ACTIVATION OF THE CLASSICAL PATHWAY OF COMPLEMENT BY HAGEMAN FACTOR FRAGMENT*

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There is a striking similarity between the mechanisms involved in the activation of the complement system and those of the Hageman factor-dependent pathways that lead to blood coagulation, fibrinolysis, and kinin formation Both systems require an initial contact with an activator which in turn triggers a cascade of reactions involving the sequential interactions of a series of enzymes and regulators. Furthermore, certain proteins seem to operate as regulators of both systems. The serum C1 inactivator (C1-INA),[1] an inhibitor of activated C1 that is absent in patients with hereditary angioedema (1), is known to inhibit the activities of plasmin (2, 3), activated Hageman factor (2–5), anaphylatoxin inactivator (2), Factor XIa (6), kallikrein (2, 7), C1r, and C1s (2, 3) through the formation of stoichiometric complexes. In the absence of C1-INA, activated Hageman factor may activate the kinin system by conversion of prekallikrein to kallikrein, which in turn cleaves bradykinin from high molecular weight kininogen. Since bradykinin is a potent mediator of enhanced vascular permeability, the pathogenesis of hereditary angioedema may involve interactions between the Hageman factor-dependent pathways and the classical pathway of complement activation. Previous evidence has suggested that C1 activation in this disease is Hageman factor dependent (8). Plasmin has been shown to be capable of activating C1 (9) and may represent one such enzyme.

In the following report, we present evidence that Hageman factor fragment (HFf) efficiently activates the complement system via the classical pathway. Native Hageman factor is a single-chain β-globulin (80,000 mol wt) circulating in plasma in a zymogen form at a concentration of ~20–45 μg/ml (10). When activated, Hageman factor is converted to HFa (Factor XIIa), a serine protease composed of a heavy chain and a light chain held together by disulfide bonds (11). Another form of activated Hageman factor is the 28,000-mol wt fragment designated HFf (12). HFf is a weak coagulant enzyme because it lacks the binding site for initiating surfaces; on the other hand, it is a potent activator of prekallikrein (12).

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[1] *Abbreviations used in this paper:* C1-INA, serum C1 inactivator; C2D, serum genetically deficient in C2; EA, sheep erythrocytes sensitized with rabbit antibody; GPS-EDTA, guinea pig serum diluted 1:50 with 0.04 M EDTA-GVB; GVB, veronal-buffered saline containing 0.1% gelatin; HF, Hageman factor; HFa, activated Hageman factor; HFf, Hageman factor fragment; NHS, normal human serum; SDS, sodium dodecyl sulfate.

Materials and Methods

Buffers. The following buffers were used: VB, isotonic veronal-buffered saline, pH 7.4; VB^{++}, VB containing 0.15 mM $CaCl_2$ and 0.5 mM $MgCl_2$; GVB^{++}, VB^{++} containing 0.1% gelatin; GVB-E, VB containing 0.1% gelatin and 0.01 M EDTA; $SGVB^{++}$, GVB^{++} containing 2.5% (wt/vol) sucrose; Mg-EGTA, 0.1 M $MgCl_2$, 0.1 M EGTA adjusted to pH 7.0; and Mg-GVB, VB containing 0.5 mM $MgCl_2$ and 0.1% gelatin was prepared according to published procedures (13).

Purified Proteins. Macromolecular C1 was purified according to a procedure described by Nelson et al. (14), from serum that was made free of plasminogen by passage over a Lysine-Sepharose 6B immunoadsorbent column (15) equilibrated with veronal-buffered saline, pH 7.0, containing 0.15 M NaCl, 0.02 M sodium barbital, 0.01 M $MgCl_2$, and 3 mM $CaCl_2$. Highly purified Hageman factor (HF), activated Hageman factor (HFa), and Hageman factor fragment (HFf), were prepared according to the method of Silverberg et al. (16), which consisted of the following steps: dialysis of highly purified HF against 10 mM sodium phosphate, pH 8.0, containing 0.15 M NaCl, followed by digestion of HF with 4.0 IU of kallikrein activity/mg of HF for 16 h at 37°C in plastic tubes. The mixture was applied to a QAE-Sephadex A-50 equilibrated with sodium phosphate buffer, pH 8.0. Kallikrein was recovered in the effluent, and HFa and HFf were eluted with a NaCl concentration gradient, with 10 mM sodium phosphate buffer, pH 8.0, containing 0.6 M NaCl as limit buffer. C1 (17), C2 (18), C3 (19), C4 (20), and C5 (21) were isolated according to published procedures.

Purification and Aggregation of IgG. Human IgG was isolated from serum by a single-step chromatography on a column of DEAE Affi-Gel Blue, according to the manufacturer's specifications (Bio-Rad Laboratories, Richmond, Calif.). The column (3 × 25 cm) was packed with a total bed volume of 7 ml/ml of serum to be processed, and equilibrated with starting buffer containing 0.02 M K_2HPO_4 and 0.02% NaN_3 at pH 8.0. After dialysis against the same buffer for 10 h at 4°C, the serum was applied to the column and eluted with two bed volumes of starting buffer. The IgG-containing fractions (first peak) were pooled and concentrated.

Aggregation of such purified IgG was performed by incubation at 63°C for 20 min at 10 mg/ml concentration. Particulate aggregates were removed by centrifugation at 200 *g* for 5 min. The supernate was used as soluble aggregated IgG.

Antisera. Monospecific antisera to C1q, C1r, C1s, C2, C3, C4, and Factor B were raised in rabbits.

Immunochemical Analyses. Radial double or single immunodiffusion analyses were carried out in 0.8% agarose in barbital-buffered saline, pH 7.2, containing 2.5 mM $CaCl_2$ and 0.09 M NaCl at 4°C for 48 h as described (22). Immunoelectrophoresis was performed in 1% agar or 0.8% agarose in barbital-buffered saline, pH 8.6, containing 0.09 M NaCl and 2.5 mM calcium, at 4.7 V/cm at 4°C for 2 h. Sodium dodecyl sulfate (SDS) (9%) polyacrylamide gel electrophoresis was performed according to Laemmli (23).

Sera. Fresh human serum was obtained from healthy donors. Alternatively, serum was obtained from outdated plasma (purchased from Intercounty Blood Services, Melville, N. Y.) by clotting for 12 h at 4°C upon addition of $CaCl_2$ to a final concentration of 20 mM, and centrifugation at 10,000 rpm (Sorvall RC-5B, DuPont Instruments–Sorvall Biomedical Div., Newtown, Conn.). C2-deficient serum (C2D) was a generous gift from Dr. Max Hamburger of our laboratory. Guinea pig serum was made into a 1:50 dilution with 0.04 M EDTA-containing GVB (GPS-EDTA).

Preparation of Potassium Thiocyanate (KSCN)-Treated Serum. For titrations of C3 and C5, C6–C9 serum was prepared by incubation of guinea pig serum with an equal volume of cold 1 M KSCN for 20 h at 4°C (24, 25). After addition of hydrazine hydrate to a final concentration of 0.015 M, the mixture was incubated for 45 min at 37°C, dialyzed against veronal-buffered saline, pH 7.4, with several changes of buffer, and used in experiments.

Preparation of Complement-containing Cellular Intermediates. The preparation of sheep erythrocytes sensitized with rabbit antibody (EA), and complement-carrying EA, EAC1, EAC14 (17), EAC142 (26), and EAC1423 (27) has been previously described. Briefly, 5 ml of EA (1×10^9/ml) was incubated with 0.5 ml of C1 for 15 min at 37°C. The cells were washed once with warm GVB^{++} (kept at 37°C) and resuspended to original volume as EAC1. Then 1 ml EAC1 (1×10^9/ml) cells were further processed to form EAC4 by incubation with 100 μg of isolated

and fully active C4 for 30 min at 37°C, washed with 0.01 M EDTA-GVB, followed by two washes with GVB^{++} and resuspended in GVB^{++}. EAC14 cells for C2 titration were prepared by further incubation of EAC4 cells with C1 as described above. EAC142 cells were prepared by incubation of EAC14 cells with C2 for 10 min at 30°C and EAC1423 by incubation with C3 as described elsewhere (27).

Hemolytic Assays. Various concentrations of HF, HFf, or Hfa were tested for their effect on serum complement by incubation with 10 μl of normal human serum (NHS) for 60 min at 37°C. Then 0.2 ml of sensitized sheep erythrocytes (EA, 5×10^8/ml) were added, the volume was adjusted to 0.4 ml with 0.1% gelatin containing veronal buffer (GVB^{++}), and further incubated for 60 min at 37°C. The reaction was stopped by addition of 1.6 ml of cold GVB^{++}, the mixture was centrifuged, and free hemoglobin was measured in the supernate at 412 nM.

Determination of CH_{50} (28) and individual complement titrations of C1 (13), C2 (18), C3 (26), C4 (17), and C5 (25) were performed according to previously published methods.

Hemolytic Assay for the Alternative Pathway. To determine the effect of HFf on the alternative pathway of complement activation, C2D serum or NHS containing 5 mM Mg-EGTA was incubated with either 50 μg/ml HFf or GVB for 60 min at 37°C. Then 5×10^7 rabbit erythrocytes (E_R) were incubated with 25 μl of such treated serum for 60 min at 37°C in a total volume of 0.2 ml Mg-GVB. The reaction was stopped with 1 ml cold GVBE and the released hemoglobin was determined after centrifugation.

Kinetics of HFf-dependent Activation of Complement. NHS (1 ml) was incubated with 50 μg HFf for various lengths of time ranging from 0–60 min. Then 0.1-ml aliquots were removed at different time intervals and assayed for residual complement hemolytic activity as described above.

Results

Depletion of Complement Hemolytic Activity by HFf. In all our experiments, highly purified HF, HFa, or HFf were used. A representative preparation of HFf and HFa is shown in Fig. 1. NHS (10 μl) was incubated with or without increasing, equimolar concentrations of HF, HFa, and HFf for 60 min at 37°C. Then, 0.2 ml EA (5×10^8/ml) was added and further incubated for 30 min at 37°C in a final volume of 0.4 ml GVB^{++}. The reaction was stopped with 1.6 ml cold GVB^{++} and centrifuged, and the amount of hemolysis was determined spectrophotometrically at 412 nm. The data shown in Fig. 2 show that HFf was capable of abrogating complement hemolytic activity in a dose-dependent fashion. The reduction of hemolytic activity was maximal upon addition of 5 μg HFf. On the other hand, no activity was observed with equimolar concentrations of either the native form of HF, or the two-chain activated form of HF (HFa). Depletion of hemolytic activity as a function of time of incubation at 37°C is shown in Fig. 3. Maximal consumption of complement occurred at an incubation period of 60 min at a 50 μg/ml HFf. Fig. 4 depicts the result of a representative experiment in which NHS human serum (0.1 ml) was incubated with 5 μg HFf or 2 mg/ml aggregated IgG, which under similar conditions is known to deplete complement hemolytic activity (22). After incubation the mixture was made into a 1:100 dilution with GVB^{++}, and samples ranging from 0.5–1 ml were mixed with 0.2 ml EA (5×10^8/ml) in a total volume of 1.5 ml GVB^{++} and further incubated for 60 min at 37°C. The tubes were then centrifuged and the degree of hemolysis in the supernatant fluids was determined spectrophotometrically at 412 nm. The total hemolytic complement activity was found to be completely abrogated in the serum pretreated with HFf or aggregated IgG.

Effect of HFf on the Alternative Pathway of Complement. To determine the effect of HFf on the alternative pathway, C2D serum, or NHS in 5 mM Mg-EGTA that had been pretreated with 50 μg/ml HFf, or GVB was incubated with 5×10^7 rabbit erythrocytes

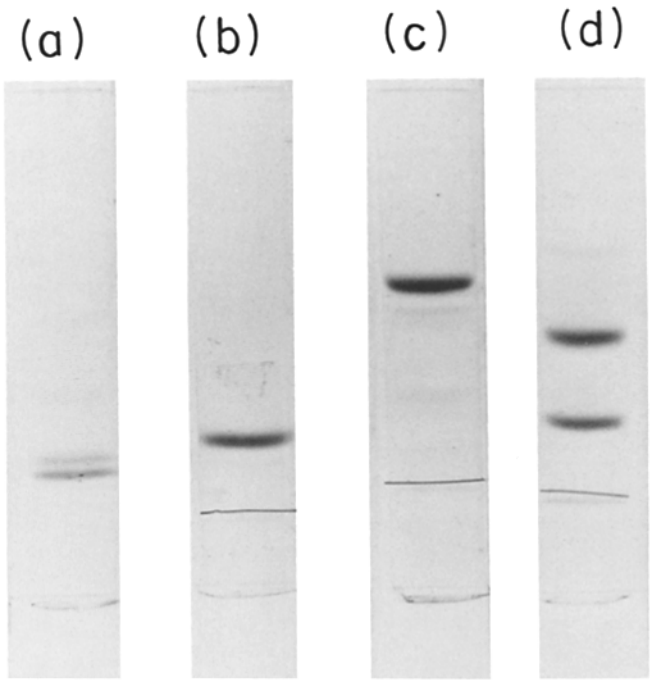

FIG. 1. SDS-polyacrylamide gel electrophoretic analysis of HFf (a and b) and HFa (c and d) in the absence (a and c), and in the presence (b and d), respectively, of 1% mercaptoethanol. HFf is typically a doublet under nonreducing conditions (a), but a single band is seen upon reduction. HFa upon reduction gives a characteristic heavy and light chain.

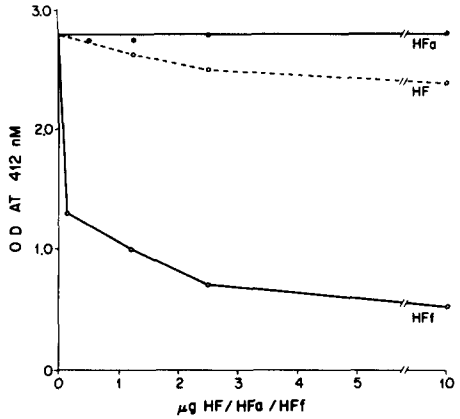

FIG. 2. Depletion of complement hemolytic activity after incubation with HFf. NHS (10 μl) was incubated with or without equimolar concentrations of HF, HFa, or HFf for 60 min at 37°C. Then, 0.2 ml EA (5×10^8/ml) was added and further incubated for 30 min at 37°C in a final volume of 0.4 ml GVB^{++}. After incubation, the reaction was stopped with 1.6 ml GVB^{++}, centrifuged, and the percent hemolysis was calculated from o. d. at 412 nm.

for 60 min at 37°C in a total volume of 0.2 ml Mg-GVB. After incubation, the reaction was stopped by cold GVB-E and centrifuged, and the amount of hemoglobin released was determined spectrophotometrically. As summarized in Table I, pretreatment of C2D or NHS containing Mg-EGTA with HFf did not abrogate the hemolytic

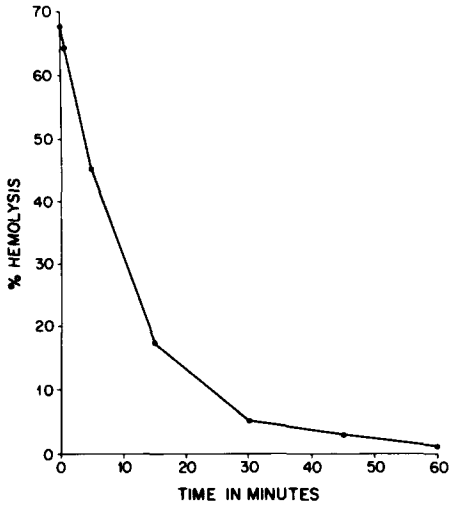

FIG. 3. Consumption of complement by HFf as a function of time of incubation. NHS (1 ml) was incubated with 50 μg HFf. At various time intervals, 0.1 ml was removed and assayed for residual complement hemolytic activity as described in Materials and Methods. Incubation was carried out up to 60 min.

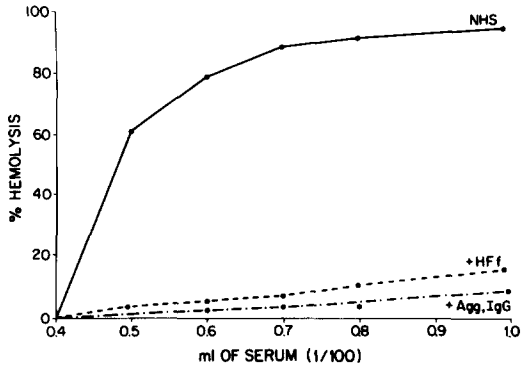

FIG. 4. Comparison of effects of aggregated IgG and HFf on serum complement. 0.1 ml of NHS was incubated with either HFf (5 μg) or aggregated IgG (2 mg/ml) for 60 min at 37°C. After incubation the mixture was made into a 1:100 dilution with GVB^{++}, and samples ranging from 0.5–1 ml were mixed with 0.2 ml EA (5×10^8/ml) in a total volume of 1.5 ml GVB^{++} and further incubated for 30 min. The tubes were then centrifuged and the degree of hemolysis was determined spectrophotometrically.

activity, indicating that HFf under these conditions does not initiate the alternative pathway of complement activation. In addition, Factor B or C3 in such treated serum were shown to be intact as assessed by immunoelectrophoretic analysis (data not shown).

Consumption of Individual Complement Components by HFf. To determine whether HFf abrogates the hemolytic activity of complement by sequential activation of the classical complement components or inactivation of individual components, titrations of C1, C2, C3, C4, and C5 were performed on serum (0.1 ml) that had been preincubated with 5 μg HFf for 60 min at 37°C. The results of representative titration experiments are shown in Fig. 5 for C1 and Fig. 6 for C4. Depletion of components

TABLE I

Effect of HFf on the Alternative Pathway

Reaction mixture	Lysis	Inhibition
	%	%
E_R + C2D	68	—
E_R + (C2D + HFf)	70	0
E_R + NHS-Mg-EGTA	75	—
E_R + (NHS-Mg-EGTA + HFf)	67	11

* C2D or NHS containing 5 mM Mg-EGTA was preincubated with 50 μg/ml HFf or GVB. Then 25 μl of such treated serum was incubated with 5×10^7 E_R for 60 min at 37°C. Residual hemolytic complement was then determined as described in Materials and Methods.

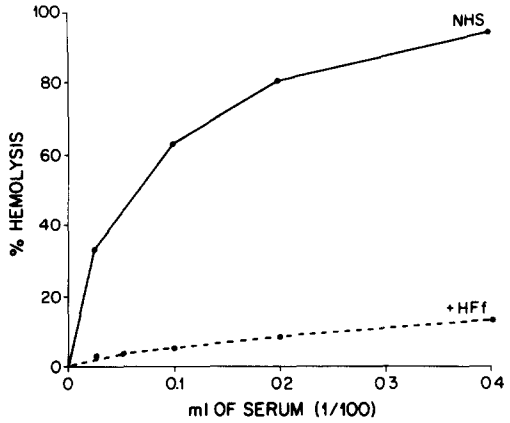

FIG. 5. Reduction of the activity of C1 in human serum after incubation with HFf. Different amounts of a 1:100 dilution, HFf-treated or nontreated serum was incubated with 0.1 ml EAC4 (5×10^8/ml) for 10 min at 30°C in a total volume of 0.5 ml $SGVB^{++}$. Then 0.05 ml of C2 (20 μg/ml) was added and incubated for 10 min at 30°C. The reaction mixture was further incubated for 60 min at 37°C after addition of 1:50 dilution of GPS-EDTA (GVB containing 0.04 M EDTA) hemolysis in supernate was determined spectrophotometrically at 412 nm after centrifugation.

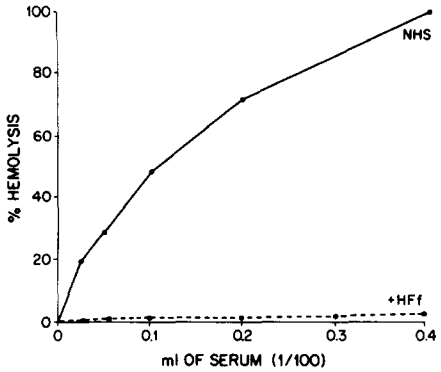

FIG. 6. Reduction of C3 hemolytic activity in serum after incubation with HFf. Experimental conditions were as those described in legend to Fig. 5 except that EAC1 (5×10^8/ml) was used.

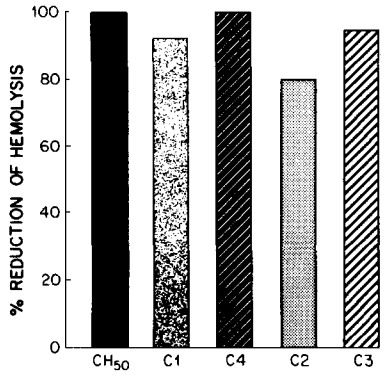

FIG. 7. Reduction of the activity of total complement and complement components in human serum after incubation with HFf. Experimental conditions are as described in Materials and Methods.

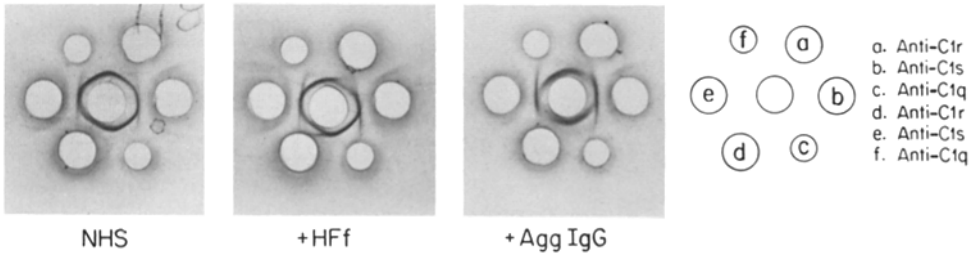

FIG. 8. Radial double immunodiffusion analysis of macromolecular C1 in serum incubated with either GVB^{++}, HFf, or aggregated IgG. Immunodiffusion analysis was carried out in 0.8% agarose in barbital-buffered saline, pH 7.2, containing 2.5 mM $CaCl_2$ and 0.09 M NaCl for 48 h at 4°C using specific antibodies to C1q, C1r, and C1s.

ranged from 99% for C1 and C4 and 91% for C3 as shown in Fig. 7. C5 was also depleted by 85% (data not shown).

In other experiments, the effect of HFf and aggregated IgG on C3 and C4 in serum was tested by immunoelectrophoresis. Human serum (0.1 ml) was incubated with either 5 μg HFf or 10 mg/ml aggregated IgG for 60 min at 37°C. After incubation, the volume was adjusted to 0.2 ml with GVB and immunoelectrophoresis was performed in 1% agar in barbital buffer, pH 8.6, for 2 h at 4°C. The ability of HFf to cleave C3 and C4 was analogous to that of aggregated IgG as judged by the shift in mobility and appearance of cleavage products (data not shown).

Effect of HFf on the C1 Macromolecule. The direct effect of HFf on macromolecular C1 was studied by the double immunodiffusion method using anti-C1q, anti-C1r, and anti-C1s as described by Ziccardi and Cooper (22). NHS (0.1 ml) was incubated with or without 30 and 60 μl of HFf (20 μg/ml) or 1 and 2 mg/ml aggregated IgG (10 mg/ml) for 60 min at 37°C. Immunodiffusion analyses of such treated NHS were then carried out in 0.8% agarose in barbital-buffered saline, pH 7.2, containing 2.5 mM $CaCl_2$ and 0.09 M NaCl for 48 h at 4°C. As shown in Fig. 8, an increase in HFf concentration resulted in the dissociation of C1, indicating activation of the C1 macromolecule. Neither HF nor HFa had any effect on C1 macromolecule. Similar experiments were performed using plasminogen-free macromolecular C1 and the results obtained (data not shown) were comparable to those found using NHS. In

addition, using C1q-depleted serum and purified C1s, an attempt was made to elucidate the mechanism of C1 activation. When C1q-depleted serum was incubated with HFf and analyzed by radial immunodiffusion techniques using antiserum to C1q, C1r, and C1s, no spur formation or disappearance of C1r antigenicity was observed, which indicates that C1 was not activated. Furthermore, incubation of C1s with HFf did not result in activation of C1s as assessed by immunoelectrophoretic analysis (data not shown).

Discussion

The interaction between the complement system and the cascade that leads to blood coagulation and kinin formation has been documented (28–30). Plasma kallikrein has been shown to inactivate C1 (31) and plasmin was found to activate it (9). The data presented here demonstrate that HFf activates the serum complement system in a manner that is entirely analogous to complement activation by antigen-antibody complexes. Upon addition of HFf to human serum, total complement hemolytic activity was depleted in a dose-dependent fashion (Fig. 2). The activity of the individual complement components was also found to be reduced (Fig. 7), with maximal depletion occurring at a dose of 50 μg of HFf/ml of serum added and an incubation period of 60 min at 37°C. That the activation of complement proceeded via the classical pathway is indicated by the ability of HFf to directly activate and dissociate the C1 macromolecule with concomitant release of C1q and C1s and disappearance of C1r antigenicity as assessed by single and double radial immunodiffusion studies using antisera to C1q, C1r, and C1s. Native, macromolecular C1 gives a continuous line of precipitation with antisera to C1q, C1r, and C1s in double diffusion as was previously shown by Ziccardi and Cooper (22). Upon incubation of serum with complement activators, however, spurring of the C1s precipitin line over that of macromolecular C1 occurred, indicating release of C1s from activated C1 (22). Furthermore, the hemolytic and immunoelectrophoretic experiments used demonstrated depletion of C2, C4, and C3. In addition, when the effect of HFf on the alternative pathway was directly investigated using C2D serum or NHS in the presence of 5 mM Mg-EGTA and rabbit erythrocytes, no reduction of hemolytic activity was observed. This suggests that HFf does not, under these conditions, initiate the alternative pathway of complement. In the HF-dependent pathway of blood coagulation, fibrinolysis, and kinin formation, the native molecule HF is bound to appropriate surfaces and activated, resulting in two cleavage products, HFa and HFf, both of which possess activity (10). In the present studies, however, neither the native molecule nor HFa was capable of depleting complement hemolytic activity.

Although kallikrein was used in the digestion of native HF to produce HFa and HFf, the preparations of HFf used in our studies were free of kallikrein (16) as assessed by SDS gel analysis and functional analysis using a variety of synthetic substrates (16). Furthermore, the sequential activation of C4, C2, and C3 suggests that activation, not inactivation, of C1 has taken place. It has been reported recently that the major effect of kallikrein on C1 is inactivation (31). The mechanism of this HFf-dependent complement activation is not yet clear. However, preliminary experiments using C1q-depleted serum have revealed that the intact C1 macromolecule may be required for C1 activation in serum. When C1q-depleted serum was incubated with HFf or GVB and then analyzed by radial double immunodiffusion techniques using monospecific

antisera to C1q, C1r, and C1s, no detectable spur formation or disappearance of C1r antigenicity was observed. In addition, incubation of highly purified C1s with HFf did not significantly shift the mobility of C1s as assessed by immunoelectrophoretic analysis (B. Ghebrehiwet, manuscript in preparation).

The biological implications of the described phenomena could be of great significance in view of the participation of the complement system and the HF-dependent pathways in the production and release of mediators of vascular permeability. HF plays a pivotal role in the activation of plasma prekallikrein to kallikrein which in turn cleaves high molecular weight kininogen to produce bradykinin, a potent mediator of vascular permeability (10). Activation of the complement system also leads to the production and release of vasoactive peptides C3a, C4a, and C5a, which constitute the complement-derived anaphylatoxins (32). In addition, a heat-stable kininlike peptide which has the ability to induce vascular permeability has been generated in the plasma of hereditary angioedema patients (HAE). In normal serum, $\overline{C1}$-INA, an inhibitor of activated C1, is also known to inhibit the functions of activated HF (4, 5). However, in sera of HAE patients which are known to be genetically deficient in $\overline{C1}$-INA (1), uninhibited activation of C1 may lead to the activation of the complement system elaborating thereby pathogenic peptide(s) which enhance vascular permeability. It has recently been reported by this laboratory (16) that HF autoactivated upon binding to surfaces if as little as one active molecule in 5,000 is present. The possibility of such autoactivation of HF in vivo cannot be determined at this point. However, it is possible that tissue injury initiates autoactivation of HF to release HFf, which in turn can activate the complement system. This kind of activation may thus represent one mechanism by which vasoactive peptides that play a role in HAE and other inflammatory reactions could be released. Furthermore, in patients with gouty inflammation, monosodium urate crystals are believed to initiate the pathogenesis of the disease (33, 35). In addition to activation of the classical pathway of complement, (35, 36), urate crystals have been shown to activate HF in human plasma and synovial fluid (37). That complement participates in the pathogenesis of gout has been reported previously (33, 34). In view of these findings, it is possible to postulate that activated HF may participate in the pathogenesis of this disease by either converting high molecular weight kininogen to produce bradykinin or activating the complement system to produce vasoactive peptides. Using highly purified C1q, C1r, and C1s, further studies are underway to elucidate the mechanisms by which HFf activates the complement system. More work is also necessary to elaborate on the biological implication of the described phenomenon.

Summary

A fragment of activated Hageman factor (HFf) has been demonstrated to activate the classical pathway of complement in a manner that is analogous to complement activation by antigen-antibody complexes or aggregated IgG. Thus C1, C4, C2, C3, and C5 were found to be depleted on addition of HFf to serum. The reduction of serum hemolytic activity was maximal upon addition of 5 μg HFf and an incubation time of 60 min at 37°C. Consumption of the total complement activity and of the individual components proceeded in a dose-dependent fashion. No comparable activity was observed when equimolar concentrations of either the native Hageman factor

(HF) or the two-chain activated form of Hageman factor (HFa) were incubated with serum. Further, the ability of HFf to convert serum C3 and C4 was similar to that of aggregated IgG as assessed by immunoelectrophoresis. This function of HFf appeared to be independent of plasminogen (or plasmin) since plasminogen-free serum was indistinguishable from normal serum. Radial double immunodiffusion experiments using antiserum to C1q, C1r, and C1s on HFf-treated serum demonstrated the dissociation of the C1 trimolecular complex, with concomitant reduction of C1r antigenicity that is indicative of C1 activation. Thus, HFf appears to lead to C1 activation upon incubation with serum or when incubated with partially purified C1. This may represent a control link between activation of the intrinsic coagulation-kinin pathway and the initiation of the classical complement cascade.

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