Biochemical Pharmacology, Vol. 51, pp. 797–804, 1996.

ISSN 0006-2952/96/$15.00 + 0.00
SSDI 0006-2952(95)02398-4

Investigation of Mechanism-Based Inhibitors of Complement Targeting the Activated Thioester of Human C3

*Arvind Sahu and Michael K. Pangburn**

DEPARTMENT OF BIOCHEMISTRY, UNIVERSITY OF TEXAS HEALTH SCIENCE CENTER, TYLER, TX 75710, U.S.A.

ABSTRACT. An intramolecular thioester bond in complement protein C3 is vital for covalent attachment of C3b (the proteolytically activated form of C3) to biological surfaces and for activation of the complement system. Proteolytic removal of C3a from C3 activates the thioester in the C3b fragment. Activated C3b primarily forms ester bonds with hydroxyl groups of carbohydrates on complement activating surfaces, but it has also been shown to react with the hydroxyl group of tyrosine and with specific Ser and Thr residues on IgG and on complement protein C4b. To examine the reactivity of the thioester, several families of hydroxylated compounds were examined. Reactivity of a series of substituted phenols varied over two orders of magnitude and demonstrated a linear correlation between reactivity and the Hammett substituent constants. Hydroxylated drugs including members of the L-DOPA/epinephrine family and hydroxamic acids also were examined. Compounds were identified that were 20,000 times more reactive than carbohydrates. These compounds were found to inhibit both the classical and alternative pathways of complement activation. Although the specificity of the thioester for its natural biological targets appears to be determined by many structural features, the data presented here demonstrate that increasing the nucleophilic character of the target hydroxyl group can increase the potency of a synthetic inhibitor many orders of magnitude. BIOCHEM PHARMACOL 51;6:797–804, 1996.

KEY WORDS. complement inhibitors; complement C3; autoimmune diseases; immune complex diseases; transplantation; ischemia; DOPA

The complement component C3† (M_r 186,000) plays a central role in the classical, alternative, and lectin [1] pathways of complement activation. Attachment of C3b (the proteolytically activated form of C3) to targets undergoing complement attack is required for all complement functions including generation of the anaphylatoxin C5a and formation of the C5–9 membrane attack complex. C3b attaches covalently to hydroxyl groups on carbohydrates and proteins on the surface of targets. Proteolytic activation of native C3 initiates attachment and results in the generation of a low molecular weight peptide C3a (M_r 9000) and an opsonic fragment C3b (M_r 176,000). Newly formed C3b possesses a short-lived (100 μsec), highly reactive, thioester bond [2, 3]. This bond is formed between Gln^{1013} and Cys^{1010} in C3 by a spontaneous post-translational modification [3, 4]. The increase in reactivity of the thioester during the activation of C3 to C3b has been estimated to be 10^{10}-fold [5]. The activated thioester of metastable C3b preferentially reacts via transesterification with hydroxyl groups rather than amino groups, resulting in ester bonds. Reaction of the carbonyl group of Gln^{1013} with hydroxyl groups on the receptive surface allows C3b to attach itself covalently to targets during complement activation.

An internal thioester bond is also present in complement protein C4 and in the protease inhibitor α_2-macroglobulin (α_2M). Unlike C3, α_2M primarily binds to amino groups [6]. Isotypes of C4 differ in their reactivity; C4A shows strong reactivity with amino groups, whereas C4B exhibits a preference for hydroxyl groups [7–9]. Although the structural basis for this specificity has been investigated, it is not yet defined [9–11].

Covalent binding of C3 to various carbohydrates and serum proteins (C3b, C4b, IgG_1 and CR2) has been examined in detail [12–20]. In one of our previous studies, we found that the thioester of metastable C3b displays a high degree of specificity for certain carbohydrates and for certain positions on these carbohydrates [12]. Metastable C3b has also been shown to react with specific Ser or Thr residues in proteins. In human IgG_1, we recently demonstrated that the acceptor site is the hydroxyl of Thr^{144} [15], and in human C4b others have shown that the site is the hydroxyl of Ser^{1217} [16]. In the present study, we examined the reactivity of the activated thioester of C3 with synthetic compounds in an effort to reveal more about the chemistry of the reaction at this site. The results demonstrate that compounds as much as 20,000 times more reactive than the natural targets, carbohydrates, were found easily and that some of these were natural metabolites, whereas others were drugs currently in use. These observations raise the ques-

* Corresponding author. Tel. (903) 877-7663. FAX (903) 877-5882.
Received 10 April 1995; accepted 12 October 1995.

† *Abbreviations:* C3, native, hemolytically active C3; ANS, 8-anilino-1-napthalenesulfonic acid; NHS, normal human serum; EA, sheep erythrocytes coated with antibodies; E_R, rabbit erythrocytes; VBS, veronal-buffered saline; GVB, VBS containing 0.1% gelatin; and GVBE, GVB containing 10 mM EDTA.

tion of whether inhibition of complement activation could play a role in the efficacy of some hydroxylated drugs.

MATERIALS AND METHODS

Purified Proteins

Complement protein C3 [21, 22] and Factors B [23] and D [24] were purified from human plasma as described. For C3b attachment inhibition experiments, C3 was repurified on a Mono S column (Pharmacia, Piscataway, NJ, U.S.A.) [22] and radiolabeled with ^{125}I using Iodogen (Pierce Chemical Co., Rockford, IL, U.S.A.). The specific activity of C3 was between 0.5 and 1.5 μCi/μg.

Buffers and Other Immunologic Reagents

GVB: 5 mM sodium veronal, 145 mM NaCl containing 0.1% gelatin, pH 7.4; GVB^{2+}: GVB with 0.5 mM $MgCl_2$ and 0.15 mM $CaCl_2$; GVBE: GVB with 10 mM EDTA; HBS: 20 mM HEPES, 140 mM NaCl, and 0.02% sodium azide, pH 7.4. ZymC3b was made by coating zymosan particles with C3b using C3, factor B, factor D and Ni^{2+} (approximately 10^5 C3b/zymosan) as described [12].

Inhibitors

Glucose, threonine, tyrosine, tyrosine analogues, L-DOPA, acetohydroxamic acid, salicylhydroxamic acid, and dopamine derivatives were purchased from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). Catechin hydrate and substituted phenols were from the Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). Dopamine was from Abbott Laboratories (North Chicago, IL, U.S.A.).

Hemolytic Assays

Inhibition of classical and alternative pathway-mediated lysis of erythrocytes was determined as described below. To define the effect of various inhibitors on the classical pathway, 160 μL of NHS (1:100 dilution) was mixed with 10 μL of EA (1×10^9/mL) and various concentrations of inhibitor in a total volume of 500 μL of GVB^{2+}. The reaction mixture was incubated for 1 hr at 37° and tubes were centrifuged. The percentage of lysis was determined by measuring the absorbance at 414 nm. The NHS used in these tests was pooled from several donors and contained approximately equal amounts of C4A and C4B. The alternative pathway-mediated activity was measured by incubating 7 μL of NHS with 10 μL of rabbit erythrocytes (1×10^9/mL) and various concentrations of an inhibitor in a final volume of 100 μL GVB containing 5 mM MgEGTA. The reaction mixture was incubated at 37° for 20 min, and lysis was stopped by adding 400 μL of GVBE. The percentage of lysis was determined as above. The data generated were normalized by setting 100% lysis equal to the lysis that occurred in the absence of an inhibitor.

C3b Attachment Inhibition Assay

Inhibition of C3b attachment to zymosan was performed as described [12]. In short, particles coated with C3 convertase (ZymC3b,Bb) were made by mixing 10^7 ZymC3b with 5.0 μg factor B and 0.2 μg factor D in 200 μL GVB containing 1 mM $NiCl_2$. The reaction mixture was allowed to incubate at 22° for 5 min and stopped by adding GVBE. The C3 convertase ZymC3b,Bb (10 μL) was mixed immediately with 10 μL of ^{125}I-C3 (0.08 μCi) and various concentrations of inhibitors in a total volume of 40 μL and incubated with mixing for 15 min at 37°. The reaction mixture was then centrifuged through 20% sucrose in GVBE to separate bound from free ^{125}I-C3b. Nonspecific binding was determined by incubating ^{125}I-C3 with zymosan lacking C3 convertase (ZymC3b). Data obtained was normalized by considering 100% bound equal to the amount of ^{125}I-C3b bound in the absence of an inhibitor. The binding data were plotted and fit by non-linear regression analysis (GraFit, Erithacus Software, Staines, U.K.) using the equation for single site inhibition:

$$\% \text{ C3b Bound} = 100/(1 + (\text{Inhibitor concn})/IC_{50}) \quad (1)$$

The concentration of inhibitor required to inhibit 50% of C3b attachment to zymosan is reported as IC_{50}. The reactivity of inhibitors relative to water was calculated as previously described [12]. Data are expressed as means ± SD of IC_{50}. Statistical analysis was done using a *t*-test ($N \geq 10$) and SigmaStat software (Jandel Scientific Software).

C3 Convertase Assay

The alternative pathway C3 convertase C3b,Bb activity was determined by employing a spectrofluorometric assay [25]. The enzyme was prepared with 4 μg C3b, 46 μg factor B, and 0.5 μg factor D in 25 μL VBS containing 5 mM MgEGTA at 22°. After 3 min of incubation, enzyme formation was stopped with 75 μL of 100 mM EDTA. Immediately thereafter, 75 μL of the above enzyme was added to a prewarmed cuvette containing 235 μg C3, 40 μM ANS, and the appropriate concentration of inhibitor in 1225 μL HBS at 37°. The change in fluorescence was recorded for 600 sec, and then 10 μg trypsin was added to convert the remaining amount of C3 to C3b. The enzyme activity was calculated by measuring the initial slope. The excitation wavelength was 386 nm with 4 nm band-pass and the emission wavelength was 472 nm with 4 nm band-pass. The fluorescence emission of ANS was measured in an SLM Aminco 8000C spectrofluorometer (SLM Instrument Inc., Rochester, NY, U.S.A.).

RESULTS

Reactivity of C3b with Tyrosine and Tyrosine Analogues

In a previous study [26], we showed that the reactivity of the thioester of metastable C3b with tyrosine and phenol was much greater than with its previously known natural targets: carbohydrates, serine, and threonine. The reactivity of hydroxylated compounds was assessed as described in Fig. 1. In

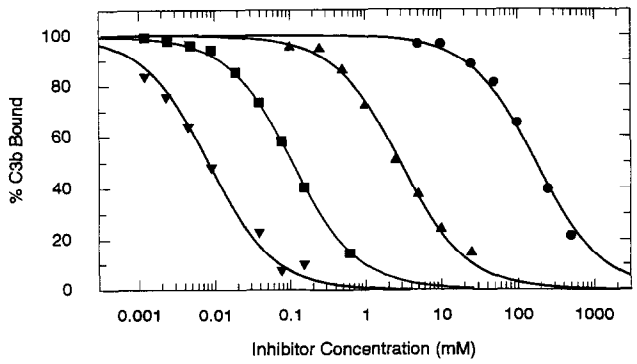


FIG. 1. Inhibition of ^{125}I-C3b attachment to zymosan. Zymosan particles coated with surface-bound C3 convertase (C3b, Bb) were incubated with ^{125}I-C3 and graded concentrations of inhibitors for 15 min at 37°. Bound and free C3b were separated by centrifugation of particles through 20% sucrose. The data were normalized by setting 100% bound equal to the average binding of C3b in the absence of an inhibitor. Binding curves were generated by non-linear regression analysis using the software GraFit and equation 1 for single site competitive inhibition. Key: (●) glucose; (▲) phenol; (■) catechin hydrate; and (▼) salicylhydroxamic acid.

these assays, attachment of radiolabeled C3b to a biological particle was blocked by increasing concentrations of a fluid phase competitor. Inhibition of C3b attachment was shown to be dependent on the concentration of the competitor and on its reactivity with the activated thioester [12, 15]. Table 1 shows the results of reactivity assays done on many additional hydroxylated and non-hydroxylated compounds. While tyrosine was highly reactive, O-methyltyrosine and phenylalanine showed no reactivity even at the highest concentration used. Therefore, inhibition was due to the hydroxyl group not the amino group. Addition of a second OH group at position 3 in the phenyl ring (see L-DOPA, Table 1) resulted in a 3-fold increase in reactivity. This may be due to an increase in electron density as will be described below. Removal of the amino or the carboxyl group from L-DOPA enhanced the reactivity approximately an additional 2-fold. Addition of an OH group at position 5 in the phenyl ring of dopamine (see 5-hydroxydopamine) did not change the reactivity; however, substitution at position 6 in the phenyl ring (6-hydroxydopamine) or at the β-carbon atom (norepinephrine) reduced the reactivity 4.3- and 1.7-fold, respectively.

Reactivity of C3b with Substituted Phenol Derivatives

To examine the correlation between the electron density in the phenyl ring and reactivity with the thioester of C3b, phenol derivatives with known Hammett substituent constants were tested [27, 28]. The Hammett constant is an empirically determined measure of the electron-donating or -withdrawing ability of a group in a particular position on the ring. Figure 2 shows that the reactivity of substituted phenols varied over 84-fold, depending on the nature and position of the group on the phenyl ring. Substituted phenols with strong electron-withdrawing groups (e.g. -CHO and -NO_2) were less reactive than phenols with electron-donating groups (e.g. -NH_2 and -OH), suggesting that the nucleophilic character of the -OH group is a major determinant of reactivity. The most reactive compound in this series was *p*-aminophenol with an IC_{50} of 0.64 mM, while the least reactive compound was *p*-nitrophenol with an IC_{50} of 54 mM. The correlation coefficient of the data was 0.74, indicating a significant correlation ($P < 0.006$).

Effect of Hydroxylated Compounds on C3 Convertase Activity

In the C3b attachment inhibition assays, the observed inhibition has been interpreted to be due to covalent binding of inhibitors to the thioester of C3b as has been demonstrated for sugars, polysaccharides, and tyrosine [12, 15, 26], but the same findings would result from inhibition of the C3 convertase (C3b,Bb) on the cell. To rule out the second possibility, the compounds in the present study were tested for their effect on C3 convertase activity (Table 2). The enzyme activity was measured in a fluorometric assay [25]. All inhibitors except glucose were tested at a concentration that was five times their IC_{50} in the C3b attachment assay. No significant inhibition of C3b,Bb activity was observed with any compound in this study. Benzamidine, which is a known inhibitor of C3b,Bb [25], served as a positive control and showed 71% inhibition at a concentration of 50 mM.

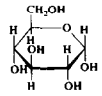
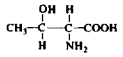
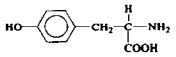
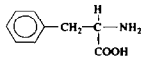
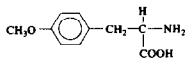
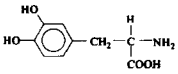
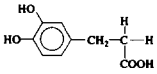
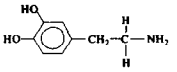
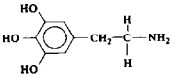
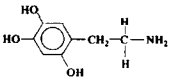
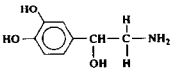
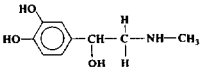
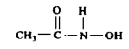
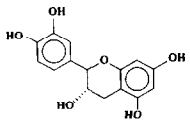
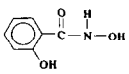
Examination of Reactivity of C3b with Hydroxylated Drugs and Other Compounds

Many drugs contain hydroxyl groups. These aid solubility, often allow membrane permeability, and are generally unreactive. However, metastable C3b specifically reacts with hydroxyl groups, and its natural targets are the largely unreactive hydroxyl groups on polysaccharides. Our previous work on the specificity of the thioester made us suspect that hydroxylated drugs may be capable of inhibiting complement activation by blocking C3b attachment. Drugs of the L-DOPA family contain hydroxylated phenyl rings and are often given in very high doses. Table 1 shows that all of these di- and tri-hydroxylated compounds were highly reactive with the thioester of metastable C3b. Reactivities as much as eight times greater than that of tyrosine were found. Others have reported that hydroxamic acids are highly reactive with the C3b thioester [29]. Table 1 shows that acetohydroxamic acid had an IC_{50} of 270 μM. Salicylhydroxamic acid exhibited the lowest IC_{50} in this study at 8.3 μM. Its reactivity was approximately 20,000-fold greater than the normal targets of C3b attachment, carbohydrates, and it was 6.6 million-fold more reactive than water.

Inhibition of Complement Activation

Compounds that inhibit C3b attachment would be expected to block complement activation. Figure 3 shows the concentration dependence of inhibition of complement by hydroxylated compounds. Inhibition of the classical pathway was mea-

TABLE 1. Reactivity of hydroxylated compounds with the activated thioester of C3b

Compound		IC_{50}* (mM)	Relative reactivity per mol†	Relative reactivity per OH†
Water	H_2O		1‡	1‡
Glucose		161 ± 18	336	67
Threonine		35 ± 2	1,580	1,580
Tyrosine		3.2 ± 0.1	17,000	17,000
Phenylalanine		>50§		
O-Methyltyrosine		>50§		
Phenol		2.9 ± 0.4	19,100	19,100
L-DOPA		0.95 ± 0.06	58,200	29,100
Deamino-DOPA (Hydrocaffic acid)		0.41 ± 0.02	135,000	67,400
Dopamine		0.60 ± 0.07	91,700	45,900
5-Hydroxydopamine		0.53 ± 0.03	104,000	34,800
6-Hydroxydopamine		2.56 ± 0.20	22,800	7,610
Norepinephrine		1.0 ± 0.08	55,800	18,600
Epinephrine		0.73 ± 0.06	75,400	25,100
Acetohydroxamic acid		0.27 ± 0.02	207,000	207,000
Catechin hydrate		0.089 ± 0.006	732,000	146,000
Salicylhydroxamic acid		0.0083 ± 0.0007	6,660,000	3,330,000

* The IC_{50} values were determined as described in Materials and Methods and reported as means ± SD; N = ≥10.
† Relative to the reactivity of water with the thioester of C3b [12].
‡ Value for water is 1 by definition [12].
§ Maximum concentration of compound that could be used in the assays.

sured with antibody-coated sheep erythrocytes (Fig. 3A) and NHS. Lysis of rabbit erythrocytes by NHS containing MgEGTA was used to measure inhibition of the alternative pathway of complement activation (Fig. 3B). All of the hydroxylated compounds inhibited lysis, and the IC_{50} concentrations correlated well with the values determined with the C3b attachment inhibition assay. The concentration required to inhibit 50% complement activity is listed in Table 3. With four of the six compounds the concentration required to inhibit activation of the alternative pathway was lower than that

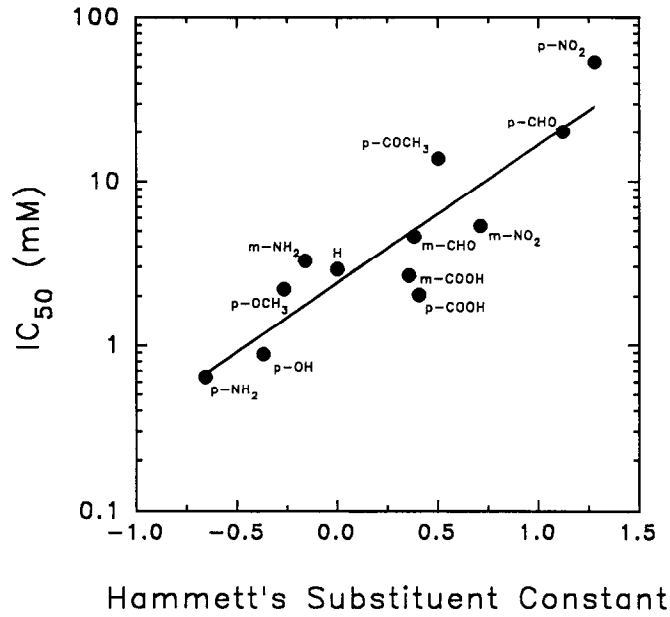


FIG. 2. Correlation of inhibitory capacity (IC_{50}) of different substituted phenols with Hammett's substituent constant. Phenol (H) was substituted at meta (*m*) and para (*p*) positions. The substituted groups were: -NH_2, amino; -OH, hydroxyl; -OCH_3, methoxy; -COOH, carboxyl; -CHO, carbonyl; -NO_2, nitro; and -$COCH_3$, acetyl. There was a significant correlation between IC_{50} and the Hammett substituent constant (r = 0.74).

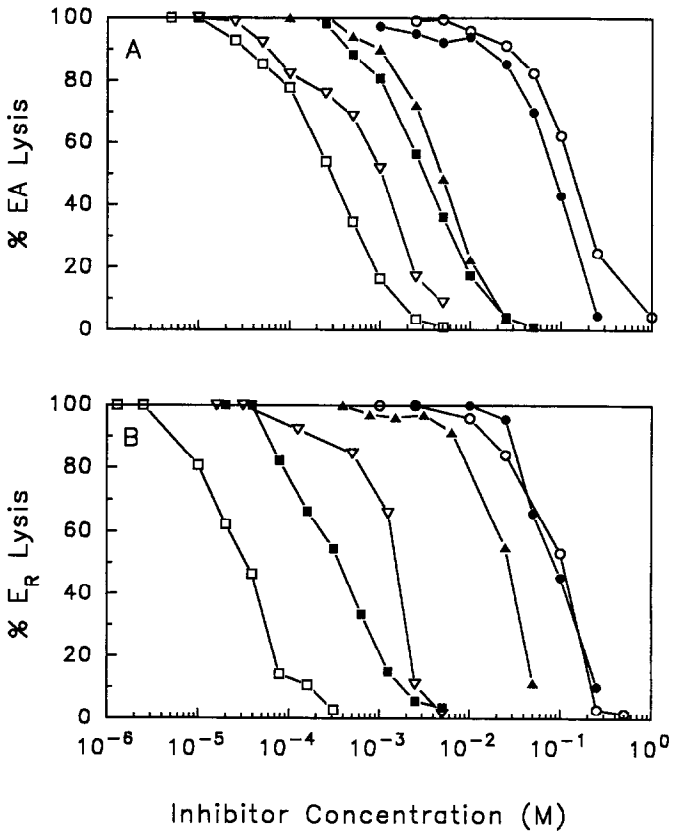


FIG. 3. Inhibition of classical and alternative pathway-mediated lysis of erythrocytes by different hydroxylated compounds. Panel A: Inhibition of classical pathway-mediated lysis of antibody-coated sheep erythrocytes. Panel B: Inhibition of alternative pathway-mediated lysis of rabbit erythrocytes. Key: (○) glucose; (●) threonine; (▲) phenol; (□) salicylhydroxamic acid; (■) acetohydroxamic acid; and (▽) catechin hydrate.

required to inhibit the classical pathway, possibly because the alternative pathway is more dependent on C3b deposition. However, the classical pathway was more sensitive than the alternative pathway to the two phenolic compounds. Although the serum used in these assays contained both C4A and C4B, the lysis of sheep erythrocytes is largely dependent on the C4B isotype of C4. Thus, these results suggest that C4B is particularly sensitive to phenolic compounds [28]. A homozygous C4A serum might give a very different response.

DISCUSSION

Inhibition of C3b attachment has the direct result of inhibiting complement activation and, consequently, of preventing generation of the anaphylatoxins C3a and C5a. Inhibition could reduce complement-mediated pathology in many diseases. Our previous reports have demonstrated the effectiveness of free carbohydrates, amino acids, and peptides in blocking attachment of C3b to surfaces of complement activating particles [12, 15, 26]. The present report describes the effectiveness of structurally related compounds and drugs.

The reactivity of carbohydrate hydroxyl groups with C3b is

TABLE 2. Effects of various hydroxylated compounds on C3 convertase activity

Compound	Concentration tested (mM)	Inhibition of C3b attachment IC_{50}(mM)	Inhibition of C3 convertase* (%)
Glucose	161	161	9.8
Threonine	175	35	3.8
Phenol	15	2.9	6.0
Acetohydroxamic acid	1.4	0.27	−8.3
Catechin hydrate	0.38	0.089	3.8
Salicylhydroxamic acid	0.042	0.0083	1.5
Benzamidine	50	ND†	71

* A fluorometric assay was used to determine the C3 convertase (C3b,Bb) activity [25].
† ND = not determined.

TABLE 3. Effects of various hydroxylated compounds on complement activation

Compound	C3b Attachment IC_{50} (mM)	Classical pathway* IC_{50} (mM)	Alternative pathway† IC_{50} (mM)
Glucose	161	140	105
Threonine	35	86	84
Phenol	2.9	4.8	27
Acetohydroxamic acid	0.27	3.0	0.35
Catechin hydrate	0.089	1.1	1.5
Salicylhydroxamic acid	0.0083	0.28	0.033

* Classical pathway activity was measured using EA lysis assays with pooled NHS containing approximately equal amounts of C4A and C4B.

† Alternative pathway activity was measured using E_R lysis assays.

extremely weak [12], as exemplified here by glucose which has an IC_{50} of 161 mM. Calculations show that on the surface of complement activating particles local concentrations of carbohydrates can reach or exceed this level. Attachment efficiencies on biological particles have been measured between 8 and 26% during complement activation in plasma [12]. Figure 3 and Table 3 show that complement activation in serum can be inhibited by hydroxylated compounds and that for the most part the concentrations required corresponded to the IC_{50} values measured in the attachment inhibition assays (Table 1). There were some notable exceptions to this relationship, however. The concentrations of the hydroxamic acids required to inhibit the alternative pathway were close to the IC_{50}. However, 10-fold higher concentrations were required to inhibit the classical pathway. In contrast, phenol inhibited the classical pathway with an IC_{50} similar to that found for inhibition of C3b attachment, but 6-fold higher concentrations were required to inhibit the alternative pathway. These results suggest the possibility of designing pathway-specific drugs. The ability to specifically inhibit the pathway, causing a particular pathology without completely shutting down the immune defense capabilities of complement, would be highly desirable. For example, it would be advantageous to inhibit alternative pathway-mediated tissue damage following strokes, heart attacks, and other ischemia–reperfusion events [30–35] while leaving the classical pathway intact to handle immune complex processing. Analogously, in antibody-mediated autoimmune diseases it would be desirable to inhibit classical pathway-mediated tissue damage while retaining the infection-fighting capability of the alternative pathway. Clearly, more information on the specificity pockets surrounding the thioester sites of C3 and C4 is needed.

The reactivity of compounds examined in this study varied over 20,000-fold. Both the nucleophilic character of the hydroxyl and other neighboring structural features affected reactivity with the thioester of metastable C3b. The effect of the former is demonstrated most clearly in Fig. 2 where the nucleophilic character of twelve substituted phenols is plotted against the effectiveness of these compounds in inhibiting C3b attachment. Electron-withdrawing groups decrease the electron-donating potential of the hydroxyl group, increase the Hammett substituent constant, and reduce the reactivity with the activated thioester of C3b. Electron-donating groups had the opposite effect with the result that the *p*-nitro and *p*-amino derivatives differed 84-fold in their reactivity with C3b. The specificity pocket surrounding the thioester was predicted to be narrow and to possess hydrophobic regions [15]. This would be consistent with the preference for phenyl rings found here. Studies of threonine derivatives [15] also demonstrated a strong (27-fold) preference for an amino group on the carbon neighboring the hydroxylated carbon, and peptides from IgG have demonstrated that the sequence surrounding Thr^{144} affects the reactivity of this residue with C3b [15]. No attempt was made in the present study to incorporate additional specificity features into the model compounds, but these observations suggest that additional improvements in specificity and reactivity are possible.

The finding that the neuroactive compounds L-DOPA, epinephrine, and norepinephrine are potential inhibitors of complement raises the possibility that these compounds may limit complement activation *in vivo*. This is especially interesting in light of recent reports of complement involvement in Alzheimer's disease and other brain diseases [36–38]. Also relevant is the observation that glial and other cells in the brain have been shown to make most if not all of the complement components. This eliminates the problem associated with passage of these proteins across the blood/brain barrier and has led recently to consideration of complement as a source of pathology in numerous neurological diseases [39].

The problems associated with complement activation in strokes [33], heart attacks [35], antibody-mediated autoimmune reactions, xenotransplantation [40–42], extracorporeal dialysis, and blood oxygenation [43–46] all emphasize the need for effective complement inhibitory drugs. None currently exist. The work presented here targets the reactive site of the central molecule common to all pathways of complement activation. We have shown that several off-the-shelf compounds or drugs are up to 20,000 times more effective (i.e. salicylhydroxamic acid) in thioester transesterification than are natural targets (i.e. carbohydrates). The compound acetohydroxamic acid has been approved in the United States for treatment of renal stones, and it is 1800 times as effective as sugars in blocking C3b attachment. Even the widely used drug dopamine is 270 times more effective than common carbohydrates. Whether the complement regulatory properties of these compounds might contribute to their efficacy is unknown.

The authors express their appreciation to Nicole S. Narlo and Kerry L. Wadey-Pangburn for their excellent technical assistance. This research was supported by National Institutes of Health Research Grant DK-35081.

References

1. Holmskov U, Malhotra R, Sim RB and Jensenius JC, Collections: Collagenous C-type lectins of the innate immune defense system. *Immunol Today* **15:** 67–74, 1994.
2. Tack BF, Harrison RA, Janatova J, Thomas ML and Prahl JW, Evidence for presence of an internal thiolester bond in third

component of human complement. *Proc Natl Acad Sci USA* **77:** 5764–5768, 1980.
3. Pangburn MK, Spontaneous reformation of the intramolecular thioester in complement protein C3 and low temperature capture of a conformational intermediate capable of reformation. *J Biol Chem* **267:** 8584–8590, 1992.
4. DeBruijn MHL and Fey GH, Human complement component C3: cDNA coding sequence and derived primary structure. *Proc Natl Acad Sci USA* **82:** 708–712, 1985.
5. Pangburn MK and Müller-Eberhard HJ, The alternative pathway of complement. *Springer Semin Immunopathol* **7:** 163–192, 1984.
6. Barrett AJ and Starkey PM, The interaction of α_2-macroglobulin with proteinases. Characteristics and specificity of the reaction and a hypothesis concerning it molecular mechanism. *Biochem J* **133:** 709–724, 1973.
7. Law SKA, Dodds AW and Porter RR, A comparison of the properties of two classes, C4A and C4B, of the human complement component C4. *EMBO J* **3:** 1819–1823, 1984.
8. Dodds AW and Law SKA, Structural basis of the binding specificity of the thioester-containing proteins, C4, C3 and alpha-2-macroglobulin. *Complement* **5:** 89–97, 1988.
9. Isenman DE and Young JR, The molecular basis for the difference in immune hemolysis activity of the Chido and Rogers isotypes of human complement component C4. *J Immunol* **132:** 3019–3027, 1984.
10. Carroll MC, Fathallah DM, Bergamaschini L, Alicot EM and Isenman DE, Substitution of a single amino acid (aspartic acid for histidine) converts the functional activity of human complement C4B to C4A. *Proc Natl Acad Sci USA* **87:** 6868–6872, 1990.
11. Reilly BD, Skanes VM and Levine RP, Evidence showing that the 1105 and 1106 isotypic residues of the fourth component of human complement, C4A, are not involved in amide bond formation. *Mol Immunol* **31:** 761–769, 1994.
12. Sahu A, Kozel TR and Pangburn MK, Specificity of the thioester-containing site of human C3 and its significance to complement activation. *Biochem J* **302:** 429–436, 1994.
13. Kinoshita T, Takata Y, Kozono H, Takeda J, Hong K and Inoue K, C5 convertase of the alternative complement pathway: Covalent linkage between two C3b molecules within the trimolecular complex enzyme. *J Immunol* **141:** 3895–3901, 1988.
14. Shohet JM, Pemberton P and Carroll MC, Identification of a major binding site for complement C3 on the IgG_1 heavy chain. *J Biol Chem* **268:** 5866–5871, 1993.
15. Sahu A and Pangburn MK, Covalent attachment of human complement C3 to IgG: Identification of the amino acid residue involved in ester linkage formation. *J Biol Chem* **269:** 28997–29002, 1994.
16. Kim YU, Carroll MC, Isenman DE, Nonaka M, Pramoonjago P, Takeda J, Inoue K and Kinoshita T, Covalent binding of C3b to C4b within the classical complement pathway C5 convertase. Determination of amino acid residues involved in ester linkage formation. *J Biol Chem* **267:** 4171–4176, 1992.
17. Takata Y, Kinoshita T, Kozono H, Takeda J, Tanaka E, Hong K and Inoue K, Covalent association of C3b with C4b within C5 convertase of the classical complement pathway. *J Exp Med* **165:** 1494–1507, 1987.
18. Kozono H, Kinoshita T, Kim YU, Takata-Kozono Y, Tsunasawa S, Sakiyama F, Takeda J, Hong K and Inoue K, Localization of the covalent C3b-binding site on C4b within the complement classical pathway C5 convertase, C4b2a3b. *J Biol Chem* **265:** 14444–14449, 1990.
19. Mold C, Nemerow GR, Bradt BM and Cooper NR, CR2 is a complement activator and the covalent binding site for C3 during alternative pathway activation by Raji cells. *J Immunol* **140:** 1923–1929, 1988.
20. Marquart HV, Svehag S and Leslie RGO, CR2 is the primary acceptor site for C3 during alternative pathway activation of complement on human peripheral B lymphocytes. *J Immunol* **153:** 307–315, 1994.
21. Hammer CH, Wirtz GH, Renfer L, Gresham HD and Tack BF, Large scale isolation of functionally active components of the human complement system. *J Biol Chem* **256:** 3995–4006, 1981.
22. Pangburn MK, A fluorometric assay for native C3. The hemolytically active form of the third component of human complement. *J Immunol Methods* **102:** 7–14, 1987.
23. Götze O and Müller-Eberhard HJ, The C3-activator system: An alternative pathway of complement activation. *J Exp Med* **134:** 90s–108s, 1971.
24. Lesavre PH, Hugli Te, Esser AF and Müller-Eberhard HJ, The alternative pathway C3/C5 convertase: Chemical basis of factor B activation. *J Immunol* **123:** 529–534, 1979.
25. Pangburn MK and Müller-Eberhard HJ, The C3 convertase of the alternative pathway of human complement. Enzymatic properties of the biomolecular proteinase. *Biochem J* **235:** 723–730, 1986.
26. Sahu A and Pangburn MK, Tyrosine is a potential site for covalent attachment of activated complement component C3. *Mol Immunol* **32:** 711–716, 1995.
27. Roberts JD and Caserio MC, *Basic Principles of Organic Chemistry*, pp. 954–962. W. A. Benjamin, New York, 1965.
28. Baba AS and Tamura N, Influence of aromatic compounds on the interaction of activated C4 with EAC1. *Immunology* **32:** 251–256, 1977.
29. Sim RB, Twose TM, Paterson DS and Sim E, The covalent-binding reaction of complement component C3. *Biochem J* **193:** 115–127, 1981.
30. Hill J, Lindsay TF, Ortiz F, Yeh CG, Hechtman HB and Moore FDJ, Soluble complement receptor type 1 ameliorates the local and remote organ injury after intestinal ischemia–reperfusion in the rat. *J Immunol* **149:** 1723–1728, 1992.
31. Pemberton M, Anderson G, Vetvicka V, Justus DE and Ross GD, Microvascular effects of complement blockade with soluble recombinant CR1 on ischemia/reperfusion injury of skeletal muscle. *J Immunol* **150:** 5104–5113, 1993.
32. Rubin BB, Smith A, Liauw S, Isenman DE, Romaschin AD and Walker PM, Complement activation and white cell sequestration in postischemic skeletal muscle. *Am J Physiol* **259:** H525–H531, 1990.
33. Vasthare US, Rosenwasser RH, Barone FC and Tuma RF, Involvement of the complement system in cerebral ischemic and reperfusion injury. *FASEB J* **7:** A424, 1993.
34. Moore FD Jr, Therapeutic regulation of the complement system in acute injury states. *Adv Immunol* **56:** 267–299, 1994.
35. Kilgore KS, Friedrichs GS, Homeister JW and Lucchesi BR, The complement system in myocardial ischaemia/reperfusion injury. *Cardiovasc Res* **28:** 437–444, 1994.
36. McGeer PL, Rogers J and McGeer EG, Neuroimmune mechanisms in Alzheimer disease pathogenesis. *Alzheimer Dis Assoc Disord* **8:** 149–158, 1994.
37. Jiang H, Burdick D, Glabe CG, Cotman CW and Tenner AJ, β-Amyloid activates complement by binding to a specific region of the collagen-like domain of the C1q A chain. *J Immunol* **152:** 5050–5059, 1994.
38. Aisen PS and Davis KL, Inflammatory mechanisms in Alzheimer's disease: Implications for therapy. *Am J Psychiatry* **151:** 1105–1113, 1994.
39. Walker DG and McGeer PL, Complement gene expression in human brain: Comparison between normal and Alzheimer disease cases. *Brain Res Mol Brain Res* **14:** 109–116, 1992.
40. Wang MW, Johnson PS, Wright LJ, Lim SM and White DJ, Immunofluorescent localization of pig complement component 3, regardless of the presence or absence of detectable immunoglobulins, in hyperacutely rejected heart xenografts. *Histochem J* **24:** 102–109, 1994.
41. Leventhal JR, Matas AJ, Sun LH, Reif S, Bolman RM3, Dal-

masso AP and Platt JL, The immunopathology of cardiac xenograft rejection in the guinea pig-to-rat model. *Transplantation* **56:** 1–8, 1993.

42. Bach FH, Turman MA, Vercellotti GM, Platt JL and Dalmasso AP, Accommodation: A working paradigm for progressing toward clinical discordant xenografting. *Transplant Proc* **23:** 205–207, 1991.
43. Nilsson L, Storm KE, Thelin S, Bagge L, Hultman J, Thorelius J and Nilsson U, Heparin-coated equipment reduces complement activation during cardiopulmonary bypass in the pig. *Artif Organs* **14:** 46–53, 1990.
44. Videm V, Svennevig JL, Fosse E, Semb G, Osterud A and Mollnes TE, Reduced complement activation with heparin-coated oxygenator and tubings in coronary bypass operations. *J Thorac Cardiovasc Surg* **103:** 806–813, 1992.
45. Cheung AK, Complement activation as index of haemodialysis membrane biocompatibility: The choice of methods and assays. *Nephrol Dial Transplant* **9:** 96–103, 1994.
46. Johnson RJ, Complement activation during extracorporeal therapy: Biochemistry, cell biology and clinical relevance. *Nephrol Dial Transplant* **9:** 36–45, 1994.