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Inhibition of Complement by Covalent Attachment of Rosmarinic Acid to Activated C3b

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ABSTRACT. Rosmarinic acid has been reported to inhibit complement activation *in vivo* as well as *in vitro*. Previous studies suggested that the inhibitory effect was due to inhibition of C3/C5 convertases, but inhibition of C3b attachment would yield the same results. Recent work in our laboratory demonstrated that compounds with polyhydroxylated phenyl rings are highly reactive with the thioester bond in nascent C3b. These compounds block complement activation by preventing attachment of C3b to the activating surface. Because rosmarinic acid contains two 3,4-dihydroxyphenyl groups, the current study was undertaken to re-examine the mechanism of inhibition by analyzing the effect of rosmarinic acid on C3b attachment. In assays using purified complement proteins, rosmarinic acid inhibited covalent attachment of C3b to cells with an IC_{50} = 34 μM. Inhibition of C5 convertase activity required 1500 μM rosmarinic acid, and no significant inhibition of the C3 convertase enzyme, which produces C3b from C3, was observed at 10,000 μM. In hemolytic assays using human serum, rosmarinic acid was shown to inhibit activation of both the classical (IC_{50} = 180 μM) and the alternative (IC_{50} = 160 μM) pathways of complement. Rosmarinic acid concentrations up to 10,000 μM did not cause direct inactivation of C3. Radioiodination of rosmarinic acid was used to demonstrate covalent activation-dependent incorporation of rosmarinic acid specifically into the thioester-containing α′-chain of nascent C3b. These findings indicate that inhibition of complement activation by rosmarinic acid is due to the reaction of rosmarinic acid with the activated thioester of metastable C3b, resulting in covalent attachment of the inhibitor to the protein. BIOCHEM PHARMACOL **57**;12:1439–1446, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. complement; C3; thioester; complement inhibitors; rosmarinic acid

Rosmarinic acid is a naturally occurring hydroxylated compound with anti-inflammatory activity. It is isolated from herbal balmmint plants such as *Rosmarinus officinalis* and *Melissa officinalis*. Its structure first suggested that it might act as a scavenger of toxic oxygen products [1], and studies on its anti-inflammatory activities led to its discovery as an inhibitor of complement [2]. It has been shown to inhibit both the classical and the alternative pathways of complement activation [3, 4]. *In vivo* it is known to inhibit CVF†-induced paw edema [2, 3], immune complex-mediated passive cutaneous anaphylaxis [3], and complement-dependent stimulation of prostacyclin synthesis [5]. It blocks opsonization [6], can be absorbed efficiently through the skin [7], and possesses antioxidant properties due to its ability to scavenge oxygen free radicals [8]. Previous studies have reported that rosmarinic acid inhibits complement C3 and C5 convertases. Englberger *et al.* [3] reported the inhibition of classical C3 convertase at 10 μM as measured by inhibition of lysis of EAC142 cells, whereas Peake *et al.* [4] showed only 15% inhibition of EAC142 at 2.6 mM and 35% inhibition of CVF,Bb at 3 mM, as measured by inhibition of C3a generation. The later study also reported a significant inhibition of lysis of EAC43b cells and C5a generation at 1 mM and concluded that rosmarinic acid inhibits the C5 convertase [4].

Recently we have studied the reactivity of a series of complement inhibitors targeting the activated thioester site of nascent C3b [9, 10]. We found that tyrosine and other hydroxylated phenyl compounds react with the thioester of proteolytically activated C3, resulting in inhibition of C3b attachment to complement activating surfaces. Rosmarinic acid is structurally related to these compounds. Thus, we sought to re-examine the mechanism of inhibition of complement by rosmarinic acid. In this communication evidence is provided to show that rosmarinic acid inhibits complement activation predominately by reacting covalently with the activated thioester in nascent C3b, thus blocking C3b attachment to complement-activating surfaces.

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† *Abbreviations:* C3, native, hemolytically active C3; C3b, proteolytically activated product of C3; CVF, cobra venom factor; CVF,Bb, cobra venom factor-derived C3 convertase; EA, sheep erythrocytes coated with antibodies; Er, rabbit erythrocytes; VBS, veronal buffered saline; GVB, VBS containing 0.1% gelatin; GVB^{2+}, GVB containing 0.5 mM $MgCl_2$ and 0.15 mM $CaCl_2$; GVBE, GVB containing 10 mM EDTA; NHS, normal human serum; ZymC3b, zymosan particle-bound C3b; and ZymC3b,Bb zymosan particle-bound C3 convertase.

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MATERIALS AND METHODS

Reagents and Materials

Stock solutions of 20 mM rosmarinic acid (2-[[3-(3,4-dihydroxyphenyl)-1-oxo-2-propinyl]-oxy]-3-(3,4-dihydroxyphenyl)-propionic acid) (ICN Pharmaceuticals) were prepared in PBS adjusted to pH 7.4. These solutions were protected from light and kept frozen until used. Dilutions were made in buffers appropriate to the assay being performed. C3-deficient human serum was purchased from the Sigma Chemical Co. ZymC3b was prepared by depositing C3b on zymosan particles (10^5 C3b/particle) as described [11]. Buffers used were GVB, 5 mM sodium veronal, 145 mM NaCl containing 0.1% gelatin, 0.02% NaN_3, pH 7.4; GVB^{2+}, GVB containing 0.5 mM $MgCl_2$ and 0.15 mM $CaCl_2$; GVBE, GVB containing 10 mM EDTA; PBS, 10 mM sodium phosphate, 140 mM NaCl, pH 7.4.

Purified Proteins

C3 [12, 13], factor B [14], factor D [15], and C5 [12] were purified from normal human plasma as described. Complement component C3 was repurified on a Mono S column (Pharmacia) [13]. CVF and CVF,Bb were prepared as previously described [16].

Radioiodination

C3 was radiolabeled with ^{125}I using Iodogen (Pierce Chemical Co.) to a specific activity between 0.5 and 1.5 μCi/μg. Similarly, rosmarinic acid (0.55 mM) was labeled with 500 μCi ^{125}I in a final volume of 100 μL VBS in an Iodogen-coated tube for 30 min on ice. The iodination was terminated by removing the sample from the Iodogen-coated tube and used without further purification.

Radioactivity in samples electrophoresed through SDS-PAGE gels was measured with a 3-hr exposure of a phosphor screen, and the exposure was quantitated with the PhosphorImager instrument from Molecular Dynamics.

Hemolytic Assays

Inhibition of classical pathway-mediated hemolysis was measured using antibody-coated sheep erythrocytes (EA). EA (10 μL of 1×10^9/mL) were mixed with 330 μL of various concentrations of rosmarinic acid in GVB^{2+} and 160 μL of NHS diluted 1:100 in GVB^{2+}. The reaction mixtures were incubated for 1 hr at 37°. To determine the extent of hemolysis, reaction mixtures were centrifuged and optical densities of supernatants were determined at 414 nm. Inhibition of alternative pathway-mediated hemolysis was determined by measuring lysis of rabbit erythrocytes (Er). Different concentrations of rosmarinic acid diluted in 78 μL of GVB were mixed with 5 μL of 50 mM MgEGTA, 7 μL of NHS (undiluted), and 10 μL of Er (1×10^9/mL). The reaction mixtures were incubated at 37° for 20 min, and the reaction was stopped by adding 400 μL of cold GVBE. The tubes were centrifuged, and the percentage of hemolysis was determined as described above.

C3 was assayed for functional activity using C3-deficient human serum (2 μL) and antibody-sensitized sheep erythrocytes (2×10^7 EA/mL) in a final volume of 100 μL GVB^{2+} at 37° for 30 min. The lysis reaction was stopped by adding 400 μL of cold GVBE. After spinning out the cells, the extent of hemolysis was determined from the absorbance of the supernatant at 414 nm.

C3b Attachment Inhibition Assay

Inhibition of C3b attachment to zymosan by rosmarinic acid was measured as previously described [11]. Briefly, particle-bound C3 convertase (ZymC3b,Bb) was made by incubating 10^7 ZymC3b (10^5 C3b/zymosan) with 5.0 μg factor B and 0.2 μg factor D in 200 μL GVB containing 1 mM $NiCl_2$ at 22° for 5 min. The reaction was stopped by adding 200 μL GVBE. ZymC3b,Bb (10 μL) was mixed immediately with a mixture of 10 μL of ^{125}I-labeled C3 (0.08 μCi) and 20 μL of various concentrations of inhibitor in GVB. The reaction mixture was incubated with mixing for 15 min at 37° and centrifuged through 20% sucrose in GVBE. The pellet and supernatant were counted, and the percent C3b attached was calculated. Nonspecific binding was determined by incubating ^{125}I-labeled C3 with ZymC3b devoid of enzyme. Data obtained were normalized by considering 100% bound equal to the amount of ^{125}I-labeled C3b bound in the absence of an inhibitor. The binding data was plotted and fit by non-linear regression analysis (GraFit, Erithacus Software) using the equation for single site inhibition:

$$\%\ \text{C3b Bound} = 100/(1 + (\text{Inhibitor Conc.})/\text{IC}_{50}) \quad (1)$$

The concentration of inhibitor required to block 50% of C3b attachment to zymosan is reported as the IC_{50}. Data are expressed as means ± SD of IC_{50} for all the determinations reported here.

C3 Convertase Assays

Particle-bound C3 convertase (ZymC3b,Bb) was prepared by incubating 100 μL ZymC3b (2×10^9 particles/mL and 150,000 C3b/particle) with 2.4 μg factor B and 0.4 μg factor D in a final volume of 108 μL of GVB containing 0.1 mM $NiCl_2$ at 22° for 5 min. Enzyme formation was stopped by the addition of EDTA to give a final concentration of 10 mM. Aliquots (20 μL) of the ZymC3b,Bb were added immediately to reaction mixtures (40 μL) containing 11 μg C3 and various concentrations of rosmarinic acid. After 15 min at 37° the reaction mixtures were centrifuged to remove the zymosan-bound enzyme, and the concentration of C3 in the supernatant was quantitated using the hemolytic assay described above.

C5 Convertase Assay

In this assay a preformed particle-bound C5 convertase was used and the enzyme activity was determined by measuring the amount of C5a released. C5a was quantitated using a commercial human complement C5a RIA assay (Amersham Life Science). Particle-bound C5 convertase was made by mixing 4×10^8 ZymC3b particles with 20 μg of factor B and 5 μg of factor D in 250 μL GVB containing 2 mM $NiCl_2$. The reaction mixture was incubated at 22° for 3 min. Fifty microliters of ZymC3b,Bb was mixed with 5 μL of C5 (1 μg) and different concentrations of rosmarinic acid in 45 μL GVB. The reaction mixture was incubated at 37° for 15 min and centrifuged at 10,000 *g* for 2 min. Supernatant (80 μL) was mixed with 45 μL of human plasma containing 20 mM EDTA (to enhance precipitation of uncleaved C5 in the assay, which interferes with the assay of C5a). Following the addition of 125 μL of precipitating reagent (provided with the RIA kit) the mixture was left at 22° for 15 min. Thereafter, samples were centrifuged and 50 μL of supernatant was utilized to quantitate the amount of C5a released. In controls lacking rosmarinic acid, less than 10% (4.7 ng C5a) of the C5 was cleaved, indicating that C5 was not limiting. The data obtained were normalized by setting the amount of C5a released in the absence of inhibitor equal to 100%.

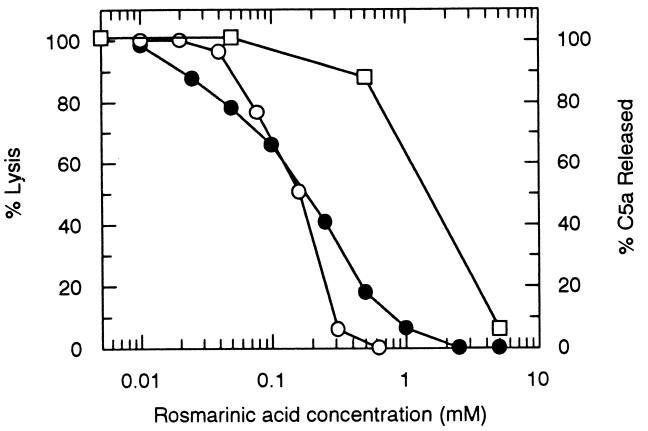


FIG. 1. Comparison of rosmarinic acid inhibition of the classical pathway (●), of the alternative pathway (○), and of C5 convertase activity (□). EA (classical pathway) or Er (alternative pathway) were incubated at 37° with normal human serum in the presence of the indicated concentrations of rosmarinic acid and Ca^{2+} and Mg^{2+} or MgEGTA, respectively. Percent hemolysis was normalized by setting 100% lysis equal to the lysis obtained in the absence of rosmarinic acid. The C5 convertase activity (□) was determined by measuring the amount of C5a released when a particle-bound C5 convertase [ZymC3b,Bb(Ni^{2+})] was incubated for 15 min with purified C5 and different concentrations of rosmarinic acid. The amount of C5a released was determined by a radioimmunoassay as described in Materials and Methods.

RESULTS

Effect of Rosmarinic Acid on Complement-Mediated Lysis of Erythrocytes

Figure 1 shows the effect of rosmarinic acid on the classical and the alternative pathways of complement activation. Rosmarinic acid inhibited both the pathways in a concentration-dependent manner. An IC_{50} of 180 μM was determined for rosmarinic acid inhibition of the classical pathway using lysis of antibody-coated sheep erythrocytes by normal human serum. The alternative pathway exhibited an almost identical IC_{50} of 160 μM rosmarinic acid. These values were similar to those reported earlier by Englberger *et al.* [3].

Effect of Rosmarinic Acid on C5 Convertase Activity

Previously, it has been reported that inhibition of complement by rosmarinic acid involves inhibition of C5 convertase function [4]. To verify these results we studied the effect of rosmarinic acid on C5 convertase. A pre-formed C5 convertase was used in these assays because of the possibility that rosmarinic acid inhibits C5 convertase formation by blocking C3b attachment. The alternative pathway C5 convertase was formed on zymosan particles bearing large numbers of attached C3b molecules by incubating the particles with purified factors B and D and $NiCl_2$. Incubation of these particles with purified C5 in the presence of increasing concentrations of rosmarinic acid demonstrated (Fig. 1, open squares) that the concentration of rosmarinic acid required to inhibit the enzyme was much higher than that required to inhibit hemolysis. Fifty percent inhibition of C5 convertase activity was observed at 1500 μM rosmarinic acid, which was 9-fold higher than that required to inhibit classical or alternative pathway-mediated hemolysis of erythrocytes (Fig. 1). A similar IC_{50} was found when C5 cleavage by the cobra venom factor-derived C5 convertase [17] was measured in the presence of rosmarinic acid (not shown).

Inhibition of Covalent Attachment of C3 by Rosmarinic Acid

When C3 is cleaved by a protease, its thioester becomes activated and it either forms an ester bond with hydroxyl groups on sugars on the surface of organisms or it reacts with water and fails to attach [18]. Soluble compounds containing hydroxyl groups compete with surface carbohydrates and block attachment of C3b to the surface [11, 19]. Inhibition of C3b attachment by a soluble competitor can be used to measure the reactivity of that compound with the activated thioester of metastable C3b. The reactivity of rosmarinic acid was measured by employing the C3b attachment inhibition assay used in our previous studies [11, 19]. This assay measures the effect of increasing concentrations of a competitor on attachment of radiolabeled C3b to zymosan particles bearing a C3 convertase. Figure 2 shows the inhibition curve for rosmarinic acid generated using this assay. The data fit well with the theoretical curve for single site competitive inhibition (Fig. 2, solid line) and

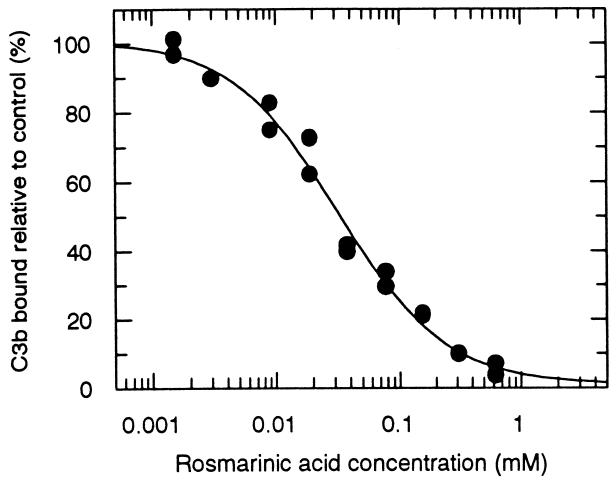


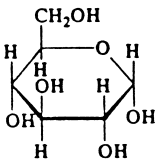
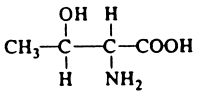
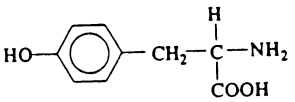
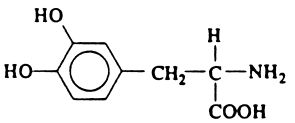
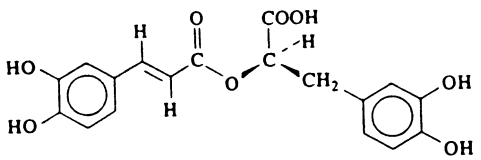
FIG. 2. Inhibition of ^{125}I-labeled C3b attachment to zymosan by rosmarinic acid. Zymosan particles bearing surface-bound C3 convertase (C3b,Bb) were incubated with ^{125}I-labeled C3 and various concentrations of rosmarinic acid for 15 min at 37°. The percentage of C3b bound was determined by centrifuging particles through 20% sucrose. The data were normalized by setting 100% bound equal to the C3b bound in the absence of rosmarinic acid. The data were fit to the curve for single site competitive inhibition (solid line) using equation 1 and the software program GraFit (Erithacus Software).

indicated an IC_{50} of 34 μM rosmarinic acid. Comparison (Table 1) of the reactivity of a set of previously characterized hydroxylated compounds showed that rosmarinic acid was a highly reactive member of this family. The structures of rosmarinic acid and several other inhibitory compounds are given in Table 1. The results show that rosmarinic acid was approximately 5000-fold more reactive than sugars (e.g. glucose), which are the primary natural acceptors for C3b attachment on the surface of microorganisms.

Effect of Rosmarinic Acid on C3 and on the C3 Convertase

It could be argued that the observed inhibition of C3b attachment was the result of direct inactivation of C3 or of inhibition of the C3 convertase. The data presented in Figs. 3 and 4 rule out these two alternative mechanisms. To test for direct inactivation of C3, C3 was preincubated with various concentrations of rosmarinic acid up to 10 mM (Fig. 3) under conditions similar to those used in Fig. 2, which demonstrated inhibition of C3b attachment. After 15 min, the C3 was diluted 2000-fold and the remaining functional C3 was titered. Figure 3 shows that minimal direct inactivation of C3 occurred upon incubation of C3 with rosmarinic acid. Figure 4 demonstrates that the alternative pathway C3 convertase was not inhibited by rosmarinic

TABLE 1. Reactivity of the activated thioester of C3b with rosmarinic acid and other hydroxylated compounds

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	1,575	1,575
Tyrosine		3.2 ± 0.1	17,273	17,273
L-DOPA		0.95 ± 0.06	58,202	29,101
Rosmarinic acid		0.034 ± 0.007	1,636,079	409,020

*IC_{50} values were determined as described under Materials and Methods. Data are reported as means ± SD of IC_{50} (N = 6–11).
†Relative to the reactivity of water [11].
‡Value for water is 1 by definition [11].

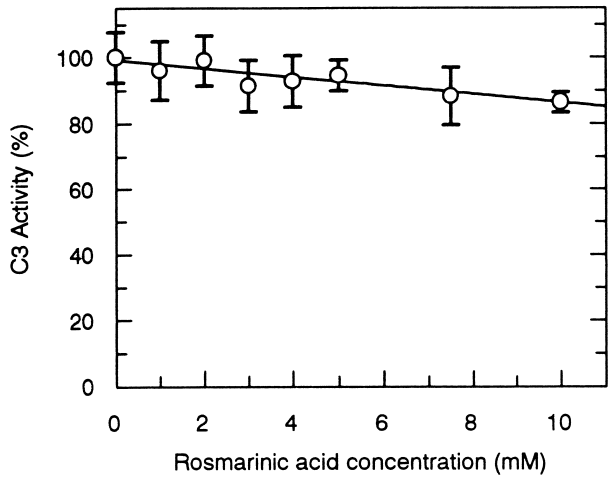


FIG. 3. Effect of rosmarinic acid on C3 functional activity. Purified C3 was incubated for 15 min at 37° with the indicated concentrations of rosmarinic acid in GVB^{2+} containing 2.5 mg/mL bovine serum albumin. The functional activity remaining was determined by diluting the samples 2000-fold and titrating the C3 in a hemolytic assay using C3-depleted serum and antibody-sensitized sheep erythrocytes (EA) as described in Materials and Methods. Data are reported as means ± SD (N = 3) where the initial C3 activity reported as 100% was 49% lysis of EA.

acid up to a concentration of 10 mM rosmarinic acid. In addition, two other tests were performed to verify that C3 was cleaved in the presence of rosmarinic acid. A fluorimetric assay [20] that allows real-time measurement of C3

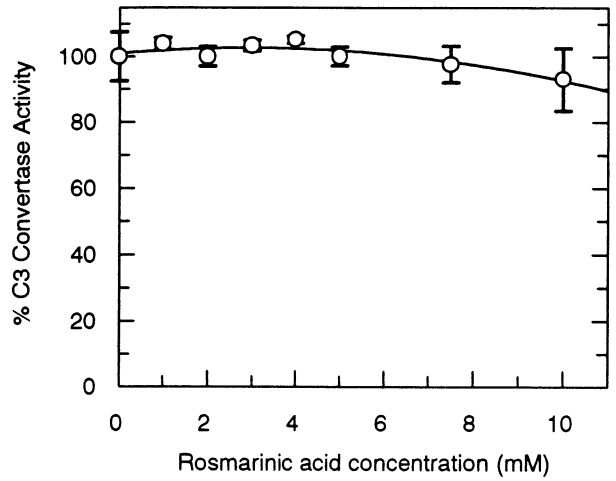


FIG. 4. Effect of rosmarinic acid on C3 convertase activity. Zymosan particles were prepared bearing surface-bound C3 convertase (C3b,Bb made with Ni^{2+}). ZymC3b,Bb then was incubated in the presence of 10 mM EDTA with C3 and various concentrations of rosmarinic acid for 15 min at 37°. The percentage of C3 cleaved was determined by titering the remaining C3 using a hemolytic assay using C3-depleted serum and antibody-sensitized sheep erythrocytes as described in Materials and Methods. The data were normalized by setting 100% C3 convertase activity equal to the C3 consumed in the absence of rosmarinic acid. Data are reported as means ± SD (N = 3) where the initial C3 activity reported as 100% was 48% lysis of EA.

convertase activity was used to show that rosmarinic acid had no effect on C3 cleavage at 7-fold over the concentration required to inhibit C3b attachment (not shown). In addition, no inhibition of C3 cleavage by the cobra venom factor-derived C3 convertase (CVF,Bb) was observed with up to 500 μM rosmarinic acid in assays measuring the production of C3a by RIA (not shown). The results demonstrate that rosmarinic acid did not inhibit either C3 or the C3 convertase and that C3 was cleaved normally by the C3 convertase in the presence of approximately 300 times the concentration of rosmarinic acid needed to block C3b attachment (Fig. 2; Table 1).

Covalent Incorporation of Rosmarinic Acid into the α′-Chain C3b

Previous studies have demonstrated that a variety of hydroxylated compounds react with the activated thioester of nascent C3b, forming a covalent ester bond with Gln1013 in the α′-chain of C3b [21–24]. Figure 5 shows that radiolabeled rosmarinic acid specifically incorporated into the α′-chain of C3b. No incorporation into the β-chain was observed, indicating the specificity of the reaction. Native C3 (lanes 2 and 4, Fig. 5) showed minimal incorporation, suggesting that activation to C3b was required. The label incorporated into the α-chain of C3 may be due to direct attack of rosmarinic acid on the thioester, as suggested by the 10–15% inhibition shown in Fig. 3. Pre-activated C3b showed little or no incorporation of radiolabel, indicating a requirement for the presence of rosmarinic acid during C3 activation. Some label was incorporated into high molecular weight material (Fig. 5, top of gel), which is probably due to α-chain dimer formation as has been reported by others [25].

DISCUSSION

Much effort is currently being directed at discovering drugs capable of controlling complement activation. Approaches include soluble forms of membrane-bound regulatory proteins [26, 27], such as sCR1 [28], antibodies capable of blocking activation, such as anti-C5 [29, 30], inhibitory synthetic peptides, one of which blocks C3 activation [31, 32], and protease inhibitors, which block enzymes involved in key activation steps, such as C1s, factor D, and the C3/C5 convertases [27, 32, 33]. C3b attachment is an essential event in all three pathways of complement activation and is a prerequisite for C5 activation and C5a anaphylatoxin release at sites of inflammation. The results in Fig. 2 demonstrate that rosmarinic acid was a very effective inhibitor of C3b attachment.

Other studies which have examined regulation of complement by rosmarinic acid have attributed inhibition to mechanisms other than blocking of C3b attachment. Englberger *et al.* [3] concluded that rosmarinic acid inhibits complement activation by inhibiting the C3 convertase. Antibody-coated sheep erythrocytes were prepared with the

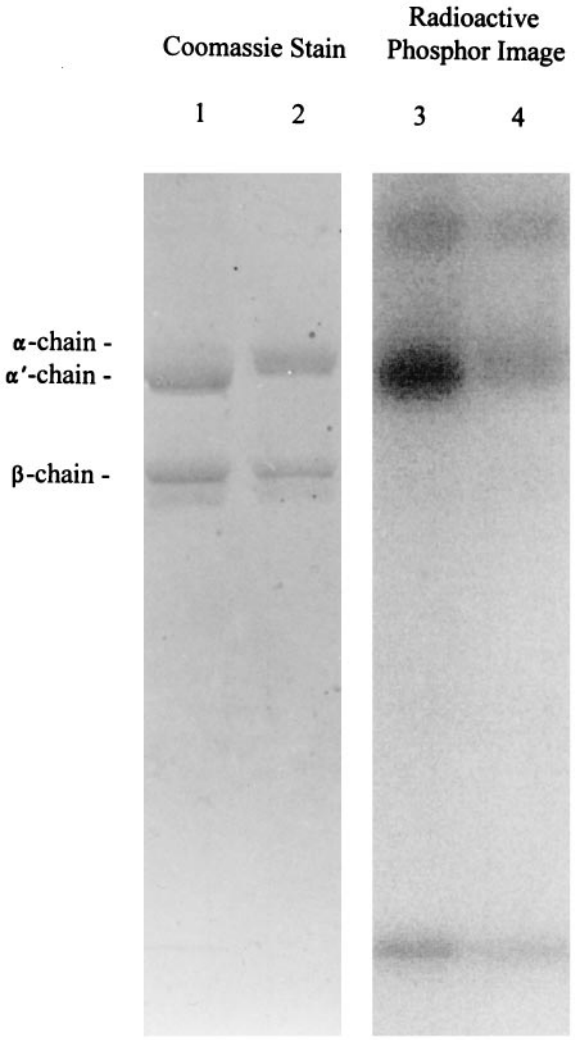


FIG. 5. Covalent incorporation of radioiodinated rosmarinic acid into the α′-chain of C3b. Rosmarinic acid was directly labeled with ^{125}I by the same procedure ordinarily used to label phenolic groups of tyrosine. Columns 1 and 3: C3b prepared from C3 by a C3 convertase in the presence of ^{125}I-labeled rosmarinic acid, dialyzed, dissolved in SDS-PAGE sample buffer, and electrophoresed through a 10% polyacrylamide gel in the presence of reducing agent to separate the α′- and β-chains of C3b. Columns 2 and 4: C3 treated identically except that no C3 convertase was present to convert it to C3b. The gel was stained with Coomassie blue, destained, and photographed. Radioactivity of the gel shown in lanes 1 and 2 was quantitated with a Phosphorimager from Molecular Dynamics, and the resulting image is shown on the right.

classical pathway C3 convertase on their surface (EAC142 cells). These cells were then converted to C5 convertase-bearing cells by incubation with purified C3 and subsequently were assayed for conversion by measuring lysis with a source of C5–C9. Conversion of a C3 convertase to a C5 convertase requires proteolytic activation of C3 *and* attachment of the C3b to the surface. Because conversion to C5 convertase activity was inhibited by rosmarinic acid, the authors concluded that C3 activation was inhibited. This conclusion was a reasonable one based on the assay performed and the lack of knowledge about the reactivity of polyhydroxyl compounds with the activated thioester of nascent C3b [9, 10]. However, it is now clear from the data in Fig. 2 that rosmarinic acid can block C3b attachment, and this alternative mechanism would give the same result. Assays for C3 convertase activity demonstrated that rosmarinic acid did not cause any inhibition of the C3 convertase even at 300-fold over the concentration required for inhibition of C3b attachment (34 μM). Consistent with this finding, Peake *et al.* [4] reported less than 50% inhibition of C3 convertase activity (i.e. release of C3a) at 3000 μM. Figure 3 demonstrates that C3 itself was not affected by rosmarinic acid concentrations up to 10 mM. Figure 1 shows that lysis of erythrocytes via the alternative pathway was inhibited 50% at 160 μM. It is, therefore, unlikely that inhibition of complement activation by rosmarinic acid is due to inhibition of C3 or the C3 convertase. The effective concentration needed to inhibit complement activation in serum was higher (160 μM) than that found in assays with purified components (34 μM), probably due to the fact that lysis of cells by complement is not linearly related to C3b attachment. That is, 50% inhibition of cell lysis would be expected to require a higher percentage of inhibition of C3b attachment. The effective concentration of rosmarinic acid also may have been lowered by binding to serum proteins such as albumin.

It has also been reported that rosmarinic acid inhibits C5 convertase activity [4]. We have confirmed (Fig. 1) the findings of Peake *et al.* [4], who measured inhibition of C5a release by a preformed C5 convertase. However, the concentrations of rosmarinic acid required to block C5 convertase activity (Fig. 1) were high compared to the concentrations that inhibited complement activation by either pathway. The concentrations of rosmarinic acid found by Peake *et al.* [4] to inhibit C5a generation and hemolysis (1000–2000 μM) were nearly identical to the level we found (1500 μM) to inhibit C5 convertase activity (Fig. 1). This concentration was, however, 8-fold higher than that required to inhibit hemolysis by either pathway of complement in our assays. The results suggest that the observed inhibition of complement is primarily due to reduced C3b attachment. Nevertheless, C5 activation would be reduced by two mechanisms. First, fewer C3b would yield fewer C5 convertase sites on the activator surface, and second, those sites that do form would be partially inhibited by rosmarinic acid. Interestingly, although C3 and C5 are cleaved by the same active site on the C3/C5 convertase, rosmarinic acid inhibits only C5 cleavage. While the mechanism of inhibition is unknown at this time, the lack of inhibition of C3 cleavage suggests a C5-specific effect, such as binding to C5 in a region critical for catalysis.

Table 1 shows the structures of a family of polyhydroxylated compounds that have been shown to be effective inhibitors of C3b attachment. All inhibit complement activation at or near the concentrations that inhibit C3b attachment. The structures at each end of rosmarinic acid are identical to the ring structure of L-DOPA, yet rosma-

rinic acid is 28-fold more effective in its reactivity with the activated thioester of C3b. This suggests that structural features of rosmarinic acid other than the reactivity of the dihydroxyphenyl groups themselves enhance the binding and reactivity of this compound with the thioester site of C3b. Radiolabeled glucose and several other hydroxyl-containing compounds have been shown to incorporate into the thioester site in the α′-chain of C3b during C3 activation [22]. Figure 5 demonstrates that rosmarinic acid also became covalently attached specifically to the α′-chain of C3b, suggesting a similar mechanism.

Rosmarinic acid is derived from a small flowering herb and has been shown to possess anti-inflammatory activities *in vivo* [5, 34, 35] including suppression of endotoxin-induced complement activation and shock [34]. Although this compound may have multiple physiological effects (i.e. it is a weak antioxidant), its IC_{50} of 34 μM for C3b attachment represents the lowest level inhibition of any biological reaction yet described for this compound. Thus, the reaction of rosmarinic acid with the activated thioester of C3b should be considered when mechanistic explanations for its biological effects are examined.

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