 5.0 ml of chilled isotonic saline were added, and the tubes centrifuged at 0°C.

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[1] *Abbreviations used in this paper:* EA, sensitized sheep erythrocytes; EDTA, ethylenediaminetetraacetic acid; DEAE, diethylaminoethyl; TBS, triethanolamine-buffered saline.

In each case, the oxyhemoglobin in the supernate was determined spectrophotometrically, at 541 mμ in the first method and 412 mμ for the second.

Assay of Human C1, C4, C2, and C3–9.—These activities were determined according to methods previously described (6).

Sucrose Density Gradient.—Linear sucrose gradients were prepared using 5%–20% (w/v) sucrose in TBS with Ca^{++} or with EDTA allowing the gradient to form under gravity flow from the mixing chamber. Immediately preceding each run, 0.1 ml of serum was layered on the gradient.

A Beckman Model L-2 preparative ultracentrifuge and a swinging bucket rotor (SW 39 L) were used for all experiments, and centrifugation was performed at 35,000 rpm for 15 hr. Gradient fractions were obtained by placing a 25 gauge needle into the bottom of the tube and with a constant flow pump, filling the tube from the bottom with a 50% sucrose solution. This gently moves the entire gradient up through a monitor which records optical density at 280 mμ; twenty 0.3 ml fractions were collected, diluted in 3.0 ml of chilled 0.15 M NaCl solution, and dialyzed at 0°–4°C overnight against TBS buffer.

Protein Quantitations.—Complement component protein concentrations were determined by radial immunodiffusion method of Mancini et al. (8).

C1q.—Antibody to this component was prepared by a modification (9) of the method described by Morse and Christian (10).

C1s.—These determinations were performed using an antiserum to purified C1s prepared as previously described (11).

C3.—The protein concentration of this component was determined by using commercial antibody to C3 obtained from Hyland Laboratories, Los Angeles, Calif.

C4.—Antiserum to this component was obtained from Hyland Laboratories.

RESULTS

The serum which provides the basis for this study was obtained from a 13-yr old child with a form of chronic glomerulonephritis. The severe limitation in the ability of this serum to lyse optimally sensitized sheep erythrocytes is due to a deficiency of hemolytic C1 (Table I). In addition to showing that the C1-deficient serum has normal hemolytic activity of C2 and C3–9, Table I shows that, in the same serum, the concentration of C1q and C3 proteins are normal while the concentration of C1s is approximately 30%–40% of normal. In contrast, C4 protein concentration (Table I) and hemolytic activity (Table I and Fig. 1) and C1 esterase inhibitor activity are substantially greater than normal. The basis for the increase in amount and function of C4 is not clear, but it is possible that C1r and/or C1s play a regulatory role in the normal catabolism or synthesis of C4. In order to establish whether the deficiency in hemolytic function is entirely attributable to the deficiency of C1, the following experiments were performed. Equal volumes of EA, EAC1,[2] or EAC4[3] were added to appropriate dilutions of serum at 30°C. The cells were kept in even suspension by constant gentle agitation, and samples of 1.0 ml (7.7×10^7 cells) were taken at intervals and added to 1.5 ml of chilled guinea pig complement (1/37.5) in 0.04 M EDTA. Incubation

[2] Prepared by adding approximately 100 effective molecules of partially purified guinea pig C1 per cell.

[3] EAC14 prepared from these cells showed maximum reaction with C2 to form EAC142 in 8 min.

for 90 min at 37°C was followed by addition of 5.0 ml of 0.15 M NaCl solution, centrifugation at 0°C, and determination of supernate oxyhemoglobin spectrophotometrically at 412 mμ.

The results as shown in Fig. 2 a emphasize the marked deficiency of this serum, even at high concentrations, in forming EAC142 from EA or EAC4. Fig. 2 b shows that provision of C1 in the form EAC1 provides conditions which permit a rate and degree of EAC142 formation from this serum that is essentially the same as observed with normal serum.

Studies of Hemolytic Activity of the C1 Subcomponents.—Euglobulin prepared from this serum by methods previously described (1, 2) was found to be hemo-

TABLE I

Hemolytic Complement Component Activities and Selected Component Protein Concentrations in C1-Deficient and Normal Human Serums

	CH_{50}*	C1‡	C4‡	C2‡	C3-9‡	C1q protein	C1s protein	C4 protein	C3 protein	C1-esterase inhibitor§
						μg N/ml	*μg/ml*	*μg N/ml*	*mg/100 ml*	*units/ml*
CC‖ (patient)	<1	~100	60,000	500	120	18	16¶	95	125	18–24
Nml (1)	45	21,000	18,000	450	100	18	32.8**	30	110	6–8
Nml (2)	52	27,000	25,000	620	135	24	32.8**	41	155	6–8
Nml (3)	58	30,000	21,000	550	110	22	32.8**	35	125	6–8

* CH_{50} units/ml.

‡ CH_{50} units/0.5 ml.

§ These values were obtained through the courtesy of Dr. J. Pensky. The units expressed are those defined in reference 20.

‖ CC, C1 deficient serum; Nml, normal human serum.

¶ Mean of six determinations. Lowest values was 13, the highest was 31 over a period of 1 yr.

** Normal mean. The normal range (± 2 SD) = 20.4–45.2.

lytically inactive when assayed for C1. Combination of the euglobulin with preparations of hemolytically active C1q, C1r, or C1s (Table II) indicated that the addition of C1r alone reconstituted the hemolytic activity. When similar combinations of subcomponents were added to whole serum the total hemolytic complement activity was restored to normal by C1r alone. Addition of C1q, C1s, or combination of these subcomponents made no appreciable difference in total serum complement activity.

Chromatography on DEAE-cellulose substantiated this observation. Fig. 3 b is a representative elution pattern of protein and subcomponent activities derived from resolution of a normal euglobulin on DEAE-cellulose. Fig. 3 a shows the resolution of a euglobulin preparation from the C1-deficient serum. Hemolytically active C1q and C1s are clearly present in the latter though C1s

activity is relatively less than normal. C1r hemolytic activity could not be detected in any of the fractions from two column runs. Fractions from the C1q range (fraction 12) and the C1s range (fraction 115) of one of these two columns produced no C1 activity when combined with any of the other fractions from

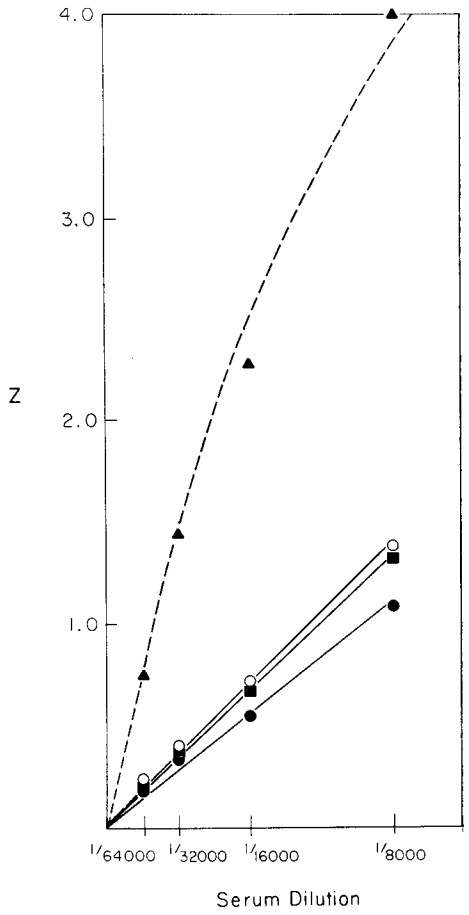

FIG. 1. The dose response curve of C4 hemolytic activity in three representative normal human serums (○——○, ●——●, ■——■) and in the C1-deficient human serum (▲——▲) are shown.

the same column but led to substantial formation of C1 when combined with known C1r (Table III).

Since the three subcomponents of C1 appear to be bound together in a macromolecular complex in normal serum, it was of interest to know what relationship two of the three subcomponents might have to each other in the apparent ab-

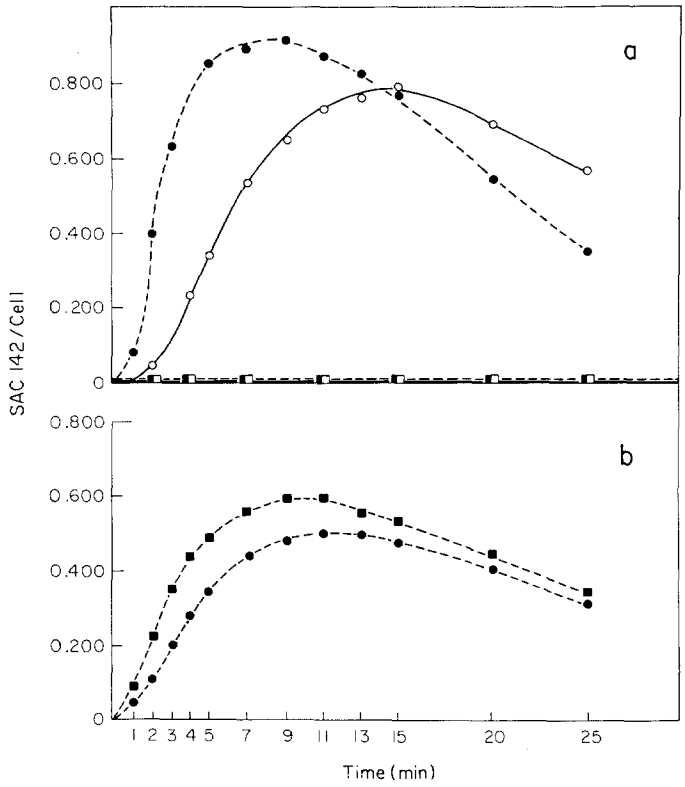

FIG. 2. (a). Rate and degree of SAC142 formation by normal human serum (1/300 dilution) from EA ○——○ and EAC4 ●——●; and by C1-deficient human serum (1/10 dilution) from EA □——□ and EAC4 ■——■. (b). Rate and degree of SAC142 formation by normal human serum (1/500 dilution) ●——● and C1-deficient human serum (1/500 dilution) ■——■ from EAC1.

TABLE II

Reconstitution of Hemolytic C1 Activity in the Euglobulin from C1-Deficient Human Serum

Euglobulin (1/50)	C1q (1/100)	C1r (1/100)	C1s (1/100)	OD (541 mμ)
ml	*ml*	*ml*	*ml*	
0.5	0.5	—	0.5	0.030
1.0	—	0.5	—	1.040
0.5	—	0.5	0.5	1.110
1.5	—	—	—	0.020

These reagents were added to EA, incubated for 10 min at 37°C, and resulting EAC1 were lysed with human serum Na MgEDTA as described in Methods.

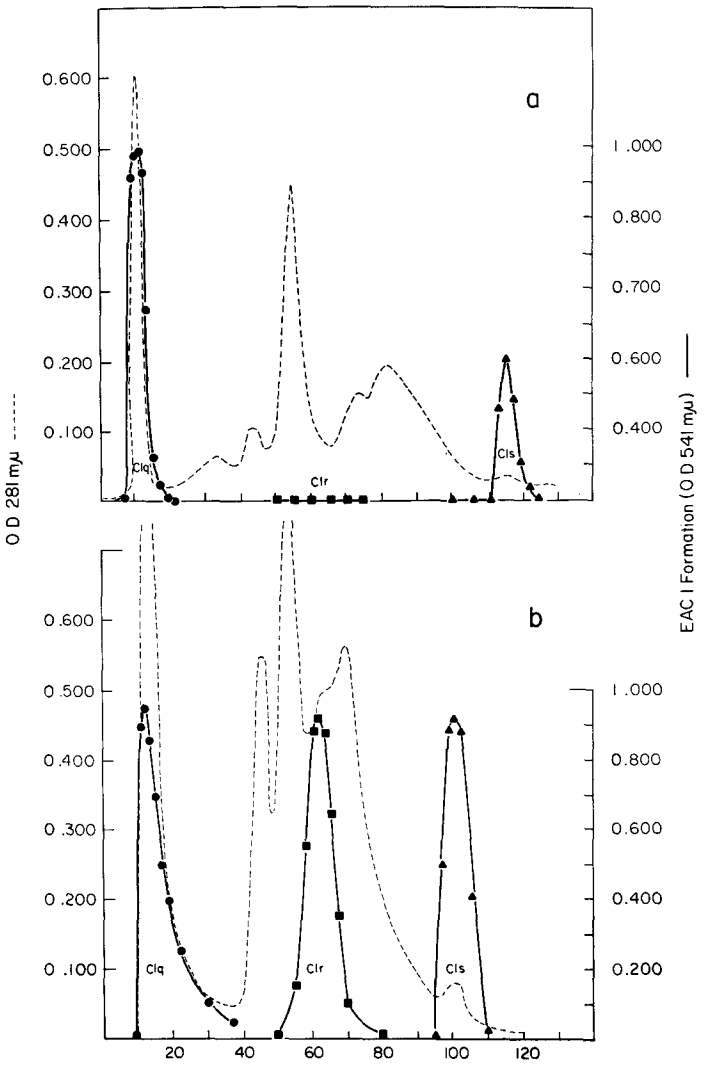

FIG. 3. (a). The elution pattern from DEAE-cellulose chromatography of euglobulin prepared from C1-deficient human serum. The starting phosphate buffer (ionic strength 0.15) and the limiting buffer (ionic strength 0.5) contained 10^{-3} M Na_3 EDTA. The gradient was begun at fraction 21. These conditions were identical for all DEAE columns. The hemolytic activities of C1q (●——●) and C1s (▲——▲) were observed in the fractions indicated. No C1r (■——■) activity was detected. (b). The elution pattern from DEAE-cellulose chromatography of euglobulin prepared from normal human serum. The hemolytic activities of C1q (●——●), C1r (■——■), and C1s (▲——▲) are present in the fractions as shown.

sence of the third. The C1-deficient serum, with no detectable C1r function, was fractionated by sucrose density gradient ultracentrifugation in an attempt to obtain information pertaining to this question.

It has been shown previously that when normal human serum is subjected to centrifugation in a sucrose gradient, hemolytically active C1 sediments near the 19S region (2). Fig. 4 a shows that when normal human serum was centrifuged in a sucrose gradient containing Ca^{++}, whole C1 activity was greatest in the fractions near the 19S marker. No subcomponents were detectable in lighter fractions. However, when C1r-deficient serum was centrifuged simultaneously, there was no whole C1 activity: C1q activity was greatest in fraction 11, and C1s activity was maximum between fractions 7 and 8, lighter

TABLE III

Recombination of Subcomponents Derived from C1r-Deficient Euglobulin

Fraction No. 12* (C1q)	Fraction 115* (C1s)	C1r‡	TBS	Other fraction Nos.§	OD
+	+	−	+	−	0.012
+	+	−	−	70	0.015
+	+	−	−	72	0.013
+	+	−	−	74	0.011
+	+	−	−	76	0.014
+	+	−	−	78	0.011
+	+	+	−	−	0.534

* 0.5 ml of a 1/50 dilution of the fractions taken from the column as shown in Fig. 2 a.

‡ 0.5 ml of a normal C1r preparation (1/100 dilution).

§ Representative fractions from the region where C1r would normally elute (70, 72, 74, 76, and 78) have been used as a possible source of C1r.

than the 7S marker (Fig. 3 b). The location of maximum C1q and C1s activity were identical when C1-deficient serum was centrifuged in the presence of Ca^{++} or in EDTA, and under both conditions there was minimal C1q–C1s activity in fractions 8 and 9 where these two subcomponents overlapped in the gradient. The very small amount of C1s activity in the gradient fractions might be the result of two circumstances; namely, reduced concentration of C1s protein (Table I) and the presence of the C1-esterase inhibitor which sediments in the same region as C1s on sucrose gradient (2).

These experiments suggest that the relative absence of hemolytic C1 activity in this human serum is due predominantly to a deficit in C1r function, though clearly, C1s protein concentration and function are substantially less than normal. The lack of detectable C1r function may be due to an actual deficiency of C1r or to a structural defect in the molecule such that it cannot bind with either C1q or C1s.

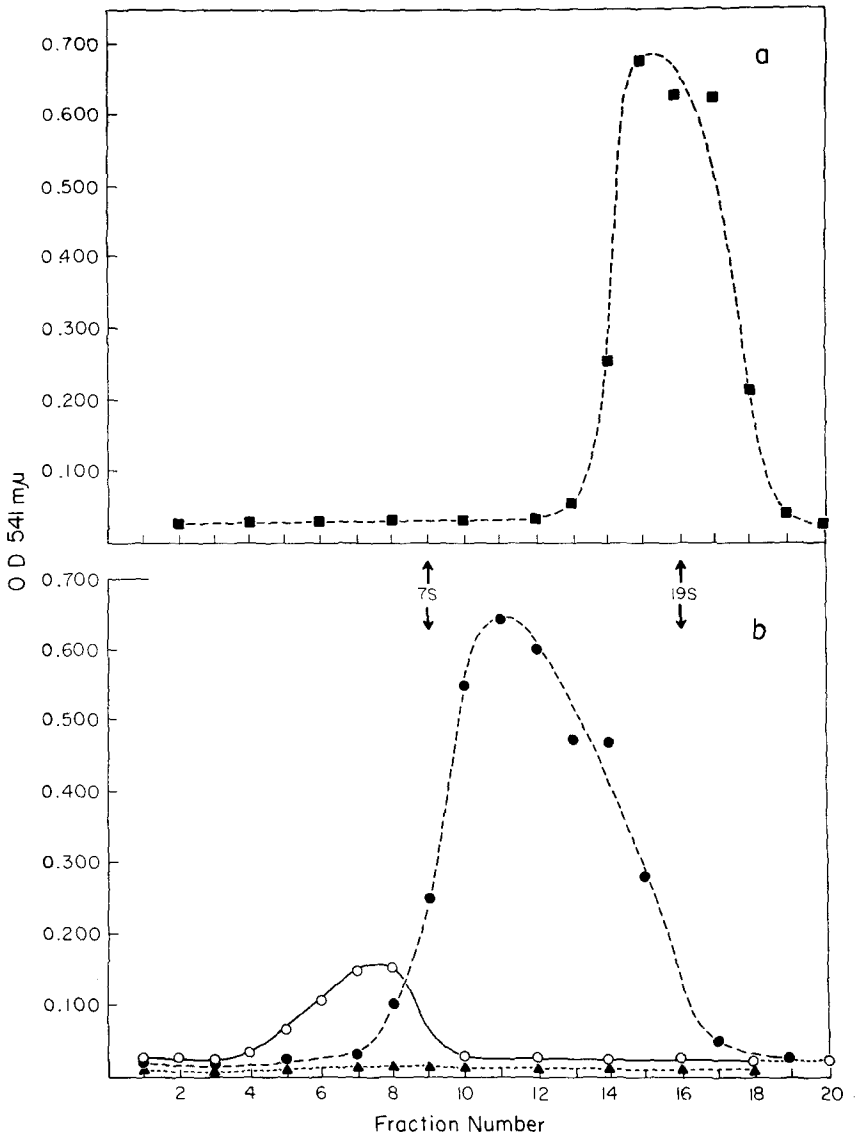

FIG. 4. (a). The sedimentation pattern of hemolytic C1 (■——■) activity after sucrose density gradient ultracentrifugation of normal human serum in the presence of calcium ions. (b). After sucrose density gradient ultracentrifugation of C1-deficient human serum in the presence of calcium ions the subcomponents C1q (●——●) and C1s (○——○) sediment independently as indicated. No C1r activity is detectable in any of the fractions (▲——▲).

DISCUSSION

The studies presented here raise important points concerning the basis for the severe deficiency of C1r hemolytic activity and the moderate deficiency of C1s hemolytic activity in the presence of normal C1q, the meaning of this defect in relationship to synthesis of the subcomponents and the relationship of the subcomponents C1q and C1s to each other in the relative absence of C1r.

The lack of other family members with a similar deficiency make it impossible to know whether this abnormality is related to a primary or secondary deficit in ability to synthesize C1r and to some extent C1s.

It is of interest that this serum was obtained from a child with chronic glomerulonephritis. The association of the C1 deficiency with this disease is important in view of the association of other serum complement aberrations in patients with nephritis (9, 12). However, the mechanisms which lead to these serum complement deficiencies in such patients may be varied as suggested by observations supporting decreased synthesis (13), increased consumption (13)[4], and increased urinary loss of complement components (14). Therefore, the fact that this serum is obtained from a patient with chronic glomerulonephritis does not provide any explanation for the basic mechanism which has produced such a severe deficit of hemolytic C1.

Though there is no precedent for selective C1 deficiency in nephritis, hemolytic C1 and C1q protein concentration have been found reduced in some forms of hypogammaglobulinemia (15–17), disorders known to be associated with defective synthesis of immunoglobulins (18). It has been proposed that these C1 and C1q deficiencies are likely related to decreased ability to synthesize C1q (3, 15–17). In addition, Pondman et al. (19) described a child with a selective deficiency of hemolytic C1 activity related to deficiencies of C1s and C1r. The hemolytic activity of the serum was restored by providing purified C1s but not by C1q or C1r. Though their patient was presented with a diagnosis of systemic lupus erythematosus, there was no mention of any evidence for the presence of nephritis. Their observations provide several interesting and important similarities to those presented here.

There is no precedent from either in vitro or in vivo studies for a selective activation or consumption of one C1 subcomponent and though this cannot be ignored as a possible mechanism for the component deficiency in this serum, it seems unlikely. In the serum of our patient, C1r hemolytic activity seemed completely dissociated from C1q hemolytic activity and protein concentration. The absence of C1r activity was accompanied by a lower than normal C1s activity and C1s protein concentration. Such a concurrence of deficiencies could reflect direct linkage of synthesis of C1r and C1s or a more indirect influence of C1r on C1s synthesis or degradation. The finding that our patient's serum has

[4] Pickering, R. J., R. C. Herdman, H. Gewurz, H. J. Müller-Eberhard, and R. A. Good. C3 metabolism studies in patients with chronic glomerulonephritis. Unpublished observations.

increased C4 hemolytic activity and C4 protein concentration argues that the extreme deficiency of C1r and the less profound deficit of C1s are not consequent to activation of these subcomponents of C1. Instead, this marked increase in serum concentration of the component which is a natural substrate would be compatible with deficient activation of C1s by C1r and consequent reduction of normal activation of C4. Similarly, the unusually high level of C1-esterase inhibitor activity may result from an accumulation of this protein under conditions where a subcomponent deficiency precludes normal C1 activation and, therefore, reduces utilization of the inhibitor (20). However, it is important to note that C1-esterase inhibitor levels of similar magnitude have been reported by Donaldson (21) in patients with azotemia, hypertension, and normal hemolytic serum complement. Whatever its basis, the striking elevation of C4 protein concentrations and hemolytic activity in a patient with virtual absence of C1 hemolytic activity due to deficiency of C1r stands in contrast to the normal concentrations of later acting complement components.

Increased urinary excretion as a mechanism underlying the decreased serum concentration of complement proteins in renal disease has seemed the least likely possibility though recently this issue has been raised again (14).

It seems most likely then, that the C1 deficiency observed in this serum is related to an inability, either primary or secondary, of the patient to synthesize adequate amounts of C1r and probably C1s. The fact that C1q concentration remains normal in the face of a lack of detectable C1r function and reduction of C1s activity and C1s protein concentration suggests that at least two cell types are responsible for synthesis and possibly for assembly of macromolecular C1. Observations of a similar dissociation in serum from patients with lymphopenic hypogammaglobulinemia (22) and one patient with systemic lupus erythematosus (19) support this notion.

The concept of a macromolecular C1 molecule composed of three subcomponents requiring calcium ions in order to maintain functional and structural stability was first proposed by Lepow et al. (1) and Naff et al. (2). Since that time the separation of the molecule into its components has remained an in vitro phenomenon dependent on the chelation of calcium ions by EDTA. Studies of this C1r-deficient serum indicate that in the relative absence of this subcomponent, substantial amounts of the remaining two are present in the serum, apparently independent of each other. The observations presented here support the original concept of Lepow et al. (1) and Naff et al. (2) by showing that, even in the presence of calcium ions, biologic alterations can lead to conditions which favor dissociation or inefficient assembly of the macromolecule with resulting deficient hemolytic activity. It appears that all subcomponents are not only essential for C1 function, but that adequate amounts of normal C1r are essential for assembly and for maintenance of the macromolecular structure.

Whether C1r protein is absent from this serum, present in a nonfunctional

form, or in amounts too small to be detected by present functional assays remains to be determined.

SUMMARY

The experiments presented here utilize a human serum markedly deficient in hemolytic complement activity to show that: (*a*) The hemolytic deficiency is the result of a selective deficiency in hemolytic C1. (*b*) The relative absence of hemolytic C1 is due to a profound deficit in C1r function associated with less than normal C1s protein and hemolytic function and normal C1q protein concentration and function. This deficit in C1r in the face of normal C1q suggests that different cell types are responsible for the synthesis of each of these components. (*c*) Whatever the basis for the deficiency of C1r function, this defect results in an inadequate association of the remaining C1 subcomponents, C1q and C1s, even in the presence of calcium ions, thus suggesting that C1r has an important role in the assembly and/or maintenance of macromolecular C1.

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