DEMONSTRATION AND QUANTITATION OF ACTIVATION OF THE FIRST COMPONENT OF COMPLEMENT IN HUMAN SERUM*

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The first component of human complement (C1)[1] is comprised of three glycoprotein subunits, C1q, C1r, and C1s, held together in a calcium-dependent complex (1–3). The interaction of the C1q subunit with immune complexes triggers a series of intramolecular changes in the C1 molecule leading to the conversion of C1r and C1s from inactive precursor molecules to active proteases, thus initiating the classical complement pathway, and with it the numerous biological manifestations of complement (4–8). Although physicochemical changes in the individual subunits have been documented (9–12), the correlates of these events in macromolecular C1 have not been thoroughly analyzed.

We have recently reported that macromolecular C1 as well as free C1s can be directly demonstrated and quantitated in human serum by single and double immunodiffusion techniques.[2] In the course of these studies it became apparent that activated C1 gave a very different precipitation pattern than native C1 with antisera to C1q, C1r, and C1s in double immunodiffusion analyses. The progressive changes in the C1 Ouchterlony pattern, which are due to C1 dissociation and also alterations in antigenicity of the subunits, are described here. Furthermore, it is possible to measure some of these alterations by single radial diffusion techniques and thus directly quantitate C1 activation in human sera.

Materials and Methods

Materials and Proteins. Methods for the purification and characterization of C1q, C1r, and C1s from human serum and for the production of monospecific antisera to each have been published (12–14). Fresh human serum was obtained from healthy donors. Bovine serum albumin was purchased from Calbiochem (San Diego, Calif.). IgG was isolated from the pseudoglobulin fraction of human serum (5 mM ethylene-diaminetetra-acetic acid (EDTA), pH 5.4) by ion exchange chromatography on diethylaminoethyl cellulose in phosphate-buffered saline at pH 7.3.

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[1] *Abbreviations used in this paper:* agg IgG, heat aggregated IgG; C1, first component of human complement; CH_{50}, dilution of serum giving 50% lysis of sensitized erythrocytes in the test system; EDTA, ethylene-diaminetetra-acetic acid; NHS, normal human serum.

[2] R. J. Ziccardi, and N. R. Cooper. 1978. Direct demonstration and quantitation of the first complement component in human serum. *Science (Wash. D. C.).* In press.

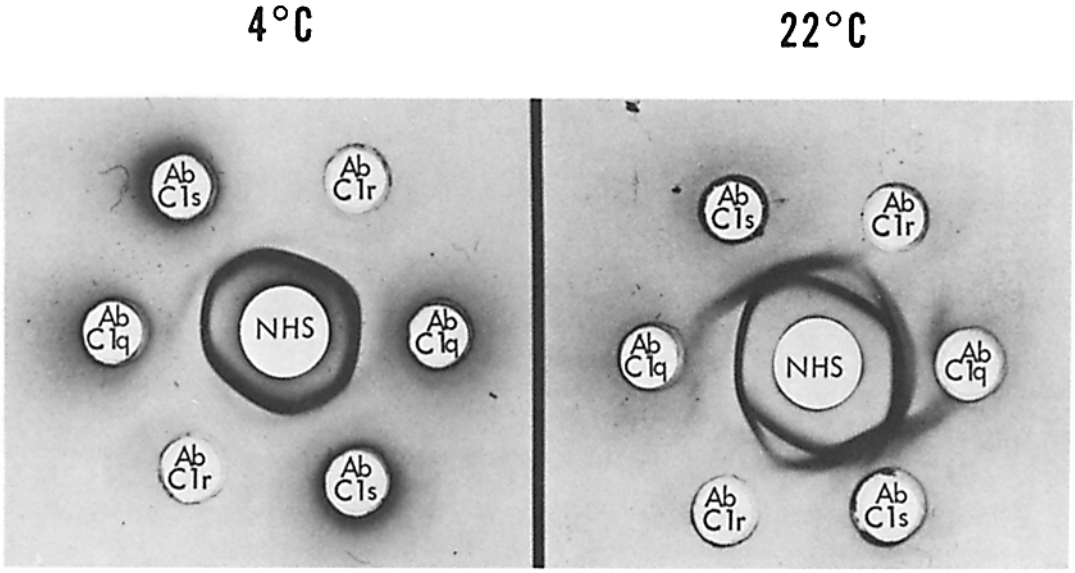

FIG. 1. Effect of temperature on the immunodiffusion pattern of C1 in human serum. Normal human serum was allowed to diffuse in agarose containing calcium for 48 h at 4°C (left panel) or 22°C (right panel). The single fused precipitin line observed at 4°C represents intact C1 as C1q, C1r, and C1s antigens are on the same molecule. A very different pattern was obtained at 22°C.

The IgG obtained was essentially pure judged by polyacrylamide gel electrophoresis in sodium dodecyl sulfate. Aggregation of IgG was achieved by incubation of a 10 mg/ml sample at 63°C for 20 min after which particulate aggregates were removed by centrifugation in a Beckman Microfuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.).

Agarose was purchased from Marine Colloids, Inc., Springfield, N. J., sodium barbital from Sigma Chemical Co., St. Louis, Mo., particulate inulin from Pfanstiehl Labs., Inc., Waukegan, Ill., and zymosan from Nutritional Biochemicals Corp., Cleveland, Ohio. Lipopolysaccharides from the heptose-deficient rough mutant Salmonella Minnesota R-595 was kindly provided by Dr. D. Morrison, Scripps Clinic and Research Foundation, La Jolla, Calif.

Hemolytic Assays. Titrations of the complement activity of serum (CH_{50}) and the hemolytic activity of C4 in human serum were carried out by published procedures (15, 16).

Immunochemical Analyses. Immunodiffusion analyses were performed in agarose in barbital-buffered saline, pH 7.2, 0.6 × physiological ionic strength containing 2.5 mM calcium as described earlier.[2] All immunodiffusion studies were carried out for 48 h at 4°C or room temperature as specified. 1 and 0.8% agarose was used for single radial and double immunodiffusion, respectively. The concentrations of anti-C1q, anti-C1r, and anti-C1s or mixtures thereof used in the single radial diffusion analyses were determined as described earlier.[2]

Immunoelectrophoresis was performed in agarose in barbital-buffered saline pH 8.6, 0.6 × physiological ionic strength, containing 2.5 mM calcium. Electrophoresis was carried out for 4 h at 20 mA per slide at 4°C.

Results

Activation of C1 in Serum during Immunodiffusion Studies at 22°C. As we have reported earlier, antisera to C1q, C1r, and C1s gave a reaction of identity when reacted at 4°C with normal human sera in double diffusion analyses in agarose containing calcium.[2] Grossly different Ouchterlony patterns were obtained, however, when identical studies were carried out at room temperature (Fig. 1). Under these conditions multiple crossing lines were

TABLE I
Serum Complement Activation by Aggregated IgG

Amount Agg IgG added to NHS	Percent complement consumption	
	C4	CH_{50}
mg/ml		
None	0	0
0.2	32	43
0.5	100	100
1.0	100	100
2.0	100	100

obtained with anti-C1q, anti-C1r, and anti-C1s. It seemed likely that the observed changes in the double diffusion patterns reflected activation of C1 secondary to interaction with agarose during the diffusion process at 22°C. Consistent with this interpretation, serum incubated for 5 h at room temperature with 50 mg/ml of agarose lost 55% of its CH_{50}, 36% of its C4, and 40% of its C2 activity, while serum not treated with agarose lost only 6, 4, and 9%, respectively, on incubation at room temperature. All values were compared to serum samples kept at 0°C for the same periods of time.

Double Immunodiffusion Analyses of Activated C1 in Human Serum. In these experiments, fresh human serum was treated for 60 min at 37°C with aggregated IgG to activate serum C1. A 10-fold range of activator input was examined. As shown in Table I, complement activation, as indicated by CH_{50} depletion, and C1 activation, as evidenced by C4 consumption, were evident after incubation of serum with 0.2 mg/ml of aggregated IgG. These samples were subjected to double immunodiffusion analyses at 4°C as depicted in Fig. 2. Whereas the control serum gave the typical fused precipitin line characteristic of C1 with anti-C1q, anti-C1r and anti-C1s, a markedly different pattern was observed on analyzing the sera containing activated C1 (Fig. 2). Changes in the precipitin lines representing C1 as well as those due to C1q, C1r, and C1s reacting with their respective antibodies were observed. The continuous C1 precipitin line produced by anti-C1q, anti-C1r, and anti-C1s reacting with C1 in serum disappeared as the dose of aggregated IgG increased. In the case of C1q, spurring of the line produced by anti-C1q over that generated by anti-C1r was evident by the second dose of aggregated IgG, 0.5 mg/ml, and the intensity of the spur increased further with the dose of aggregates. Simultaneously, the intensity of the precipitin reaction of C1r with its antiserum progressively decreased with increasing amounts of aggregated Ig and no clear C1r precipitin line was apparent by 2 mg/ml of aggregated IgG. Spurring of the anti-C1s precipitin line over the lines produced by anti-C1q and anti-C1r was evident with the lowest dose of aggregated IgG. The intensity of this spur increased with the addition of progressively larger amounts of aggregated IgG to the serum. Results very similar to these were obtained with another C1 activator, bacterial lipopolysaccharide R-595. Spurring of the C1s line was observed when serum was treated with 0.2 mg/ml R-595 and disappearance of the C1r line was evident with 2 mg/ml R-595. On the other hand, nonaggregated IgG (2 mg/ml), inulin (20 mg/ml), and zymosan (5 mg/ml) produced minimal if any changes in the C1 immunodiffusion pattern.

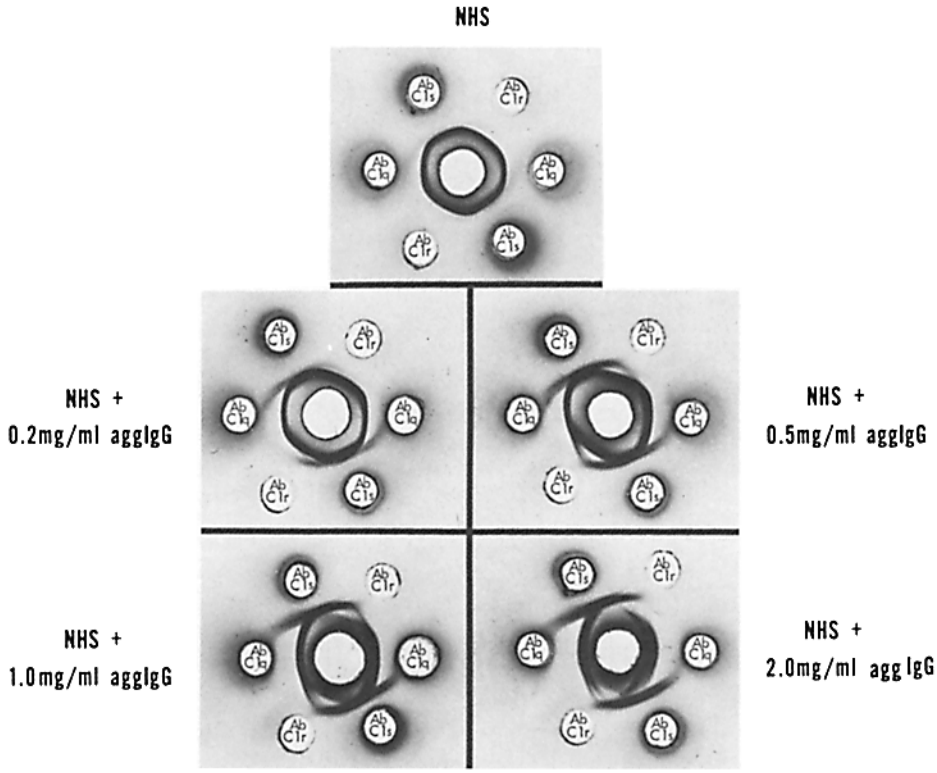

FIG. 2. Effect of activation on the immunodiffusion pattern of C1 in human serum. NHS was incubated for 1 h at 37°C alone or with various concentrations of heat aggregated IgG. The mixtures were subjected to double immunodiffusion analyses at 4°C in agarose containing calcium. Activation of C1 in serum was accompanied by a progressive change in the immunodiffusion pattern.

Dissociation of C1 in Human Serum with Activation. The most likely interpretation of the changing Ouchterlony pattern detailed above was that C1 was undergoing progressive dissociation with increasing doses of C1 activator. While single radial diffusion studies of untreated normal human serum (NHS) in agarose containing equivalent concentrations of antibodies to C1q, C1r, and C1s together yielded a single precipitin ring,[2] multiple rings (two to three) were observed in identical studies of serum treated with complement activators, strongly supporting the concept that C1 was undergoing dissociation.

Release of C1s from C1 in serum with activation was directly demonstrated by immunoelectrophoretic analyses of serum containing activated C1 in agarose in the presence of calcium (Fig. 3). Untreated normal serum or serum incubated with nonaggregated IgG showed a precipitin line with anti-C1s around the well of application, as is characteristic of native C1 in serum. After incubation with aggregated IgG, however, free C1s was detected in the serum (Fig. 3).

Quantitation of C1s Released with C1 Activation. C1s released during activation of C1 in serum was quantitated by single radial immunodiffusion analyses in agarose containing calcium. To differentiate free C1s from C1s in macromolecular C1, high concentrations of antibody to C1q were incorporated

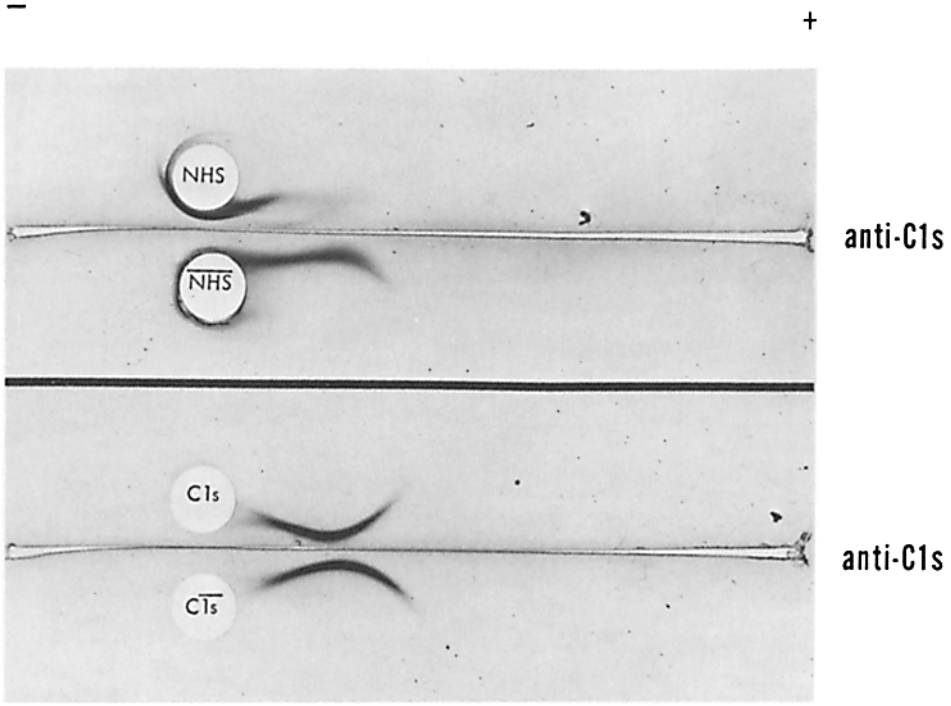

FIG. 3. Demonstration of release of C1s with activation of C1 in human serum. Human serum was incubated for 1 h at 37°C with 2 mg/ml of nonaggregated IgG (NHS) or aggregated IgG ($\overline{\text{NHS}}$), and then subjected to immunoelectrophoretic separation in agarose containing calcium. Simultaneously, C1s and $\overline{\text{C1s}}$, each at a concentration of 30 μg/ml, were analyzed. The pattern was developed with anti-C1s. Release of C1s with activation is evident.

into the agarose to confine the C1s in the C1 complex to a precipitin ring at the application well, while low concentrations of antibody to C1s were employed to quantitate free C1s. 13, 24, 32, and 39 μg/ml of free C1s were detected in serum incubated with 0.2, 0.5, 1.0, and 2.0 mg/ml aggregated IgG, respectively (Fig. 4). The highest activator concentration released all of the C1s from the C1 in this serum. No free C1s was detected in untreated serum or serum treated with the same doses of nonaggregated IgG.

Progressive Loss of C1r Antigenicity with C1 Activation. The double immunodiffusion studies described above demonstrate a diminution in intensity of the C1r precipitin line with the addition of increasing doses of aggregated IgG to serum (Fig. 2). Consistent with this finding, quantitative radial diffusion studies in the presence of EDTA showed a progressive reduction in serum C1r antigen content as greater amounts of activator were added to the serum (Table II). It should be noted that no change in C1r content was observed on addition to serum of the same concentrations of nonaggregated IgG. Thus the C1r content decreased from 79% of the untreated serum value with an activator dose of 0.2 mg/ml to 19% with a dose of 2.0 mg/ml. On the other hand, no change in total C1s content in the sera treated with the same doses of activator was observed (Table II). With the exception of the highest activator dose, C1q content also did not change. Therefore, C1 activation in serum can be detected

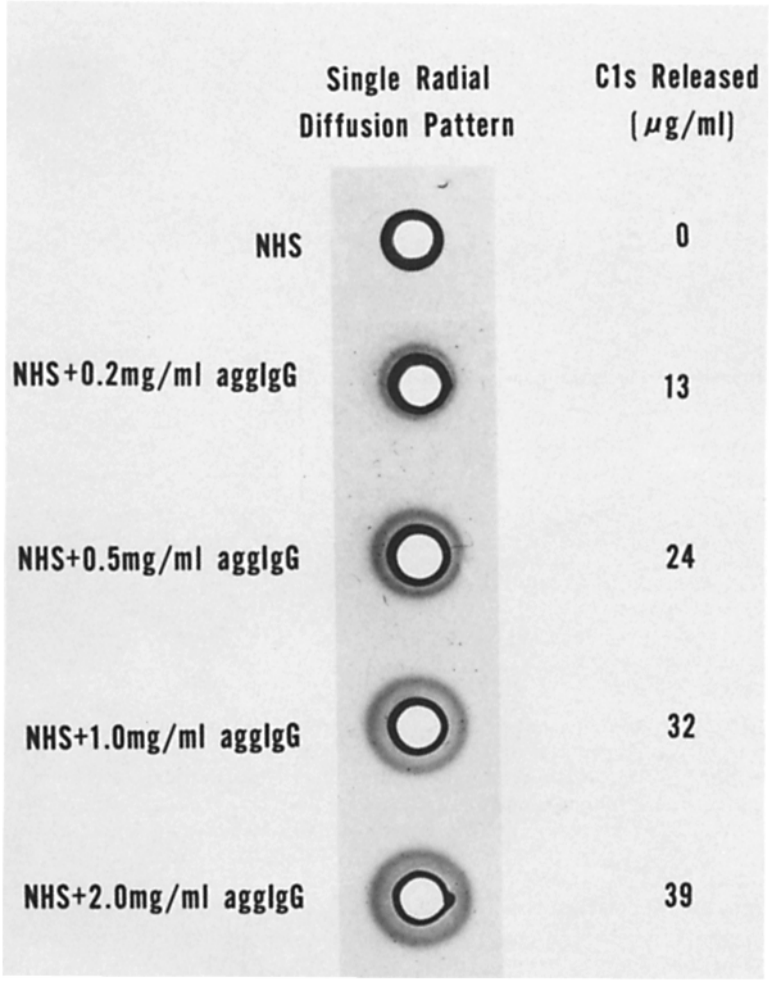

FIG. 4. Quantitation of C1s released with activation of C1 in human serum. NHS was incubated for 1 h at 37°C alone or with various concentrations of heat aggregated IgG. The mixtures were subjected to single radial immunodiffusion analyses at 4°C in agarose containing calcium and a high concentration of antibody to C1q to confine the intact C1 to a precipitin ring immediately surrounding the well of application. Low concentrations of anti-C1s were also incorporated into the agarose to permit quantitation of released C1s. The progressive release of C1s with increasing activator dose is evident (center column). The immunodiffusion plates also contained various amounts of isolated C1s (not shown) to serve as standards for quantitation of released C1s (right column). No free C1s was detected in untreated serum or serum treated with the same doses of nonaggregated IgG.

as a decrease in the C1r:C1s ratio from the value of 1.0 characteristic of normal serum (Table II).

Discussion

Macromolecular C1 can be detected in normal human serum as a continuous line of precipitation induced by anti-C1q, anti-C1r, and anti-C1s in double immunodiffusion studies performed at 4°C, as we have previously noted.[2] Very

TABLE II
Quantitation of C1q, C1r, and C1s in Human Serum Treated with Various Doses of Aggregated IgG

Amount agg IgG added to NHS*	Percent of untreated serum concentration‡			Ratio	
	C1q	C1r	C1s	C1q/C1s	C1r/C1s
mg/ml					
None	100	85	85	1.18	1.00
0.2	100	79	82	1.22	0.96
0.5	90	38	80	1.13	0.48
1.0	89	25	79	1.13	0.32
2.0	64	19	80	0.80	0.24

* All samples were incubated at 37°C for 1 h.
‡ Determined by single radial immunodiffusion in agarose containing EDTA (17).

different Ouchterlony patterns were observed when the diffusion studies were performed at room temperature (Fig.1). The double diffusion patterns at room temperature were found to be characteristic of activated C1 in serum and identical patterns were obtained at 4°C with serum previously incubated with complement activators such as aggregated IgG or R-595 lipopolysaccharide. In this regard agarose was noted to effectively activate C1 in serum at 22°C.

It is apparent that the patterns obtained with serum containing $C\bar{1}$ are similar to those obtained with serum containing C1 dissociated by addition of EDTA[2] although there are differences. Dissociation of C1q and C1s from C1 with activation is shown by the spurs of the precipitin lines produced by these components reacting with their respective antisera over that of C1 (Figs. 1 and 2). Release of C1s, as shown by the spur, is evident at the lowest activator dose examined (0.2 mg/ml) while dissociation of C1q is only apparent with higher activator doses, suggesting that C1s is released at an earlier stage in the activation process than C1q. Interestingly, the C1q precipitin line does not penetrate or cross the precipitin line due to released C1s, presumably because the released C1q bound to the C1s-anti-C1s immune complex. Release of C1s from the C1 complex with activation was also demonstrated by immunoelectrophoretic studies (Fig. 3). Additionally, single radial immunodiffusion analyses of serum containing activated C1 yielded multiple rings in agarose containing antisera to C1q, C1r, and C1s. Although dissociation of C1 with activation has not previously been directly demonstrated, Laurell et al. found C1r and C1s with various electrophoretic mobilities in the sera of patients with certain diseases (18), which is consistent with dissociation. Bartholomew and Esser postulated dissociation with activation as they were unable to find $C\bar{1}$ in sucrose density gradient analyses of activated serum.[3]

Perhaps the most surprising change in the Ouchterlony pattern of serum containing activated C1 is the progressive decrease in intensity of the C1r precipitin line with the addition of increasing amounts of aggregated IgG (or R-595) to serum (Fig. 2). In fact, no clear cut C1r precipitin line was evident on

[3] R. M. Bartholomew, and A. F. Esser. 1977. Exchange of radiolabeled C1s into serum C1 and its application in a simplified activation assay. *J. Immunol.* In press.

addition of aggregated IgG to a concentration of 2 mg/ml. There are four possible explanations for the loss in C1r antigenicity with activation. First, C1r after activation may become bound to the activating substance and thus be unable to diffuse into the agarose. This hypothesis requires that activated C1r bind firmly to each of the activators examined, i.e., aggregated IgG, LPS-R-595 and agarose, as loss of C1r antigenicity was observed with each. A second possibility is that activated C1r, like C1q and activated C1s, is released during C1 activation but then spontaneously aggregates, thus escaping detection with anti-C1r. Isolated C1r converted into $C\overline{1r}$ by incubation at 37°C does in fact aggregate (R. J. Ziccardi and N. R. Cooper, unpublished observations). Third, the antigenicity of C1r may be lost because of structural rearrangements following the peptide bond cleavage which occurs with C1r activation (9). However, C1r and $C\overline{1r}$ formed by incubation of C1r at 37°C (9) give a reaction of identity using the same anti-C1r in double immunodiffusion analyses in agarose containing EDTA with no trace of spurring and there is also no evidence of a reduction in intensity of the precipitin line of $C\overline{1r}$ reacting with the antiserum (unpublished observation). Nevertheless, it is possible that further changes occur in the C1r molecule with activation of C1 in serum leading to the production of a form of $C\overline{1r}$ different from that which was generated in vitro. A fourth possibility is that C1r antigenicity becomes completely masked by C1q, C1s, or both in $C\bar{1}$. This is not likely, as C1r antigenicity in $C\bar{1}$ is also not apparent on performing the studies in EDTA (Table II) which dissociates C1. A related possibility is that $C\overline{1r}$ binds to $C\bar{1}$ inactivator which completely masks its antigenicity. This interaction is known to be calcium independent. In this regard, Laurell et al. (18) felt C1r was present in a $C\overline{1s}$-$C\bar{1}$ inactivator complex in certain pathological sera although they were unable to show precipitation with antisera to C1r. Experiments are underway to differentiate between these hypotheses.

Although the explanation for the loss in $C\overline{1r}$ antigenicity with activation is not yet clear, this observation, along with the finding that C1s antigenicity does not change with activation, furnishes a practical approach to quantitating C1 activation in serum. The C1r:C1s molar ratio in normal human serum is known to be unity (3). A decrease in the C1r:C1s molar ratio, as determined by quantitative single radial diffusion studies, reflects the extent of activation (Table II).

An alternative approach to quantitating C1 activation in serum is based on the finding that C1s is released during the activation process (Fig. 3). Released C1s can be quantitated in the presence of macromolecular C1 by performing radial diffusion analyses in agarose containing high concentrations of anti-C1q, to confine C1 to the area around the application well, and lesser concentrations of anti-C1s to permit free C1s to produce a measurable ring of precipitation. The quantity of C1s released from C1 was found to be proportional to the dose of activator added to the serum (Fig. 4).

In another paper, techniques were presented which permit the quantitation of C1 and free C1 subunits in normal human sera,[2] while the present study describes two alternative approaches to the detection and quantitation of activation of C1 in normal human serum. Presently, intact C1 can be only measured by hemolytic techniques which involve specialized reagents and multiple steps (19) and activated C1 can be measured by more complex

hemolytic techniques (20) or by indirect approaches based on its ability to inactivate C2 and C4 (21). There is no practical published method to quantitate free C1 subunits in the presence of macromolecular C1. The techniques presented here and in the other paper[2] will thus be useful in analyzing the properties and levels of macromolecular C1 and certain aspects of the activation process.

The present approaches to quantitating C1, free C1 subunits and C1 activation in human sera have a potentially wider range of usefulness as they may be of value in following the status of the complement system in the serum of patients with certain diseases. Reduced levels of C1 are found in serum of patients with several diseases (22) and there is also evidence for the presence of free C1 subunits in some pathologic sera (18). de Bracco and Manni found several patients with decreased C1r:C1s ratios although a large pool of normals and pathological sera considered together in their studies yielded a constant C1r:C1s ratio (23). Circulating activated C1 is probably present in the serum of patients with complement consumptive process such as systemic lupus erythematosus as such sera frequently show strikingly reduced levels of C2 and C4 (22). The approaches presented here should permit a differentiation of disease related changes in synthesis of C1 subcomponents from consumptive processes. A reduction in the concentrations of C1q, C1r, and C1s with maintenance of an equimolar ratio would suggest a coordinate reduction in synthesis of the subcomponents while a selective reduction of the C1r level with no change in the C1q:C1s molar ratio would indicate activation.

It remains to be determined whether the techniques presented here will be of sufficient sensitivity to be of value in achieving the above aims with patients' sera. While these approaches appear to be relatively insensitive in detecting activation of C1 in serum induced by aggregated IgG, results obtained with this activator obviously cannot be extrapolated directly to pathologic sera containing immune complexes or other complement activators. Hopefully the techniques will prove to be of aid in monitoring the status of the complement system in patients.

Summary

Activation of the first component of human complement (C1) in human sera can be readily detected in double immunodiffusion studies with anti-C1q, anti-C1r, and anti-C1s as it produces a characteristic pattern quite different from that of precursor C1. Native macromolecular C1 gives a continuous line of precipitation with antisera to C1q, C1r, and C1s in double diffusion studies. After activation of C1 by incubation of serum with complement activators, three major changes occurred in the Ouchterlony pattern. First, spurring of the C1s precipitin line over that of macromolecular C1, indicating release of C1s from $\overline{C1}$, was observed with low doses of activator. Release of C1s was quantitated by single radial diffusion and shown to be complete with the highest activator dose examined. Second, C1q was released with larger activator doses as shown also by spurring of the precipitin line due to this component over the remaining macromolecular C1. Third, and most surprising, C1r antigenicity was progressively lost as the activator dose was increased and no C1r line remained with the highest dose of activator tested. This was not true

with C1s as there was no change in the total C1s concentration in serum incubated with various activator doses.

These observations provide two approaches to the quantitation of C1 activation in human serum. First, C1r and C1s can be quantitated by single radial diffusion. A decrease in the C1r:C1s ratio correlates with activation. Second, C1s released by the activation can be quantitated by single radial diffusion if the agarose contains high concentrations of anti-C1q to confine C1, also containing C1s, to the area near the application well, and lesser concentrations of anti-C1s to permit free C1s to produce a measurable ring. The extent of release of C1s also correlates with activation.

These immunochemical techniques to quantitate C1 activation directly in serum do not require specialized reagents. It is hoped that they will be useful in screening pathological sera and in monitoring the status of the complement system in patients.

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