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Direct Demonstration and Quantitation of the First Complement Component in Human Serum

Abstract. *The first component of complement, C1, can be demonstrated and quantitated in normal and pathological human serums by simple immunochemical techniques. All of the C1q, C1r, and C1s detected in normal serum was found to be in the C1 complex. A simple modification of these methods permitted the quantitation of free C1s in the presence of macromolecular C1, a technique which may prove useful in screening pathological serums.*

Earlier studies have indicated that the activity of the first component of complement, C1, requires the simultaneous presence of three proteins termed C1q, C1r, and C1s (*1–4*). Analyses of human serums by ultracentrifugation in sucrose density gradients have shown that these three proteins migrate together in a peak possessing C1 activity which is heavier than any of the individual subunits (*5*). Similar results were obtained on analyzing C1q, C1r, and C1s in normal serum by other techniques (*6*). Mixtures of isolated C1q, C1r, and C1s behaved similarly (*7, 8*). These data indicate that C1 consists of a complex of C1q, C1r, and C1s. It has not been possible, however, to directly demonstrate the C1 complex in human serum, nor has it been feasible to quantitate C1 except by activity assays requiring specialized reagents. Furthermore, it is not clear whether there are significant concentrations of free C1 subunits in normal human serum. We show here that the C1 complex can be directly demonstrated in human serum by immunochemical techniques. Minor modifications of these techniques permit quantitation of the C1 complex as well as free subunits in normal and pathological human serums.

Methods employed for the purification of C1q, C1r, and C1s and for the characterization of monospecific antiserums to each have been published (*2–4*). Titrations of the hemolytic activity of C1 in human serum were carried out as described (*7*). Normal human serum was obtained from healthy donors and used when fresh or frozen as specified in the text. Immunodiffusion analyses were performed in agarose in a buffer containing either calcium or ethylenediaminetetraacetic acid (EDTA) and at an ionic strength of 0.6 times the physiological strength, that is, conductance 8.4 mmho/cm. Single radial immunodiffusion studies were performed in agarose containing an appropriate amount of antibody to one or more of the C1 subunits. All immunodiffusion studies described in this report were carried out for 48 hours at 4°C. Diffusion in the cold was essential because C1 became activated and produced different patterns when the studies were performed at 22°C (*9*).

Antiserums to C1q, C1r, and C1s gave a single continuous fused precipitin line when diffused at 4°C against fresh normal human serum in immunodiffusion plates containing calcium (Fig. 1A). This pattern, which indicates that the C1q, C1r, and C1s antigens are on the same molecule, was observed with all normal human serums examined. The C1 complex demonstrated in this manner was not altered by repeated freezing and thawing of serum, and it was also observed in serum incubated for 1 hour at 37°C, stored for a week at 4°C, or frozen for a year at −20°C. A strikingly different pattern was observed when immunodiffusion analyses were performed in the presence of EDTA (Fig. 1B). Under these conditions, the C1 complex completely dissociated as shown by the crossing precipitin lines obtained with the various antiserums. This also indicates that the antiserums normally detect non-cross-reacting antigens.

The above studies indicated that the C1 complex could be detected in human serum by immunodiffusion. This result enabled us to employ single radial immunodiffusion to quantitate serum C1. For these studies, approximately equivalent amounts of antiserums to C1q, C1r, and C1s were incorporated into the agarose. The single ring of precipitation observed (Fig. 1C), which is the equivalent of the continuous line observed in the above Ouchterlony studies, represents C1. In accord with this interpretation, multiple precipitin rings were observed when the serums were analyzed in the presence of EDTA (Fig. 1D).

Eight normal serums and two normal serum pools were examined for their C1 content by single radial immunodiffusion. For purposes of quantitation

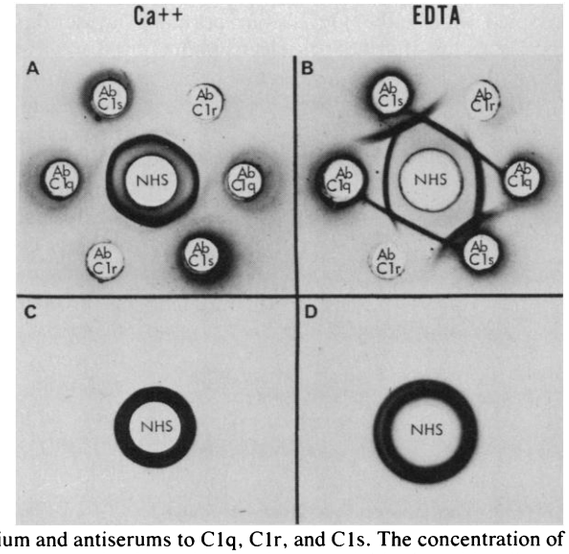

Fig. 1. Immunodiffusion analyses of C1 in normal human serum (*NHS*). Analyses were carried out in agarose containing barbital-buffered saline, *p*H 7.2, 0.6 times physiologic ionic strength for 48 hours at 4°C. Diffusion at reduced ionic strength helped to prevent C1 dissociation. (A) Double immunodiffusion analyses in 0.8 percent agarose containing 2.5 m*M* calcium. The single fused precipitin line indicates that C1q, C1r, and C1s are on the same molecule. (B) Double immunodiffusion analysis of serum containing 10 m*M* EDTA in 0.8 percent agarose containing 10 m*M* EDTA. Dissociation of the C1 complex is evident. (C) Single radial immunodiffusion in 1 percent agarose containing 2.5 m*M* calcium and antiserums to C1q, C1r, and C1s. The concentration of each antiserum used was chosen on the basis of its ability to give a precipitin ring with a diameter two to three times that of the well when reacted with physiological concentrations of the corresponding isolated antigen in single radial immunodiffusion analyses. The single ring of precipitation produced by the C1 complex in serum is evident. (D) Single radial immunodiffusion of serum containing 10 m*M* EDTA in 1 percent agarose containing 10 m*M* EDTA and antiserums to C1q, C1r, and C1s. The same concentrations of antiserums as in (C) were used. Multiple precipitin rings indicating dissociation of C1 are evident.

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the square of the diameter of the ring observed with each serum was expressed as the percentage of the mean of the square of the diameters of the eight serums (Table I). This was considered a valid technique since the squares of the diameters obtained with dilutions of serum yielded a straight line when plotted against the respective dilutions. Concentrations of C1 were also determined by hemolytic titration (Table 1). There was reasonable, although not exact, correlation between the results obtained by the two techniques.

To further demonstrate that macromolecular C1 was quantitated by immunodiffusion we conducted similar studies using agarose containing antiserum to only one of the subunits. Essentially the same results were obtained. Thus for serum RZ, values of 112, 114, 108, and 108 percent of the normal pool were obtained in single radial diffusion analyses in agarose containing antiserum to C1q, C1r, C1s, and antiserums to all three subunits, respectively. A similar close correlation was obtained with other serums.

We performed the following experiments to determine whether immunodiffusion could be used to analyze serum for free C1 subunits in the presence of macromolecular C1. To simulate this situation, purified components were added to normal human serum. No change in the double immunodiffusion pattern obtained with antiserums to C1q, C1r, and C1s was observed when C1q (75 μg/ml) or C1r (45 μg/ml) were added to serum. In contrast, added C1s subunits could be detected in serum, as shown by a line of precipitation in addition to that representing macromolecular C1. Usually this line resembled a spur emerging from the C1 precipitin line. In accord with this interpretation, single radial immunodiffusion studies revealed a C1s precipitin ring separate from that of C1 when high concentrations of antiserums to C1q and C1r and a low concentration of antiserum to C1s were incorporated into the agarose (Fig. 2). This modification was for the purpose of confining macromolecular C1 to the region around the application well. When 10, 20, and 30 μg (per milliliter) of purified C1s were added to serum, single radial immunodiffusion quantitative analyses performed in the above manner detected 13, 22, and 33 μg/ml, respectively (Fig. 2). Other studies showed that as little as 2 μg of added C1s per milliliter could be detected by these techniques.

Our studies show that C1 can be directly detected and quantitated in normal human serum by simple immunochemical techniques. The C1 concentrations determined by single radial immunodiffusion correlated reasonably well with those obtained by standard hemolytic titrations. Little variation in C1 concentrations between different normal serums was observed whether they were measured by this technique or by helmolytic methods (Table 1). Furthermore, C1 was found to be stable after long periods of time stored in the frozen state, as well as after repeated freezing and thawing. The ability to quantitate C1 by an immunochemical technique will greatly facilitate measurements of this component in various clinical conditions. The technique is simple, reproducible, and can be performed in most laboratories because it requires no specialized equipment or reagents, such as are needed for the hemolytic methods usually used. Two of the required antiserums are commercially available while the third soon will be.

Table 1. Quantitation of C1 in serum. Precipitin ring size was determined by single radial immunodiffusion with a mixture of equivalent quantities of antiserums to C1q, C1r, and C1s in the agarose. ND, not determined.

Normal serum donor	Single radial immunodiffusion		Hemolytic titration	
	Precipitin ring size (mm^2)	Percentage of mean	C1 (effective molecules per milliliter)	Percentage of mean
R.Z.	26.0	108	1.07×10^{13}	108
K.A.	25.0	104	1.06×10^{13}	107
J.P.	24.5	102	1.13×10^{13}	114
B.B.	24.5	102	ND	ND
S.H.	23.5	98	1.04×10^{13}	105
B.M.	23.5	98	0.94×10^{13}	95
K.L.	22.1	92	0.78×10^{13}	79
T.G.	21.6	90	0.91×10^{13}	92
Serum pool*	24.0	100	ND	ND
Serum pool†	25.0	104	ND	ND
Mean	24.0	100	0.99×10^{13}	100
Standard deviation	1.3		0.12×10^{13}	

*Pool of the above eight serums. †Pool of an additional ten serums.

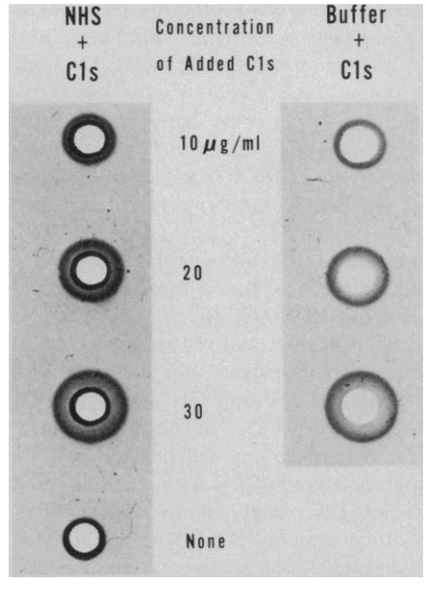

Fig. 2. Quantitation of free C1s in the presence of macromolecular C1 in normal human serum (*NHS*) by single radial immunodiffusion. Highly purified C1s was analyzed in various concentrations either alone or after addition to serum. Analyses were carried out in 1 percent agarose containing barbital buffered saline, *p*H 7.2, 0.6 times physiologic ionic strength, containing 2.5 m*M* calcium for 48 hours at 4°C. Approximately three times the amount of antiserums to C1q and C1r that were used in Fig. 1C and Table 1 were incorporated into the agarose, while the C1s antiserum concentration was unchanged. The dark precipitin rings at the application well are produced by macromolecular C1, while the larger rings are due to free C1s. It is evident that free C1s can be accurately quantitated in serum.

To determine whether free C1 subunits could be detected and quantitated by these techniques, we added isolated subunits to normal human serums. Double immunodiffusion studies indicated that free C1s could be readily detected in normal serum in the presence of macromolecular C1 because it produced a second precipitin line. As little as 2 μg of free C1s per milliliter could be detected. Added C1s could be also quantitated by single radial immunodiffusion when high concentrations of antiserum to C1q and C1r were incorporated in the agarose to confine macromolecular C1 close to the application well (Fig. 2). Added C1q and C1r could not be detected by these immunodiffusion methods for reasons that are unclear at present.

Although we were readily able to detect added C1s, our studies provided no evidence for the presence of free C1s in untreated normal human serums. This is in contrast to the electrophoretic studies of Laurell *et al.* (*6*) and our own ultracentrifugal analyses (*7*). In both of the studies evidence for free C1s, or possibly C1r-C1s complexes, was obtained. In the

present experiments C1 in serum was subjected only to the forces of diffusion, whereas in the cited studies (*6, 7*) additional stresses on the molecule, either electrical or centrifugal, were imposed. Since our earlier work has indicated that C1 has a tendency to dissociate (*7*), we think that our current studies, which indicate no free C1 subunits, are more representative of the status of C1 in normal serums. It is, however, quite likely that free C1 subunits will be found in serums from patients with various diseases, and some evidence for this has been obtained by Laurell *et al.* (*6*). The techniques presented here will permit a careful analysis of this possibility.

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Diurnal Rhythm: Effects on Hepatic Regeneration and Hepatic Regenerative Stimulator Substance

Abstract. *The effect of a controlled lighting schedule on the activity of a weanling rat liver extract that stimulates DNA synthesis in regenerating adult rat liver, and on the response of the test animals to the extract, has been investigated. Both activity of the extract and endogenous DNA synthesis in the weanling animals follow the same distinct diurnal rhythm. Reversal of the lighting schedule reverses the rhythm of endogenous DNA synthesis but activity of the extract no longer correlates with the peak of DNA synthesis. Diurnal rhythm also has a striking effect on DNA synthesis in the regenerating test animal, but the extract increases DNA synthesis to the same relative degree, regardless of the time of day the hepatectomy is performed.*

The mammalian liver possesses a remarkable capacity for hypertrophy and hyperplasia in response to a variety of stimuli (*1*). However, the mechanism or mechanisms that control the growth spurt have remained elusive (*2*).

An extract of weanling rat liver can stimulate incorporation of [methyl-^{3}H]-thymidine ([^{3}H]Tdr) into the DNA of regenerating adult rat liver (*3*). This hepatic regenerative stimulator substance (SS) was demonstrable in adult rat liver only following partial hepatectomy, when it appeared prior to the first wave of DNA synthesis and remained demonstrable for 3 days after the hepatectomy. The SS was heat-stable and acid-labile, was not dialyzable, and had a molecular weight of about 10,000 (*3, 4*).

Both normal and regenerating rodent liver display distinct diurnal rhythms of mitotic activity and [^{3}H]Tdr incorporation into DNA (*5*). Barbiroli and Potter (*6*), by controlling the periods of light and food availability, have demonstrated the existence of at least two different regulatory systems for DNA synthesis in the regenerating rat liver. Synthesis was maximal 23 hours after hepatectomy, regardless of the time of day hepatectomy was performed. However, this phenomenon was superimposed on a constant underlying rhythm of endogenous DNA synthesis that was entrained to the light: dark and feeding schedule.

We have examined the relationship of diurnal rhythms to the presence of SS in the donor animal and the responsiveness of the test animal. Male Sprague-Dawley rats were used as donors (50- to 60-g weanlings) and test animals (170- to 190-g adults). All experimental animals were caged in a windowless, air-conditioned room with lighting controlled by a timer to provide darkness from 7:00 p.m. to 7:00 a.m. For some experiments, lighting was reversed to provide darkness from 7:00 a.m. to 7:00 p.m. Food and water were freely available, and all animals were acclimatized to one of the lighting regimens for 7 to 10 days before experimentation.

To determine any diurnal variations in the presence of SS, it was prepared at 3-hour intervals over a 24-hour period from donor weanling rats. A modified extraction method produced a more purified and stable extract than that previously reported (*3*). Livers were removed from donor animals and rapidly cooled in iced saline. The pooled livers were homogenized in 0.9 percent NaCl (35 percent, weight to volume) in a chilled Omnimixer (Sorvall). The homogenate was heated at 65°C for 15 minutes in a water bath and centrifuged at 27,000*g* for 10 minutes. The supernatant solution, designated SS, was lyophilized and stored at −20°C until assayed for stimulator activity.

The assay uses 170- to 190-g male Sprague-Dawley rats, housed under the same "normal" conditions (darkness from 7:00 p.m. to 7:00 a.m.) as test animals. Between 1:00 and 2:30 p.m., the left-lateral lobe of the liver was removed from the test animals under light ether anesthesia (about 34 percent hepatectomy). Lyophilized SS was reconstituted to its original volume with water, and at 4 to 6 hours after the hepatectomy, 5 ml was injected intraperitoneally into the test animals. At 23 hours after the hepatectomy, the animals were killed, the livers were removed, and [^{3}H]Tdr incorporation into liver DNA of each rat was determined in vitro in a tissue slice assay (*3*). Liver DNA was extracted and quantified by measuring absorbance at 260 nm (A_{260}) (*7*). Incorporation of [^{3}H]Tdr was determined by adding a portion of the purified DNA to Aquasol (New England Nuclear) and assaying the radioactivity in a scintillation spectrometer. Data are expressed as disintegrations per minute (dis/min) per *A* unit of DNA. Control animals underwent an identical operative procedure except for the actual removal

Table 1. Diurnal variation in response to partial hepatectomy and injection with hepatic regenerative stimulator substance (SS). Data are expressed as means ($\bar{X}$) and standard errors of the mean (S.E.M.) and were analyzed with *t*-tests.

Time of operation	Incorporation of [^{3}H]Tdr (dis/min per *A* unit of DNA)						*P*
	Saline injection			SS injection			
	N	$\bar{X}$	S.E.M.	*N*	$\bar{X}$	S.E.M.	
9:00 to 11:00 a.m.	12	3860	555	16	9633	1658	< .01
1:30 to 3:00 p.m.	56	804	56	86	1994	210	< .01

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