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Inhibition of human complement by β-glycyrrhetinic acid

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SUMMARY

Licorice, the root extract of *Glycyrrhiza glabra* L., is used as a medicine for various diseases. Anti-inflammatory as well as anti-allergic activities have been attributed to one of its main constituents, glycyrrhizin. These activities are mainly ascribed to the action of the aglycone, β-glycyrrhetinic acid. β-Glycyrrhetinic acid has a steroid-like structure and is believed to have immunomodulatory properties. To determine whether interference with complement functions may contribute to the immunomodulatory activity of β-glycyrrhetinic acid, its effects on the classical and alternative activation pathways of human complement were investigated. We found that β-glycyrrhetinic acid is a potent inhibitor of the classical complement pathway ($IC_{50} = 35\ \mu M$), whereas no inhibitory activity was observed towards the alternative pathway ($IC_{50} > 2500\ \mu M$). The anticomplementary activity of β-glycyrrhetinic acid was dependent on its conformation, since the α-form was not active. It was also established that naturally occurring steroids, e.g. hydrocortisone and cortisone, did not inhibit human complement activity under similar conditions. Detailed mechanistic studies revealed that β-glycyrrhetinic acid acts at the level of complement component C2.

INTRODUCTION

Root extracts of *Glycyrrhiza glabra* L. (licorice) are used world-wide in traditional medicine for the treatment of inflammatory diseases including abscesses, nervous disorders, asthma and peptic ulcers. Several immunomodulatory activities have been attributed to glycyrrhizin, one of the main constituents of licorice.[1–5] For example, a preparation of ammonium glycyrrhizinate combined with L-cysteine and glycine (Strong Neominophagen C, Minophagen Pharmaceutical Co., Tokyo, Japan) has been clinically applied in the treatment of hepatitis, eosinophilic peritonitis, and more recently, human immunodeficiency virus type 1 infections.[6–8] When glycyrrhizin is administered orally, its aglycon β-glycyrrhetinic acid is the major metabolite.[9] Hence many activities of glycyrrhizin have been ascribed to β-glycyrrhetinic acid.

β-Glycyrrhetinic acid exhibits anti-inflammatory properties in different animal models.[10–12] Its mode of action, however, is as yet unknown. Previously, the mechanism of action was considered to be identical to that of glucocorticoids. This assumption was based on the structural resemblance between β-glycyrrhetinic acid and corticosteroids. Recently, it was postulated that the anti-inflammatory activity of β-glycyrrhetinic acid is probably due to the inhibition of the enzyme 11β-hydroxysteroid hydroxygenase.[13] Inhibition of this enzyme results in an accumulation of hydrocortisone, a natural steroid with anti-inflammatory properties. Oral administration of either β-glycyrrhetinic acid or glycyrrhizin was found to increase significantly plasma levels of hydrocortisone and prednisolone, respectively.[14] Because of this property, a novel application of β-glycyrrhetinic acid has been suggested based on the potentiation of the activity of glucocorticoids by inhibiting their metabolism.

A potentiation of hydrocortisone activity has been observed in skin and lung tissue after co-medication with β-glycyrrhetinic acid.[15,16] In this paper, we report that in addition to potentiation of hydrocortisone activity, β-glycyrrhetinic acid can have a direct anti-inflammatory effect by selectively inhibiting the complement cascade. A detailed mechanistic study of the anticomplementary activity is presented.

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MATERIAL AND METHODS

Samples

All test compounds were purchased from Sigma Chemicals Co. (Poole, UK).

Buffers

Five times-concentrated veronal saline buffer, pH 7·35 (VSB^0–5×), prepared according to Mayer[17], served as the

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stock solution for the preparation of VSB^0, VSB^{++} (containing 0·5 mM Mg^{2+} and 0·15 mM Ca^{2+}), EGTA-VB [containing 2·5 mM Mg^{2+} and 8 mM ethylenebis(oxyethylenenitrilo) tetra-acetic acid (EGTA, Aldrich, Bornem, Belgium)], and EDTA-VB [consisting of VSB^0 and 10 mM EDTA (Merck, Darmstadt, Germany)].

Serum

Human AB^+ blood from ten healthy volunteers was obtained from the Blood Bank, Hilversum, the Netherlands. After the blood was allowed to clot at room temperature, the serum was separated by centrifugation, pooled, and stored at −70°. This pool is further referred to as human pooled serum (HPS).

Complement reagents

R1, reagent for complement component C1, was prepared by mixing nine parts HPS with one part polyethylene glycol (PEG) 6000 (38·5% w/v) dissolved in VSB^{++}. The mixture was centrifuged at 3500 *g* (MSE Prepsin 50 ultracentrifuge with 8 × 35 Ti 431140103 rotor) and 4° for 60 min.[18] The supernatant was stored at −70° until use.

C4-deficient guinea-pig serum was used as R4. Aliquots of 200 μl were stored at −70°. Two reagents for human C2 (R2 and R2′) were used. R2 was prepared by heating HPS at 56° for 4·5 min as described by Joisel *et al.*[19] R2′ was prepared by affinity chromatography. Anti-C2 antibodies were isolated from 10 ml polyclonal rabbit anti-C2 serum (a kind gift from Dr C. W. van den Berg, Department of Biochemistry, University of Wales, College of Medicine, Cardiff, UK) by fractionated PEG 6000 precipitation. The final concentration in the first step was 4% (w/v) and centrifugation was performed at 4000 *g* and 4° for 1 hr. Next, the PEG concentration in the supernatant was increased to 15% (w/v), whereafter the mixture was centrifuged again at 4000 *g* and 4° for 1 hr. The 15%-PEG precipitate obtained in this way was further purified by Sepharose–diethylaminoethyl (DEAE) chromatography. Anti-C2 was eluted at the breakthrough of 70 mM sodium phosphate buffer (pH 6·3). Fractions containing anti-C2 were pooled and anti-C2-Sepharose was prepared by coupling to Cyanogen bromide (CNBR)-Sepharose 4B according to the manufacturer's instructions (Pharmacia, Uppsala, Sweden). Samples of human serum (1 ml) were depleted of C2 by passage over the anti-C2-column (1 × 10 cm) using VSB^{++} as eluent.

Erythrocytes

Sheep and rabbit blood diluted in citrate-buffered glucose (Alsever's solution) served as sources of sheep erythrocytes (ShE) and rabbit erythrocytes (RaE), respectively. To elute possibly adsorbed serum proteins, the erythrocytes were washed three times with 0·16 M sodium iodide before use. ShE were resuspended in VSB^{++} (4×10^8 cells/ml) and sensitized by incubation with 1:800 diluted monoclonal haemolytic amboceptor (National Institute of Health and Environmental Protection (RIVM), Bilthoven, the Netherlands) at room temperature for 10 min (the sensitized cells are further referred to as ShEA). Subsequently, the mixture was centrifuged and ShEA were resuspended in VSB^{++} (2×10^8 cells/ml). RaE were resuspended in EGTA-VB ($1{\cdot}5 \times 10^8$ cells/ml).

Haemolytic assays for human complement activation

Classical pathway (CP) and alternative pathway (AP) activities were determined using a modified version of the microassay described by Klerx *et al.*[20] Tests were performed in U-well microtitre plates (no. 651101; Greiner Labortechnik, Nurtingen, Germany). Stock solutions of test compounds were prepared in dimethyl sulphoxide (DMSO; Merck). Samples were diluted in either VSB^{++} (CP) or EGTA-VB (AP). The final concentration of DMSO in all test mixtures did not exceed 0·3%. CP activity was determined by adding 50-μl dilutions of HPS in VSB^{++} (ranging from $10^{-1.7}$ to $10^{-2.0}$) to wells containing 50 μl of the sample solution. For assessment of AP activity 25-μl dilutions of HPS in EGTA-VB ($10^{-0.2}$) were mixed with 100 μl of the sample solutions. Next the microtitre plates were preincubated at 37° for 30 min. Subsequently, 50-μl ShEA (2×10^8 cells/ml VSB^{++} (CP) or RaE ($1{\cdot}5 \times 10^8$ cells/ml EGTA-VB) (AP) were added. The plates were incubated at 37° for 60 min (CP) or 30 min (AP). Upon incubation, the plates were centrifuged at 1900 *g* for 5 min to precipitate intact cells and cell ghosts. To determine the degree of haemolysis, 50-μl amounts of supernatants were mixed with 200 μl of water in 96-well flat-bottom microtitre plates (no. 655101; Greiner). The absorbance at 405 nm was determined using an automatic enzyme-linked immunosorbent assay (ELISA) reader (STL instruments, model SF plus, Beun de Ronde, Abcoude, the Netherlands). Controls in this assay consisted of:

(1) Similarly treated supernatants of erythrocytes incubated with water (100% haemolysis);

(2) VSB^{++}, EGTA-VB, or heat-inactivated (56° for 30 min) serum dilutions (0% haemolysis controls); or

(3) Buffer supplemented with the appropriate dilution of HPS (0% inhibition).

Interference with individual complement components

A total of 50-μl HPS dilutions (ranging from 0·1 to 2 μl/ml) were mixed in U-well microtitre plates with 25-μl solutions of test compound in VSB^{++}. After preincubation at 37° for 30 min, 50-μl ShEA (2×10^8 cells/ml) and 25-μl diluted complement reagents, 1:2 for R1 or 1:10 for R2 and R4, were added. Following a second incubation step at 37° for 60 min, cell lysis was determined as described in the section haemolytic assays for human complement activities.

C1q isolation

HPS was fractionated by gradual PEG 6000 precipitation.[21] A 1:10 dilution of human AB serum in 2·5% (w/v) PEG 6000 solution in EDTA-VB was centrifuged (MSE Prepsin 50 ultracentrifuge) at 10 240 *g* and 4° for 25 min. After centrifugation, the PEG concentration of the supernatant was increased to 4·0% (w/v), whereafter the mixture was centrifuged again at 10 240 *g* and 4° for 25 min. Subsequently, the PEG precipitate was washed with 4% (w/v) PEG solution in EGTA-VB and centrifuged. The precipitate containing about 70% (w/w) C1q, was reconstituted in EDTA-VB to one-fifth of the original HPS volume.

Solid-phase C1q-binding assay

Microtitre plates (Greiner 655102) were coated overnight with 100-μl 1:150 diluted C1q preparation at 4°. The plates were washed first with EDTA–Tween 20 (2 mM EDTA, 150 mM NaCl, 0·03% Tween-20, pH = 7·35) and subsequently with 100 μl of a mixture containing aggregated IgG (WHO reference, kindly provided by Prof. Dr M. R. Daha, Leiden, the

Netherlands) and logarithmic dilutions of β-glycyrrhetinic acid. After incubation at room temperature for 30 min, plates were washed, whereafter 100 μl GAH/IgG-peroxidase labelled conjugate (Nordic, Tilburg, the Netherlands, cat. no. 3939), 1:5000 diluted in PBS/0·05% Tween-20 was added to each well. Plates were incubated at room temperature for 30 min, washed with EDTA–Tween-20 and developed with TMB/H_2O_2 substrate [0·1 M sodium acetate/citrate buffer, pH 5·5, containing 0·0025% H_2O_2 and 1% (w/v) tetramethyl benzidine (6 mg/ml TMB; Sigma, St Louis, MO, in dimethyl sulphoxide]. Absorbances values were then measured at 450 nm.

Isolation of C1s

C1s was purified by IgG–Sepharose affinity chromatography and Mono Q anion exchange chromatography according to the procedure published by Peitsch *et al.*[22] C1s was converted into C1$\bar{s}$ by incubating at 37° for 30 min.

Preparation of enriched C4

A crude C4 preparation was obtained using a modified version of the PEG precipitation procedure described by Bolotin *et al.*[23] Briefly, 5-ml samples of HPS were mixed with 20-ml buffer containing 6·25% (w/v) PEG 6000, 20 mM Tris–HCl, 150 mM NaCl, 10 mM EDTA, 5 mM benzamidine, 1 mM phenylmethylsulphonyl fluoride, and 30 mM ε-aminocaproic acid (pH 8·0). The turbid solution obtained was stirred at 0° for 30 min, after which the precipitate was removed by centrifugation (7000 *g* at 2° for 20 min). Next, the PEG concentration of the supernatant was adjusted to 8% by the addition of 25 ml of 11% PEG solution in the buffer previously described. After being stirred at 0° for 30 min, the suspension was centrifuged again (7000 *g* at 2° for 20 min). The precipitate obtained was dissolved in 30 ml VSB^0 containing 2 mM EDTA and used as a source of C4 (C4 content: 2288 IU; 4649 U/mg protein).

Measurement of C1$\bar{s}$ activity

C1$\bar{s}$ activity was measured using C4 as substrate. A mixture of 50-μl purified C1$\bar{s}$ (5 μg/ml), 50-μl C4 solution, and 50-μl dilutions of β-glycyrrhetinic acid in VSB^{++} were incubated in microtitre plates at 37° for 60 min. The reaction was stopped by adding 50-μl ice-cold EDTA-VB. Formation of C4b/c was quantified by ELISA as described by Wolbink *et al.*[24]

Measurement of C4b/c formation

Both aggregated human IgG (AHG) and ShEA were used to allow C4b/c formation, which was quantified as described in the previous paragraph.[24] For the generation of C4b/c by AHG, 20 μl of HPS and 60 μl of a β-glycyrrhetinic acid solution in VSB^{++} or only VSB^{++} were preincubated at room temperature for 10 min. Next, 20-μl samples of AHG (0·5 mg/ml) were added and the mixtures were incubated at 37° for 45 min, whereafter 100-μl ice-cold EDTA (0·5 M) was added. The generation of C4b/c by ShEA was estimated as described in the section haemolytic assays for human complement activities. Following the second incubation step at 37° for 60 min, 50-μl samples of ice-cold EDTA (1 M) were added after which the plates were centrifuged.

Determination of C1rC1s[C1-INH]$_2$

Twenty-microlitre serum samples were incubated with 60-μl β-glycyrrhetinic acid solutions in VSB^{++}. The mixtures were preincubated at room temperature for 10 min, after which 20-μl AHG (0·5 mg/ml) was added. Incubation was continued at 37° for 45 min, whereafter 100-μl samples of 0·5 M EDTA were added. Dilutions of samples (1:25) were used to determine C1rC1s[C1INH]$_2$ concentration by means of a radioimmunoassay as described previously.[25]

RESULTS

Effects of β-glycyrrhetinic acid on overall human complement activities

β-Glycyrrhetinic acid and analogues were investigated for their effects on the activation of classical and alternative complement pathways. As shown in Fig. 1, a dose-dependent inhibition of the classical pathway of human complement was observed for β-glycyrrhetinic acid. This inhibitory activity was found to be reversible, since complement activity could be restored by dialysis. No activity towards the alternative complement pathway was found. The anticomplementary activity of β-glycyrrhetinic acid appeared to be dependent on its conformation: α-glycyrrhetinic acid was less active (Table 1). Lower activity was also observed for derivates of β-glycyrrhetinic acid, i.e. glycyrrhizin, and sodium carbenoxolone. It must be stressed here that naturally occurring steroids, such as hydrocortisone and cortisone, did not show anticomplementary activity in our assays.

Since the inhibitory activity of β-glycyrrhetinic acid could be the result of complement consumption, we determined whether variation in preincubation time or temperature affected anticomplementary activity. The anticomplementary activity of β-glycyrrhetinic acid did not appear to be dependent on either the incubation time or the temperature. Thus, the inhibition of CP activity must be the result of a direct interference with a single component or a more complex enzyme.

To show that the inhibition of classical pathway activity was not due to an interaction with the binding of divalent cations Ca^{2+} or Mg^{2+}, the effects of increasing Ca^{2+} or Mg^{2+} concentrations on the inhibitory activity of β-glycyrrhetinic acid were investigated. Raising Ca^{2+} and Mg^{2+} concentrations up to eight-fold did not significantly alter the IC_{50} value of β-glycyrrhetinic acid. This suggests that the inhibitory activity

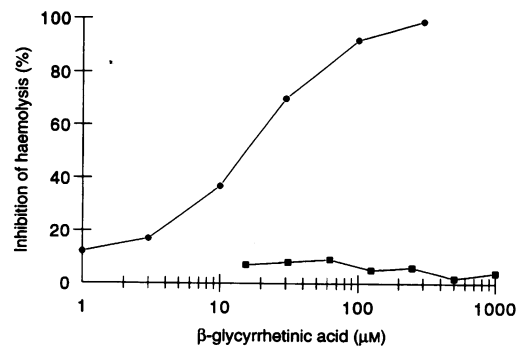

Figure 1. Effects of β-glycyrrhetinic acid on the classical (●) and alternative (■) activation pathways of human complement. Human serum was preincubated with β-glycyrrhetinic acid at 37° for 30 min, after which either antibody-coated sheep erythrocytes (classical pathway) or rabbit erythrocytes (alternative pathway) were added.

Table 1.

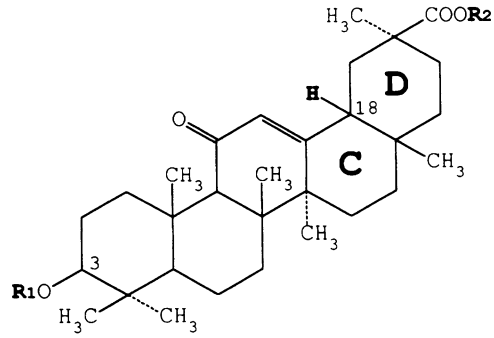

Table 1. Effect of β-glycyrrhetinic acid and analogues on classical pathway complement activation

	R1	R2	IC_{50} (μM)†
β-Glycyrrhetinic acid*	H	H	35±7
α-Glycyrrhetinic acid*	H	H	458±52
Glycyrrhizin	GlcA(1→2)GlcA‡	H	189±23
Carbenoxolone sodium	$NaOOC(CH_2)_2CO$-	Na	198±25
Hydrocortisone			>500
Cortisone			>500

* β-glycyrrhetinic acid: ring C/D *cis*, α-glycyrrhetinic acid: ring C/D *trans*.

† IC_{50} is the concentration giving rise to 50% inhibition as compared with controls. Data represent mean values±SEM ($n=8$).

‡ GlcA = Glucuronic acid.

of β-glycyrrhetinic acid must be ascribed to a more selective event. To investigate this, the effects of β-glycyrrhetinic acid on different steps in classical complement pathway activation were studied.

Effect of β-glycyrrhetinic acid on the binding of C1q to IgG

The classical pathway of complement is initiated by the binding of C1 through its C1q subunit to cell-bound antibody or, for example, endotoxin. Hence, the effects of β-glycyrrhetinic acid on the binding of C1q to immune aggregates were determined. Isolated C1q was immobilized onto a plate, and β-glycyrrhetinic acid and aggregated human IgG (AHG) were subsequently added. Next, the amount of bound IgG was detected by peroxidase-labelled anti-IgG and peroxidase substrate. As shown in Fig. 2, the binding of aggregated IgG to C1q was not inhibited by β-glycyrrhetinic acid.

Formation of C1s̄-esterase

$C1rC1s(C1\text{-}INH)_2$ complexes are formed during the activation of C1 and the subsequent inactivation of C1r and C1s by C1-INH. The amount of $C1rC1s(C1\text{-}INH)_2$ generated is considered a measure of C1s activation.[25] To determine whether β-glycyrrhetinic acid inhibited CP activation via interference with C1s̄-esterase formation, its effects on AHG-triggered $C1rC1s(C1\text{-}INH)_2$ production were assessed. β-Glycyrrhetinic acid did not affect $C1rC1s(C1\text{-}INH)_2$ formation (Fig. 3) proving that the anticomplementary activity of β-glycyrrhetinic acid is not due to interference with C1s̄ esterase formation.

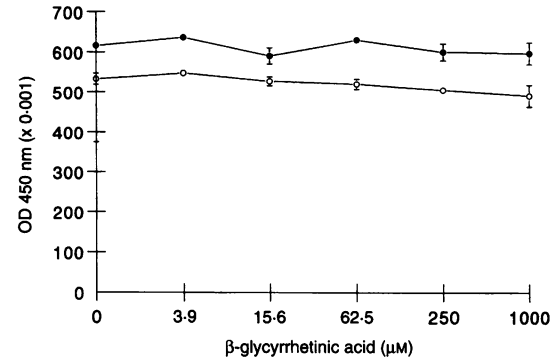

Figure 2. The influence of β-glycyrrhetinic acid on the binding of aggregated human IgG [(●) 1 μg/ml or (○) 0·3 μg/ml] to C1q-coated microtitre plates. Uncoated plates were used as control. Symbols with vertical error bars refer to the means of four independent observations±SEM.

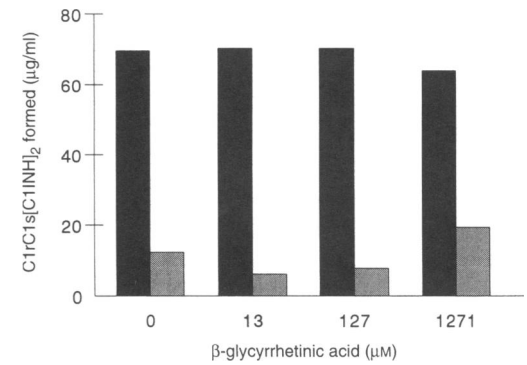

Figure 3. The effect of β-glycyrrhetinic acid on the generation of $C1rC1s[C1INH]_2$ complexes in human serum. Preincubation was performed in the presence (filled boxes) or absence (stippled boxes) of aggregated human IgG.

Effect of β-glycyrrhetinic acid on C1s̄ esterase activity

Isolated C4 was used as a substrate to assess the effect of β-glycyrrhetinic acid on C1s̄ esterase activity. C4 activation products (C4b/c) were quantified by ELISA. C1s̄ was capable of converting C4 into C4b/c under experimental conditions (Fig. 4). β-Glycyrrhetinic acid, however, did not change C1s̄-mediated formation of C4b/c. Similar results were obtained

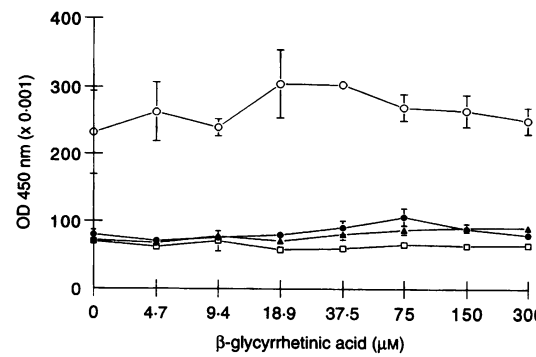

Figure 4. Absence of β-glycyrrhetinic-acid-induced interference with C4b/c formation upon incubation with C1s̄ and C4. (□) β-glycyrrhetinic acid, (●) C4+β-glycyrrhetinic acid, (▲) C1s+β-glycyrrhetinic acid, and (○) C1s+C4+β-glycyrrhetinic acid.

with fluid-phase (AHG) and corpusculate (ShEA) complement activators.

Interaction with individual complement components

The effects of β-glycyrrhetinic acid on individual complement components were studied in systems with complement reagents (R1, R4, or R2) and a limited amount of human serum. Under these conditions the complement component under investigation is the limiting factor in the complement-mediated haemolysis assay. Thus, the inhibition of haemolysis could point to an interaction of the test compound with the complement component under investigation. The inhibitory effect of β-glycyrrhetinic acid appeared most pronounced on C2 (Fig. 5). Similar effects of β-glycyrrhetinic acid on C2 were obtained using affinity-depleted R2′ (data not shown). Interference with C1- and C4-mediated lysis was also studied to further demonstrate that β-glycyrrhetinic acid does not act at the level of C1 or C4 activation. In these assays, inhibitory activity was only observed at high concentrations.

DISCUSSION

The effects of β-glycyrrhetinic acid on the human complement cascade were examined. No activity towards the alternative activation pathway was observed, whereas the classical pathway was inhibited in a dose-dependent manner. The inhibitory activity of β-glycyrrhetinic acid was shown to be connected with its conformation and the absence of substituents at the hydroxyl group at position 3 (Table 1). In addition, it was found that the anticomplementary activity of β-gly-cyrrhetinic acid is not affected by raising Ca^{2+} and Mg^{2+} concentrations. These observations suggest a selective interaction with one of the components of the classical complement pathway. Therefore, a more detailed mechanistic study was undertaken to establish the mode of action of β-glycyrrhetinic acid. These experiments revealed that β-glycyrrhetinic acid inhibits complement activation by interfering at the level of C2 rather than C1 or C4 activation.

The inhibition of C2-mediated lysis was observed in two assays. In one of these assays, C2 depletion was accomplished by heating at 56°. Joisel *et al.* reported that this method of C2 depletion is not entirely specific for C2, but results in a partial destruction of C1 as well.[19] Hence, interference with C1 had to be excluded. This was studied using C1-depleted serum instead of HPS as a source for C2. β-Glycyrrhetinic acid was also found to be inhibitory under these conditions, which points to a selective effect on C2. A second assay performed with C2-depleted serum produced by means of a Sepharose anti-C2-column confirmed our earlier findings.

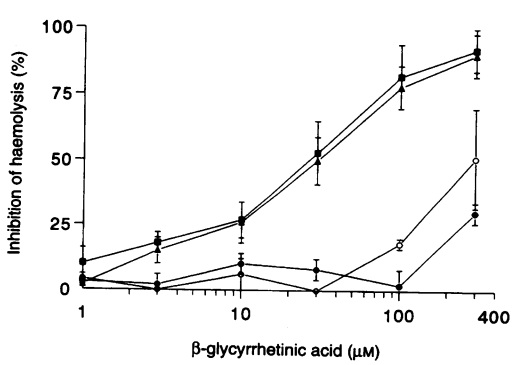

Figure 5. The inhibitory activity of β-glycyrrhetinic acid on classical complement pathway activation in human pooled serum (■) as such or under limiting concentrations of (○) C1, (●) C4, or (▲) C2.

So far, experimental anti-inflammatory activity of β-glycyrrhetinic acid has been restricted to granuloma pouch and carrageenan-induced inflammation models; inflammation induced by 12-*O*-tetradecanoylphorbol-13-acetate and arachidonic acid was not affected.[10–12,25,26] The inhibition of carrageenan-induced oedema formation by β-glycyrrhetinic acid is concordant with our findings. Carrageenan is an activator of the classical pathway of complement and systemic administration was found to result in a complete depletion of classical pathway activity.[27] Furthermore, it has been reported that complement depletion depressed carrageenan-induced oedema formation.[28] Hence, the anticomplementary activity of β-glycyrrhetinic acid is likely to contribute to its anti-inflammatory effects.

Capasso *et al.* reported that oral administration of β-glycyrrhetinic acid to rats resulted in inhibition of carrageenan-induced inflammation.[12] Since no effect on prostaglandin biosynthesis was observed, they proposed a different mechanism of action. Based on the finding that β-glycyrrhetinic acid inhibits dextran-induced leucocyte migration into the pleural space, they concluded that the anti-inflammatory activity must be ascribed to interference with cell migration. However, since dextran is a potent complement activator and inhibition of complement activation will prevent the formation of chemotactic factors, it may be assumed that migration inhibition may also be mediated by anticomplementary activity.[29] In support of this hypothesis, Inoue *et al.* observed a modulatory effect of β-glycyrrhetinic acid at least 3 hr after carrageenan treatment.[30] The oedema formed during this interval is mediated by prostaglandin release and migration of leucocytes.[31] Nakagawa and Komorita reported that the neutrophil chemotactic factors in the exudate of rat carrageenan-induced inflammation are derived from the third component of complement.[32] Thus, through inhibition of complement, β-glycyrrhetinic acid could contribute to the interference with leucocyte migration. Consequently, the anticomplementary activity of β-glycyrrhetinic acid is probably an important factor in its anti-inflammatory properties.

In comparison to β-glycyrrhetinic acid, glycyrrhizin showed only moderate activity. Mainly in Japan, glycyrrhizin is applied clinically for the treatment of a wide variety of diseases. Its therapeutic effect against chronic hepatitis was proven in a double-blind trial.[7] Since the damage to liver cells is reported to be the consequence of auto-immune reactivity, Shiki et al. investigated the effect of glycyrrhizin on antibody-mediated lysis of hepatocytes.[33] They found that glycyrrhizin suppressed the release of transaminase by isolated rat hepatocytes induced by a combination of complement and antihepatocyte-membrane antibodies. Only combinations of antibodies and complement induced transaminase release in their experiments. Therefore, it is likely that the inhibitory effect observed *in vivo* may be mediated by interference with classical complement pathway activity as well.

β-Glycyrrhetinic acid is a potent inhibitor of 11β-hydroxysteroid hydroxygenase. Inhibition of this enzyme results in

higher levels of natural as well as synthetic corticosteroids.[13] The therapeutic potential of this activity has been highlighted in several papers.[15,16] Because of this therapeutic potential, co-medication with β-glycyrrhetinic acid is considered in inflammatory disorders in which glucocorticoids can be used. Our findings suggest that in addition to an indirect anti-inflammatory effect mediated by the inhibition of glucocorticoid metabolism, β-glycyrrhetinic acid could directly interfere with inflammatory processes by inhibiting classical complement pathway activation. Schleimer proposed the use of either β-glycyrrhetinic acid or carbenoxolone as a co-medication with hydrocortisone in the treatment of inflammatory lung diseases.[15] Our results indicate that β-glycyrrhetinic acid should be favored since this compound also has complement-mediated anti-inflammatory activity.

REFERENCES

1. Kimura M., Watanabe H. & Abo T. (1992) Selective activation of extrathymic T cells in the liver by glycyrrhizin. *Biotherapy* **5,** 167.
2. Ohuchi K., Kamada Y., Levine L. & Tsurufuji S. (1981) Glycyrrhizin inhibits prostaglandin E2 production by activated peritoneal macrophages from rats. *Prostagland Med* **7,** 457.
3. Kobayashi M., Schmitt D.A., Utsunomiya T., Pollard R.B. & Suzuki F. (1993) Inhibition of burn-associated suppressor cell generation by glycyrrhizin through the induction of contrasuppressor T cells. *Immunol Cell Biol* **71,**181.
4. Zhang Y.H., Isobe K., Nagase F. *et al.* (1993) Glycyrrhizin as a promotor of the late signal transduction of interleukin-2 production by splenic lymphocytes. *Immunology* **79,** 528.
5. Kondo Y. & Takano F. (1994) Nitric oxide production in mouse peritoneal macrophages enhanced with glycyrrhizin. *Biol Pharm Bull* **17,** 759.
6. Takeda H., Ohta K., Niki H. *et al.* (1991) Eosinophilic peritonitis responding to treatment with glycyrrhizin. *Tokai J Exp Clin Med* **16,** 183.
7. Hayashi J., Kajiyama W., Noguchi A. *et al.* (1991) Glycyrrhizin withdrawal followed by human lymphoblastoid interferon in the treatment of chronic hepatitis B. *Gastroenterol Jap* **26,** 742.
8. Mori K., Sajai H., Suzuki S. *et al.* (1989) Effects of glycyrrhizin (SMNC: Stronger Neo-minophagen C) in hemophilia patients with HIV infection. *Tohoku J Exp Med* **158,** 25.
9. Yamamura Y., Kawakami J., Santa T. *et al.* (1992) Pharmacokinetic profile of glycyrrhizin in healthy volunteers by a new high-performance liquid chromatographic method. *J Pharm Sci* **81,** 1042.
10. Finney R.S.H. & Somers G.F. (1958) The anti-inflammatory activity of glycyrrhetinic acid and derivates. *J Pharm Pharmacol* **12,** 613.
11. Amagaya S., Sugishita E., Ogihara Y., Ogawa S., Okada K. & Aizawa T. (1984) Comparative studies of the stereoisomers of glycyrrhetinic acid on anti-inflammatory activities. *J Pharm Dyn* **7,** 923.
12. Capasso F., Mascolo N., Autore G. & Duraccio R. (1983) Glycyrrhetinic acid, leucocytes and prostaglandins. *J Pharm Pharmacol* **35,** 332.
13. Walker B.R. & Edwards C.R.W. (1991) 11-β-hydroxysteroid dehydrogenase and enzyme-mediated receptor protection: life after liquorice? *Clin Endocrinol* **35,** 281.
14. MacKenzie M.A., Hoefnagels W.H.L., Jansen R.W.M.M., Benraad T.J. & Kloppenborg P.W.C. (1990) The influence of glycyrrhetinic acid on plasma cortisol and cortisone in healthy young volunteers. *J Clin Endocrinol Metab* **70,** 1637.
15. Schleimer R.P. 1991. Potential regulation of inflammation in the lung by local metabolism of hydrocortisone. *Am J Respir Cell Mol Biol* **4,** 166.
16. Teelucksingh S., Mackie A.D., Burt D., McIntyre M.A., Brett L. & Edwards C.R. (1990) Potentiation of hydrocortisone activity in skin by glycyrrhetinic acid. *Lancet* **335,** 1060.
17. Mayer M.M. (1961) Complement and complement fixation. In: *Experimental Immunochemistry* (eds E.A. Kabat & M.M. Mayer), p. 133. Thomas, Springfield, IL.
18. Neoh S.H., Gordon T.P. & Roberts-Thomson P.J. (1984) A simple one step procedure for preparation of C1-deficient human serum. *J Immunol Methods* **69,** 277.
19. Joisel F., Leroux-Nicollet I., Lebreton J.P. & Fontaine M. (1983) A hemolytic assay for clinical investigation of human C2. *J Immunol Methods* **59,** 229.
20. Klerx J.P.A.M., Beukelman C.J., Van Dijk H. & Willers J.M.N. (1983) Micro-assay for colorimetric estimation of complement activity in guinea pig, human and mouse serum. *J Immunol Methods* **63,** 215.
21. Van Dijk H., van Voorst J., Jankowski I.G., Imhof J.W. & Kerckhaert J.A.M. (1984) Differential precipitation of the C1q subcomponent of the first complement component (C1) by polyethylene glycol from normal human serum and sera of patients with collagen disease. *Clin Exp Immunol* **57,** 495.
22. Peitsch M.C., Kovacsovics T.J. & Isliker H. (1988) A rapid and efficient method for the purification of the complement subcomponents C1r and C1s in zymogen form using fast protein chromatography. *J Immunol Methods* **108,** 265.
23. Bolotin C., Morris S., Tack B. & Prahl J. (1977) Purification and structural analysis of the fourth component of human complement. *Biochemistry* **16,** 2008.
24. Wolbink G.J., Bollen J., Baars J.W. *et al.* (1993) Application of monoclonal antibody against a neoepitope on activated C4 in an ELISA for quantification of complement activation via the classical pathway. *J Immunol Methods* **163,** 67.
25. Hack C.E., Hannema A.J., Eerenberg A.J.M., Out T.A. & Aalberse R.C. (1981) A C1-inhibitor-complex assay (INCA): a method to detect C1 activation in vitro and in vivo. *J Immunol* **127,** 1450.
26. Inoue H., Mori T., Shibata S. & Koshihara Y. (1989) Modulation by glycyrrhetinic acid derivatives of TPA-induced mouse ear oedema. *Br J Pharmacol* **96,** 204.
27. Ward P.A. & Cochrane C.G. (1965) Bound complement and immunological injury of blood vessels. *J Exp Med* **121,** 215.
28. Di Rosa M., Giroud J.P. & Willoughby D.A. (1971) Studies of the mediators of acute inflammatory response induced in rats in different sites by carrageenan and turpentine. *J Pathol* **104,** 15.
29. Klerx J.P.A.M., Van Dijk H., Van der Maaden W.J. & Willers J.M.N. (1985) Analytical study of differential anti-complementary effects of dextran sulphate and heparin in the mouse alternative pathway. *Int Arch Allergy Appl Immunol* **78,** 182.
30. Inoue H., Inoue K., Takeuchi T., Nagata N. & Shibata S. (1993) Inhibition of rat acute inflammatory paw oedema by dihemiphthalates of glycyrrhetinic acid derivates: comparison with glycyrrhetinic acid. *J Pharm Pharmacol* **45,** 1067.
31. Di Rosa M. (1972) Biological properties of carrageenan. *J Pharm Pharmacol* **24,** 89.
32. Nakagawa H. & Komorita N. (1993) Complement component C3-derived neutrophil chemotactic factors purified from exudate of rat carrageenin-induced inflammation. *Biochem Biophys Res Commun* **194,** 1181.
33. Shiki Y., Shirai K., Saito Y., Yoshida S., Mori Y. & Wakashin M. (1992) Effect of glycyrrhizin on lysis of hepatocyte membranes induced by anti-liver cell membrane antibody. *J Gastroenterol Hepatol* **7,** 12.