

Effect of supraphysiologic levels of C1-inhibitor on the classical, lectin and alternative pathways of complement

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Abstract

C1-inhibitor is increasingly used experimentally and clinically in inflammatory conditions like septicemia and ischemia-reperfusion injury. Several mechanisms may account for the anti-inflammatory effects of C1-inhibitor, including inhibition of complement. The aim of the present study was to investigate and compare the supraphysiologic effect of C1-inhibitor on the three complement pathways. Novel assays for specific evaluation of the classical, lectin and alternative pathways were employed using normal human serum supplemented with increasing concentrations of C1-inhibitor. Solid-phase classical- and lectin pathway activation was dose-dependently and significantly reduced up to 85% in the range of 2–28 times physiologic C1-inhibitor concentration. The lectin pathway was more potently inhibited than the classical at low doses. A functional lectin pathway assay demonstrated a significant reduction of C4 deposition up to 86% even at low concentration of C1-inhibitor and documented the effect to be at the level of MBL/MASPs. In contrast, C1-inhibitor had no effect on solid-phase alternative pathway activation, but significantly reduced cobra venom factor-induced fluid-phase activation up to 88%. The negative controls albumin and IgG had no effect on complement activation. The positive inhibitory controls compstatin (C3 inhibition), EDTA- or MBL-deficient sera reduced complement activation by 82–100%. We conclude that C1-inhibitor in high physiologic doses differentially inhibits all three-complement pathways. The inhibition pattern was strikingly different in the classical and lectin pathway, compared to the alternative. Previous studies interpreting the effects of C1-inhibitor as only due to classical pathway inhibition needs reconsideration. The data has implications for the therapeutic use of C1-inhibitor.

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1. Introduction

C1-inhibitor (C1-INH) was named according to its ability to inhibit the C1 subcomponents C1r and C1s in the classical pathway and is the only known physiologic inhibitor of these proteases (Pensky et al., 1961). Early studies of patients suffering from hereditary angioedema (HAE) shed further light on C1-INH's role in the classical pathway (Landerman et al., 1962; Donaldson and Evans, 1963). These patients' heterozygous C1-

INH deficiency leads to an increased spontaneous activation of C4 and C2 (Donaldson and Rosen, 1964). As this breakdown takes place in the fluid phase, and not on a solid surface like, e.g. bacteria, C4b is rapidly inactivated by factor I. This has been the main explanation as to why the classical C3 convertase C4bC2a does not assemble in HAE patients (Klein, 1990). A higher ratio of C4b-binding protein to C4 may also hinder activation beyond C4 and C2 (Gronski et al., 1988). Later, sensitive assays showed a very modest activation of C3 in HAE patients (Nielsen et al., 1995). During attacks of HAE even a minor increase in the terminal complement complex (TCC) was revealed (Nielsen et al., 1996).

In the lectin pathway, discovered in the late 1980's, mannose-binding lectin (MBL) or ficolins bind structures containing sugar

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on the surface of foreign particles and microbes (Kawasaki et al., 1989; Lynch et al., 2004). MBL share similarities with C1q in the classical pathway. Complexes of MBL and a C1s-like enzyme named MBL-associated serine protease (MASP)-2 become activated when bound to the target. The resulting sequential cleavage of C4 and C2 creates a C4bC2a complex indistinguishable from the C3 convertase of the classical pathway (Matsushita and Fujita, 1992; Thiel et al., 1997). MASP-2 is inhibited by C1-INH and alpha-2 macroglobulin (Matsushita et al., 2000; Terai et al., 1995). Furthermore, a role for C1-INH in regulating the alternative pathway by a non-covalent binding to C3b was recently postulated (Jiang et al., 2001).

Patients suffering from C1-INH deficiency experience bouts of edema in nearly every organ in the body (Donaldson and Evans, 1963). Many of these patients have had their edema effectively reversed by intravenous infusion of C1-INH concentrate (Agostoni et al., 1980). The concentrate is well tolerated (De Serres et al., 2003). The recognition of C1-INH's pivotal role in controlling the complement system sparked the idea of infusing supraphysiologic doses of C1-INH in conditions where complement activation contributes to the pathophysiology. Such conditions are sepsis, cytokine-induced vascular leak syndrome, acute myocardial infarction, trauma, burns, multiple organ failure and graft rejection (Caliezi et al., 2000; Kirschfink and Mollnes, 2001).

The specific effect of supraphysiologic C1-INH concentration in serum on each of the three initiating complement pathways is largely unknown and has not previously been compared under similar conditions. By use of newly developed assays we therefore examined and compared these effects. The data indicate that C1-INH has at least the same inhibitory effect on the lectin pathway as on the classical pathway. Inhibition of the alternative pathway was observed only for the fluid-phase. The data has implications for the use of C1-INH as a therapeutic reagent and for interpretation of previously published observations.

2. Materials and methods

2.1. Reagents

Sterile phosphate buffered saline (PBS) was obtained from Life Technologies®, Paisley, UK; lepirudin (Refludan®) from Hoechst, Frankfurt am Main, Germany; human albumin and IgG (Octagam) from Octapharma AG, Lachen, Switzerland; cobra venom factor from Quidel, San Diego, CA; mannan and IgG from Sigma–Aldrich, St. Louis, MA; polyclonal antibody against human C4 (A305) from Quidel, San Diego, CA. Heat aggregated immunoglobulin (HAIGG) was made by heating IgG (Kabi, Pharmacia AB, Sweden) at 63 °C for 15 min. Compstatin, a 13 amino acid cyclic peptide which binds to and inhibits cleavage of C3 has been extensively described previously (Sahu et al., 1996). C1-INH (Berinert-P®) from Aventis Behring GmbH, Marburg, Germany Berinert®-P, is separated from cryo-depleted human plasma by adsorption and precipitation steps. According to information from the company the purity is 99%. Subsequently, the purified material is pasteurized by heat treatment in solution for 10 h at 60 °C. The product is further purified by pre-

cipitation and chromatography, sterile filtrated and lyophilized in the absence of preservatives (De Serres et al., 2003).

2.2. Effect of C1-INH on solid-phase complement activation in serum

The effect of C1-INH on the function of classical, lectin and alternative pathways was examined in the Wieslab TM Complement System Screen (Euro-Diagnostica, Malmö, Sweden) (Seelen et al., 2005), which is an enzyme immunoassay (EIA) for specific detection of the three pathways with deposition of C5b-9 as a common read-out. Serum from healthy blood donors was collected and distributed into three different normal human serum pools ($n > 10$ for each). NHS was then added into tubes containing PBS, C1-INH, albumin or ethylenediaminetetraacetic acid (EDTA) whereby NHS was diluted 5/7. Final concentrations of exogenously added C1-INH were 0.5, 2.5 and 5.0 mg/ml. These concentrations of added C1-INH corresponded to 2.8 times (2.8×), 14 times (14×) and 28 times (28×) physiologic values. The ratio of C1-INH to, e.g. that of the activating component C4 is approximately the same. Corresponding final concentrations of added albumin were 0.5, 2.5 and 5.0 mg/ml. Final concentration of EDTA was minimum 20 mM. Identical experiments were done with the same serum pools, including IgG as a supplement. Concentrations of IgG were equivalent to the concentrations of C1-INH and Albumin. Further dilutions were done according to the instructions, i.e. 1:101 for the classical and lectin pathway and 1:18 for the alternative pathway.

2.3. Assessment of classical and lectin pathways at low serum dilutions by EIA

Since the Wieslab Complement System screen experiments are based on high dilution of serum for the classical and lectin pathway, we made additional experiments using close to physiologic serum concentration in newly described assays for the classical (Harboe et al., 2004) and lectin pathways (Harboe et al., 2006). Microtiter wells were coated with IgM 10 µg/ml in PBS or mannan 5 µg/ml in carbonate buffer, pH 9.6, overnight at room temperature. After washing, blocking was performed with PBS containing 1% bovine serum albumin and 0.1% Tween 20. NHS was first diluted 5/7 with PBS, C1-INH or albumin. The final concentration of exogenously added C1-INH and albumin was 0×, 2.8×, 14× and 28× physiologic values. NHS was further diluted 1/2 with veronal buffer pH 7.5 containing 0.5 mM MgCl₂, 2 mM CaCl₂, 0.05% Tween 20 and 0.1% gelatin, 50 µl of each dilution was added to the wells and incubated at 37 °C for 30 min (mannan coated wells) or 60 min (wells coated with IgM). The amount of C4 or TCC deposited on the surface of the washed wells was measured.

2.4. Effect of C1-INH on LP measured in a MBL/MASP functional assay

C4 deposition as a function of MBL/MASP activity was measured by EIA (Petersen et al., 2001). Microtiter wells were coated

with 10 $\mu\text{g/ml}$ mannan in carbonate buffer, pH 9.6, overnight at room temperature. After washing, blocking was performed with 1 mg/ml bovine serum albumin in 10 mM Tris-HCl, 140 mM NaCl, pH 7.4. A serum pool from 20 healthy donors was used as standard, diluted 1/10 and then a twofold dilution series with a buffer containing 1 M NaCl. Three different serum pools were diluted 1/50. Serum from an MBL-deficient donor was used as a control. Standards, samples and controls were added in triplicates to the wells and incubated overnight at +4 °C. A dilution series of C1-INH in concentrations of 0.25, 0.75, 2.75 or 5.25 mg/ml was made. This corresponds to 1 \times , 3 \times , 11 \times or 21 \times physiological concentration. Albumin was diluted correspondingly. After a further 1/50 dilution of either the C1-INH- or the albumin solution, corresponding to the 1/50 serum dilution in the first step, 100 μl was added to the washed wells in a cocktail with 5 $\mu\text{g/ml}$ C4 (Quidel, San Diego, CA). A baseline sample with only PBS and C4 in similar concentration was also added. After incubation at 37 °C for 1.5 h, deposited C4 was detected by the addition of HRP-conjugated anti-human C4 (04-032, Immunsytem, Uppsala, Sweden) diluted 1/1000, and incubated at room temperature for 1.5 h. The substrate solution was 0.3 mM ABTS (Sigma, St. Louis, MO) in 0.15 M acetate buffer, pH 4.0, and 0.024% hydrogen peroxide. Optical density reading at 405 nm.

2.5. Effects of C1-INH on fluid-phase complement activation in a human whole blood model

The model has previously been described in detail (Mollnes et al., 2002). All incubations were performed at 37 °C. All equipment (tubes, tips, etc.) and solutions used in the model were endotoxin-free according to information from the manufacturers. Polypropylene tubes were used to obtain low background activation of complement. Whole blood anti-coagulated with lepirudin 50 $\mu\text{g/ml}$, was collected and distributed immediately into tubes containing PBS, or exogenous C1-INH in final concentrations of 2.8 \times , 14 \times or 28 \times physiologic value, or albumin in equivalent (mg/ml) concentrations. Compstatin, 50 μM final concentration or EDTA, 20 mM were included as controls. The samples were preincubated for 4 min until PBS (baseline samples), HAIGG (0.1 mg/ml) or CVF (0.2 U/ml) were added. After activation for 2 h or 30 min (CVF-incubations), EDTA in 10 mM final concentration was added and the tubes were centrifuged for 15 min at 4000 \times g at 4 °C. The plasma was stored at -70 °C until analyzed for the terminal sC5b-9 complex (TCC) (Mollnes, 1997). As a supplement, blood from three additional donors were activated with CVF according to the guidelines above, including IgG in equivalent concentrations to C1-INH and albumin.

2.6. Detection of factor H in the C1-INH concentrate

The possible presence of factor H in the C1-INH sample was assessed using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and a sandwich ELISA. The latter was performed by coating microtiter plates with 50 μl of 2 $\mu\text{g/ml}$ monoclonal anti-factor H antibody MH22 in PBS for 2 h at room temperature. Then wells were blocked with 200 μl of 1% BSA/PBS for 1 h. Purified factor H or the C1-INH prepa-

ration were serially diluted using 1% BSA/PBS and bound factor H was detected using a 1/1000 dilution of polyclonal anti-factor H antibody.

2.7. Statistics

Effects of increasing doses of C1-INH compared to albumin were analyzed with two-way repeated measurements analysis of variance in Prism (Graphpad Software, San Diego, CA). Bonferroni post-test were used to adjust for repeated measurements. A p -value < 0.05 was considered significant.

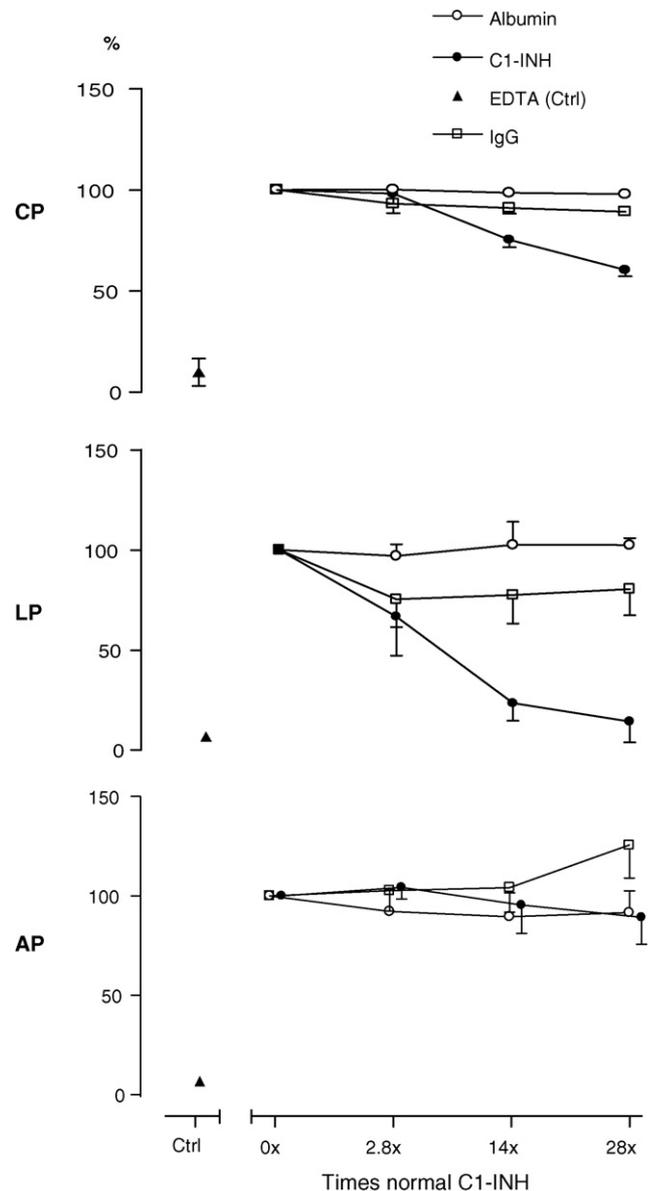


Fig. 1. Effect of C1-INH on solid-phase activation of the three complement pathways. Increasing doses of C1-INH, indicated as times the physiologic concentration in serum (x-axis), was added to normal human serum pools using albumin in equivalent microgram amounts as control. The samples were examined using the Wilelisa kit for specific evaluation of the classical (CP; upper panel), lectin (LP; middle panel) and alternative pathway (AP; lower panel). Readout was deposition of C5b-9, the normal serum control in the assay defining 100% (y-axis). Data are mean and S.E.M. of $n = 6$ experiments.

2.8. Ethics

The study was approved by the Regional Ethical Committee.

3. Results

3.1. Effect of C1-INH on classical-, lectin- and alternative pathway examined in Wielisa

3.1.1. Solid-phase classical pathway (Fig. 1, upper panel)

C1-INH reduced classical pathway mediated C5b-9 deposition dose-dependently ($p=0.0003$). The inhibition was, with Bonferroni post-tests in parentheses, 2% (n.s.), 27% ($p<0.05$) and 40% ($p<0.001$) using 2.8 \times , 14 \times or 28 \times physiologic physiologic concentration of C1-INH.

3.1.2. Solid-phase lectin pathway (Fig. 1, middle panel)

C1-INH reduced lectin pathway mediated C5b-9 deposition dose-dependently ($p<0.0001$). The inhibition was 33% ($p<0.001$), 77% ($p<0.001$) and 86% ($p<0.001$) using 2.8 \times , 14 \times and 28 \times physiologic concentration of exogenous C1-INH.

3.1.3. Solid-phase alternative pathway (Fig. 1, lower panel)

C1-INH in up to 28 \times physiologic concentration had no effect on alternative pathway mediated C5b-9 deposition.

For all the above analyses albumin in equivalent concentrations (w/v) as C1-INH had no significant effect while EDTA reduced C5b-9 deposition by 93% (Fig. 1, all panels). IgG in equivalent concentrations (w/v) reduced C5b-9 deposition in solid phase lectin pathway by 12% without a dose-response and had no significant effects in the classical or alternative pathways.

3.2. Effect of C1-INH on classical and lectin pathway at low serum dilution

Since the first sets of experiments (Wielisa) were done with serum dilution 1:101 for the classical and lectin pathway we examined the effect of C1-INH in these two pathways under low serum dilution, closely resembling physiologic conditions as recently described (Harboe et al., 2006).

3.2.1. Solid-phase classical pathway at low serum dilutions (Fig. 2, upper panel)

C1-INH reduced classical pathway mediated C5b-9 deposition dose-dependently ($p<0.0001$). The inhibition was 23% ($p>0.05$), 70% ($p<0.001$) and 85% ($p<0.001$) using 2.8 \times , 14 \times and 28 \times physiologic concentration of exogenous C1-INH. Albumin in equivalent concentrations had no significant effect, while EDTA reduced C5b-9 deposition by 89%.

3.2.2. Solid-phase lectin pathway at low serum dilutions (Fig. 2, lower panel)

C1-INH reduced lectin pathway mediated C5b-9 deposition dose-dependently ($p<0.0001$). The inhibition was 62% ($p<0.001$), 82% ($p<0.001$) and 81% ($p<0.001$) using 2.8 \times , 14 \times and 28 \times physiologic concentration of exogenous C1-INH.

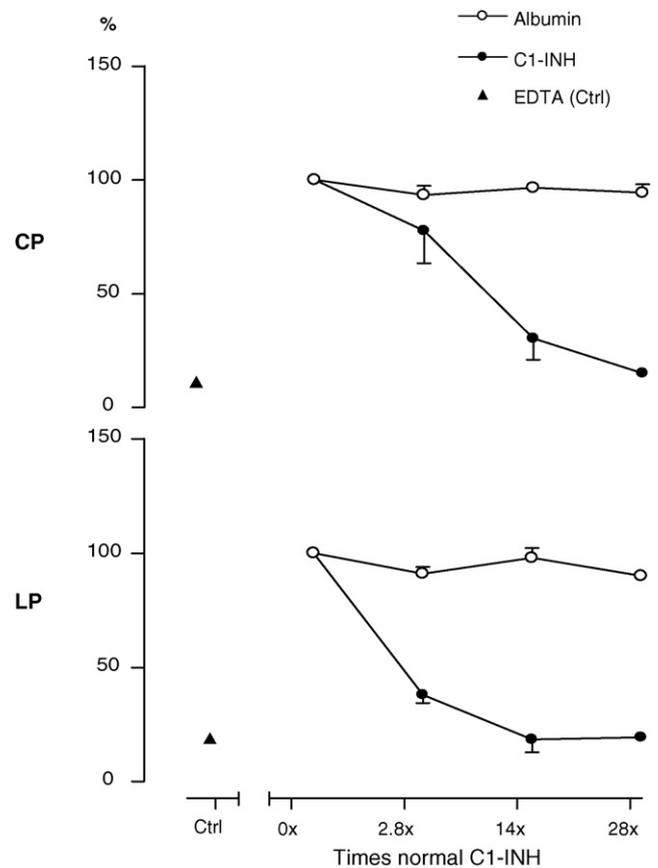


Fig. 2. Effect of C1-INH on solid-phase activation at low serum dilution. Increasing doses of C1-INH, indicated as times the physiologic concentration in serum (x-axis), was added to normal human serum pools using albumin in equivalent microgram amounts as control. The samples were examined using enzyme-immunoassays for the classical (CP; upper panel) and lectin (LP; lower panel) pathway designed for using low serum dilutions (see Section 2). Readout was deposition of C5b-9, a normal serum pool defining 100% (y-axis). Data are mean and S.E.M. of $n=3$ experiments.

Albumin in equivalent concentrations had no significant effect, while EDTA reduced C5b-9 deposition by 81%.

3.3. Functional MBL/MASP assay

C1-INH reduced MBL/MASP-mediated C4 deposition dose-dependently ($p<0.0005$) (Fig. 3). The inhibition was 77% ($p<0.001$), 83% ($p<0.001$), 86% ($p<0.001$) and 86% ($p<0.001$) using 1 \times , 3 \times , 11 \times or 21 \times physiologic concentration of C1-INH. C4 deposition in serum from a MBL-deficient (81 ng/ml) donor was reduced by 82% compared to normal human serum.

3.4. Effect of C1-INH on fluid-phase complement activation

3.4.1. Fluid-phase classical pathway (Fig. 4, upper panel)

The addition of HAIGG to serum led to a marked increase in soluble terminal C5b-9 complement complexes (TCC). C1-INH reduced HAIGG-mediated TCC generation dose-dependently ($p<0.0001$). The inhibition was -12% ($p>0.05$), 41% ($p<0.001$) and 77% ($p<0.001$) using 2.8 \times , 14 \times and

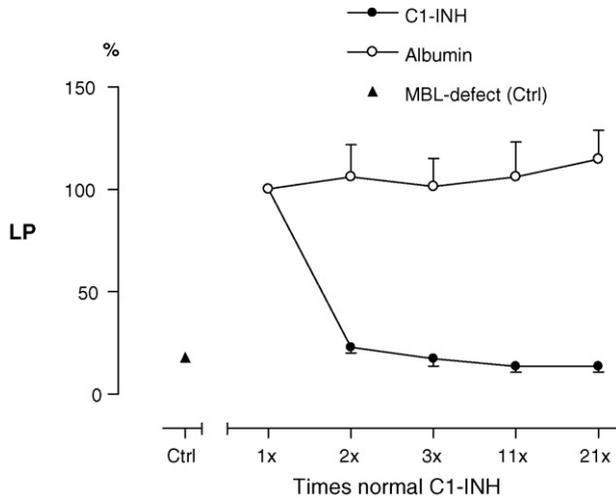


Fig. 3. Effect of C1-INH on functional lectin pathway activation. Standards, normal human serum pools and serum from an MBL-deficient donor (81 ng/ml) were incubated on mannan-coated microtiter wells in high salt concentration to enable binding of MBL/MASPs, but to avoid further activation. After washing, increasing doses of C1-INH, indicated as times the physiologic concentration in the pre-incubated serum (x-axis), were mixed with purified C4 and added to the wells, using albumin in equivalent microgram amounts as control. Readout was deposition of C4, a normal serum pool defining 100% (y-axis). Data are mean and S.E.M. of 3 × 3 experiments.

28 × physiologic concentration of C1-INH. Albumin in equivalent concentrations had no significant effect, while compstatin reduced TCC generation by 96%.

3.4.2. Fluid-phase alternative pathway (Fig. 4, lower panel)

The addition of cobra venom factor to serum led to a marked increase in TCC-generation. C1-INH reduced cobra venom factor-mediated TCC generation dose-dependently ($p < 0.0001$). The inhibition was 7% ($p > 0.05$), 59% ($p < 0.001$) and 87% ($p < 0.001$) using 2.8 ×, 14 × and 28 × physiologic concentration of C1-INH. Albumin and IgG in equivalent microgram amounts had no significant effect, while EDTA reduced TCC-generation by 99%.

3.5. Analysis of factor H in the C1-INH concentrate

The purity of the C1-INH concentrate, including a possible contamination by factor H, was assessed by SDS-PAGE and ELISA (Fig. 5). The C1-INH preparation was highly pure, containing a major protein band corresponding to the molecular weight of the active protein (105 kDa) and a weak smaller protein band corresponding to inactive C1-INH (90 kDa) (Fig. 5A). No other bands were observed; in particular no bands with molecular weight similar to factor H. Similarly, when the C1-INH preparation was tested in a sandwich ELISA for the detection of factor H, no activity was present (Fig. 5B).

4. Discussion

C1-INH's effects on the three initiating pathways of complement varied considerably. The classical pathway solid-phase

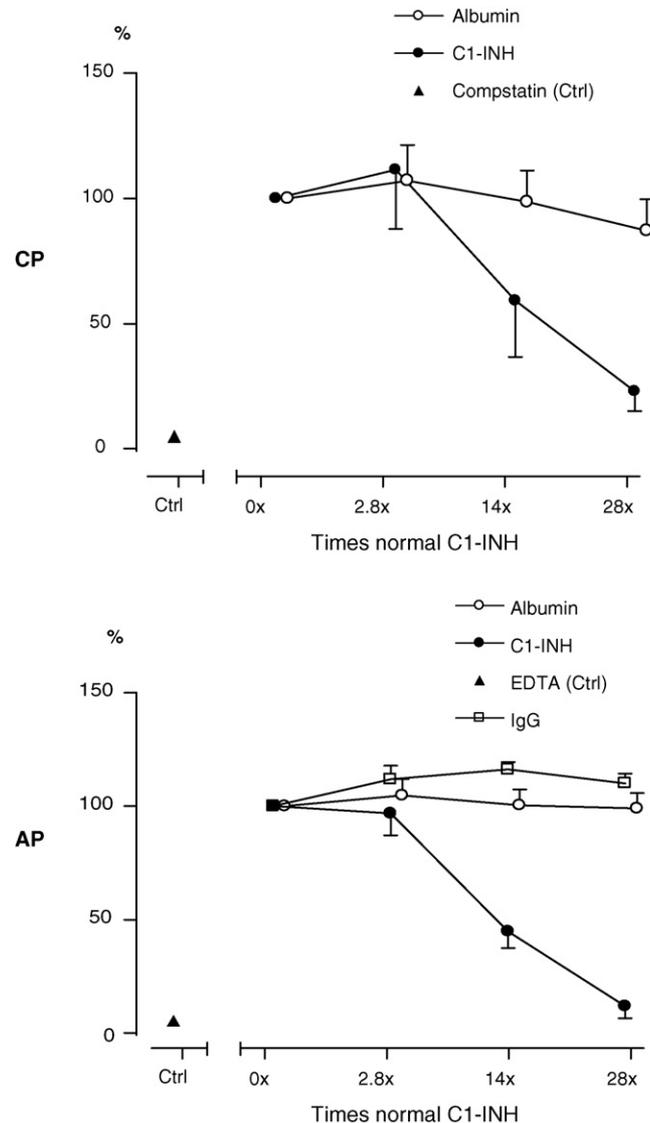


Fig. 4. Effect of C1-INH on fluid-phase complement activation. Increasing doses of C1-INH, indicated as times the physiologic concentration in serum (x-axis), was added to human whole blood and further incubated with heat aggregated IgG for classical pathway activation (CP; upper panel) and cobra venom factor for alternative pathway activation (AP; lower panel). Albumin in equivalent microgram amounts was used as control in both experiments. Readout was formation of the soluble terminal C5b-9 complement complex (TCC), the amount obtained in the absence of C1-INH defining 100% (y-axis). Data are mean and SEM of six experiments using different donors each time. IgG in equivalent microgram amounts was used as an additional control in three extra donors (AP; lower panel).

was only modestly inhibited by C1-INH when high serum dilution was used, but at low serum dilutions which are more close to the physiological conditions the effect was more pronounced. Notably, however, low C1-INH concentration only slightly reduced the activation and doses of 14–28 times physiologic concentration was needed to abolish the activation. This is somewhat surprisingly as C1-INH is the only known regulator of C1. The early works by Ziccardi (1982), however, described C1-INH as a poor inhibitor once C1 was activated by immune complexes. The speed by which immune complexes could activate C1 was believed to overwhelm C1-INH. On the other hand,

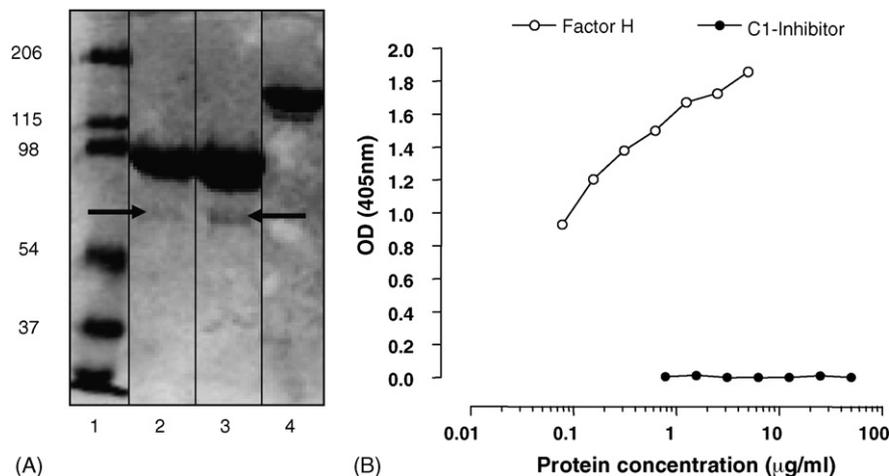


Fig. 5. Purity of the C1-INH concentrate. (A) The C1-INH inhibitor preparation in two concentrations (lane 2: 5 μ g; lane 3: 10 μ g) and purified factor H (lane 4: 5 μ g) were subjected to SDS-PAGE. C1-INH showed one major band corresponding to the active protein and one minor band (arrows) corresponding to inactivated C1-INH. No other bands were observed, in particular no corresponding to the molecular weight of factor H. lane 1: Molecular weight markers (37, 54, 98, 115 and 206 kDa). (B) The C1-INH preparation was examined in a sandwich ELISA for detection of factor H, using purified factor H as positive control. No factor H (lower detection limit below 0.1 μ g/ml in this assay) could be found in the C1-INH concentrate using concentrations up to 50 μ g/ml.

was non-immune activation of C1 efficiently controlled, and interpreted as C1-INH's primary role. The IgM coating in the wells, used in both classical pathway solid-phase assays in this paper, is a very potent and rapid activator of C1. HAIGG added to undiluted whole blood may activate C1 even more forcefully as the highest dose of C1-INH was needed to get a substantial inhibition of fluid-phase classical pathway activation. Our results fit partly in with the findings of Doekes et al. (1983) A C1-INH/C1 ratio of 8, as in normal serum, nearly abolished soluble IgG-induced C4 consumption. The effect of increasing the C1-INH/C1 ratio from 10 to 100, nearly corresponding to our dose of 14 times physiologic concentration, was marginal. The effect of increasing C1-INH/C1 ratio from zero to normal values was, on the other hand, formidable in their study. This lead Doekes et al. (1983) to suggest that C1-INH-deficient HAE patients have most of their C4 split due to small amounts of circulating non-detectable immune complexes.

Only the solid-phase of the lectin pathway can be examined due to lack of specific fluid-phase assays. We have shown an almost complete inhibition of the MBL-mediated lectin pathway activation by C1-INH. One should keep in mind, though, that ficolins were not tested. As for the classical pathway the effect of C1-INH was more pronounced when examined in low dilutions of serum. Even at three times physiologic C1-INH concentration there was an extensive reduction of lectin pathway activation. The results were even more pronounced in the functional MBL/MASP assay. The efficient inhibition of C4 deposition in this assay indicates that the effect was at the level of MASP-2. This is in accordance with previous studies showing that C1-INH inactivates this protease (Ambrus et al., 2003; Gulati et al., 2002; Petersen et al., 2000). Apparently C1-INH is a more potent regulator of the lectin pathway than of the classical pathway. If so, the fact that C1-INH is in much larger excess to MASP-2 than to C1 could play a role. In normal serum the ratio of C1-INH to MASP-2 is 250–300 while the ratio to C1q is 0.29. As opposed to the classical pathway where a reciprocal activa-

tion by C1r and C1s is in action, MASP-2 appears to autoactivate and split C4 independently of other MASPs (Presanis et al., 2004). This difference could make MASP-2 easier to control by C1-INH. Furthermore could C1-INH stabilize the MBL/MASP complex the way C1-INH does to C1, which is different from that of protease-binding.

We found a pronounced inhibitory effect of C1-INH on cobra venom factor-induced fluid phase activation of the alternative pathway at the highest C1-INH concentrations, whereas no effect was seen on solid-phase activation by lipopolysaccharide. A possible contamination with alternative pathway inhibitors should therefore be considered. However, SDS-PAGE documented a pure preparation without any indication of contaminating proteins, in accordance with information from the producer of a 99% purity, and ELISA experiments excluded the possibility that the C1-INH concentrate was contaminated with factor H. Theoretically any complement inhibitor could have influenced the activity, but factor H is in our opinion the single one candidate we needed to exclude due to its efficacy and high serum concentration compared to the other candidates. Furthermore, it is one of the complement inhibitory proteins that most frequently exists as contaminant after protein purification. Despite this, there was no factor H protein present which could explain our findings. A main reason for this is the highly purified C1-inhibitor preparation, making it even less likely that other proteins could explain some of our findings.

Until recently, C1-INH has not been regarded as a mediator of alternative pathway regulation. However, one study has shown that C1-INH in normal serum and in approximately two times above physiologic value downregulates the alternative pathway. Inhibition by C1-INH of the alternative C3 convertase activity via non-covalent binding to C3b and to cobra venom factor has been suggested (Jiang et al., 2001). Our results are in accordance with these observations and suggest an inhibitory effect of C1-INH in supraphysiologic doses on the alternative pathway.

A major part of C1-INH's therapeutic success has been in animal and human ischemia-reperfusion injuries (IRI) (Bergamaschini and Cicardi, 2003). The effect we observed on the classical pathway could have been involved in IgM-dependent and probably other classical pathway-mediated mechanisms of IRI (Weiser et al., 1996; Williams et al., 1999; Zhang et al., 2004). Recently, C1-INH's beneficial effect on cerebral IRI in mice also were found to be partly independent of C1q (De Simoni et al., 2004). The possibility of regulating the lectin pathway by C1-INH is attractive as this pathway was found to be central in, e.g. murine myocardial IRI (Jordan et al., 2001), murine renal IRI (de Vries et al., 2004) and IRI after human thoraco-abdominal aneurism repair (Fiane et al., 2003). The latter paper also described how the lectin pathway, once activated, was amplified by the alternative pathway. The role of the alternative pathway as amplification loop was recently documented also for the classical pathway, showing that more than 80% of terminal pathway activation induced by the classical pathway was indeed mediated through alternative amplification (Harboe et al., 2004). Thus, in vivo, the initial activation mechanism through classical and/or lectin pathway activation in IRI may be greatly enhanced through the alternative pathway. Furthermore, a recent study suggested an important and primary role for the alternative complement pathway in local and remote tissue injury after gastrointestinal IRI in mice (Stahl et al., 2003). Our data indicate that C1-INH in high physiologic concentration may have a selective impact on all three complement activation pathways.

Many earlier and even current demonstrations of C1-INH's efficiency in various pathophysiological and clinical conditions were often and intuitively ascribed to inhibition of the classical pathway. Our findings, suggesting a potent inhibition by C1-INH of the lectin pathway, emphasize this pathway to be considered, in particular since C4 is activated both through the classical and lectin pathway and should not be considered specific for the classical. Furthermore, our findings of inhibition of the alternative pathway also have to be taken into account when interpreting C1-INH's effects. Additionally, C1-INH may regulate processes in which proteases are not involved (Liu et al., 2005; Davis et al., 2004). Finally, complement is but one of several important biological cascades where C1-INH is an important regulator. Notably, coagulation, fibrinolysis and the kallikrein-kinin system are also involved in inflammation and IRI (Schoenmakers et al., 2005; Souza et al., 2003). In several diseases a tailored therapy of the immune system is wanted. In human diseases in which cascade system activation is undesired, C1-INH in obtainable doses could be useful.

5. Conclusions

The present data indicate that C1-INH in supraphysiologic doses in human serum inhibits solid-phase classical- and lectin pathway activation in a similar manner, but with a more pronounced effect on the lectin pathway at low doses. C1-INH also inhibited alternative pathway activation, but this was limited to fluid-phase cobra venom factor mediated activation. To our knowledge this is the first study documenting and comparing the

effect of C1-INH on all three complement pathways under similar conditions. The data have implications for the interpretation of previous studies using C1-INH to modulate the inflammatory reaction and for C1-INH as a therapeutic agent in the future.

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References

- Agostoni, A., Bergamaschini, L., Martignoni, G., Cicardi, M., Marasini, B., 1980. Treatment of acute attacks of hereditary angioedema with C1-inhibitor concentrate. *Ann. Allergy* 44, 299–301.
- Ambrus, G., Gal, P., Kojima, M., Szilagyi, K., Balczer, J., Antal, J., Graf, L., Laich, A., Moffatt, B.E., Schwaeble, W., Sim, R.B., Zavodszky, P., 2003. Natural substrates and inhibitors of mannan-binding lectin-associated serine protease-1 and -2: a study on recombinant catalytic fragments. *J. Immunol.* 170, 1374–1382.
- Bergamaschini, L., Cicardi, M., 2003. Recent advances in the use of C1 inhibitor as a therapeutic agent. *Mol. Immunol.* 40, 155–158.
- Caliezi, C., Wuillemin, W.A., Zeerleder, S., Redondo, M., Eisele, B., Hack, C.E., 2000. C1-esterase inhibitor: an anti-inflammatory agent and its potential use in the treatment of diseases other than hereditary angioedema. *Pharmacol. Rev.* 52, 91–112.
- Davis III, A.E., Cai, S., Liu, D., 2004. The biological role of the C1 inhibitor in regulation of vascular permeability and modulation of inflammation. *Adv. Immunol.* 82, 331–363.
- De Serres, J., Groner, A., Lindner, J., 2003. Safety and efficacy of pasteurized C1 inhibitor concentrate (Berinert(R) P) in hereditary angioedema: a review. *Transfus. Apheresis. Sci.* 29, 247–254.
- De Simoni, M.G., Rossi, E., Storini, C., Pizzimenti, S., Echart, C., Bergamaschini, L., 2004. The powerful neuroprotective action of C1-inhibitor on brain ischemia-reperfusion injury does not require C1q. *Am. J. Pathol.* 164, 1857–1863.
- De Vries, B., Walter, S.J., Peutz-Kootstra, C.J., Wolfs, T.G.A.M., Van Heurn, L.W.E., Buurman, W.A., 2004. The mannose-binding lectin-pathway is involved in complement activation in the course of renal ischemia-reperfusion injury. *Am. J. Pathol.* 165, 1677–1688.
- Doekes, G., Van-Es, L.A., Daha, M.R., 1983. C1 inactivator: its efficiency as a regulator of classical complement pathway activation by soluble IgG aggregates. *Immunology* 49, 215–222.
- Donaldson, V.H., Evans, R.R., 1963. A biochemical abnormality in hereditary angioneurotic edema: absence of serum inhibitor of C1-esterase. *Am. J. Med.* 35, 37–44.
- Donaldson, V.H., Rosen, F.S., 1964. Action of complement in hereditary angioneurotic edema: role of C1-esterase. *J. Clin. Invest.* 43, 2204–2213.
- Fiane, A.E., Videm, V., Lingaas, P.S., Heggelund, L., Nielsen, E.W., Geiran, O.R., Fung, M., Mollnes, T.E., 2003. Mechanism of complement activation and its role in the inflammatory response after thoracoabdominal aortic aneurysm repair. *Circulation* 108 (7), 849–856.
- Gronski, P., Bodenbender, L., Kanzy, E.J., Seiler, F.R., 1988. C4-binding protein prevents spontaneous cleavage of C3 in sera of patients with hereditary angioedema. *Complementary* 5, 1–12.
- Gulati, S., Sastry, K., Jensenius, J.C., Rice, P.A., Ram, S., 2002. Regulation of the Mannan-binding lectin pathway of complement on *Neisseria gonorrhoeae* by C1-Inhibitor and {alpha}2-macroglobulin. *J. Immunol.* 168, 4078–4086.
- Harboe, M., Garred, P., Borgen, M.S., Stahl, G.L., Roos, A., Mollnes, T.E., 2006. Design of a complement mannose-binding lectin pathway-specific activation system applicable at low serum dilutions. *Clin. Exp. Immunol.* 144, 512–520.

- Harboe, M., Ulvund, G., Vien, L., Fung, M., Mollnes, T.E., 2004. The quantitative role of alternative pathway amplification in classical pathway induced terminal complement activation. *Clin. Exp. Immunol.* 138, 439–446.
- Jiang, H., Wagner, E., Zhang, H., Frank, M.M., 2001. Complement 1 inhibitor is a regulator of the alternative complement pathway. *J. Exp. Med.* 194, 1609–1616.
- Jordan, J.E., Montalto, M.C., Stahl, G.L., 2001. Inhibition of mannose-binding lectin reduces postischemic myocardial reperfusion injury. *Circulation* 104, 1413–1418.
- Kawasaki, N., Kawasaki, T., Yamashina, I., 1989. A serum lectin (mannan-binding protein) has complement-dependent bactericidal activity. *J. Biochem. (Tokyo)* 106, 483–489.
- Kirschfink, M., Mollnes, T.E., 2001. C1-Inhibitor: an anti-inflammatory reagent with therapeutic potential. *Expert. Opin. Pharmacother.* 2, 1073–1083.
- Klein, J., 1990. Complement. In: Klein, J. (Ed.), *Immunology*. Blackwell Scientific Publication, London, pp. 188–226.
- Landerman, N.S., Webster, M.E., Becker, E.L., Ratcliffe, H.E., 1962. Hereditary angioneurotic edema. II. Deficiency of inhibitor for serum globulin permeability factor and/or plasma kallikrein. *J. Allergy* 33, 330–341.
- Liu, D., Zhang, D., Scafidi, J., Wu, X., Cramer, C.C., Davis III, A.E., 2005. C1 inhibitor prevents Gram-negative bacterial lipopolysaccharide-induced vascular permeability. *Blood* 105, 2350–2355.
- Lynch, N.J., Roscher, S., Hartung, T., Morath, S., Matsushita, M., Maennel, D.N., Kuraya, M., Fujita, T., Schwaeble, W.J., 2004. L-ficolin specifically binds to lipoteichoic acid, a cell wall constituent of gram-positive bacteria, and activates the lectin pathway of complement. *J. Immunol.* 172, 1198–1202.
- Matsushita, M., Fujita, T., 1992. Activation of the classical complement pathway by Mannose-binding protein in association with a novel C1s-like serine protease. *J. Exp. Med.* 176, 1497–1502.
- Matsushita, M., Thiel, S., Jensenius, J.C., Terai, I., Fujita, T., 2000. Proteolytic activities of two types of mannose-binding lectin-associated serine protease. *J. Immunol.* 165, 2637–2642.
- Mollnes, T.E., 1997. Analysis of in vivo complement activation. In: Herzenberg, L.A., Weir, D.M., Blackwell, C. (Eds.), *Weir's handbook of experimental immunology*. Blackwell Science, Cambridge, MA, USA.
- Mollnes, T.E., Brekke, O.L., Fung, M., Fure, H., Christiansen, D., Bergseth, G., Videm, V., Lappegard, K.T., Kohl, J., Lambris, J.D., 2002. Essential role of the C5a receptor in E coli-induced oxidative burst and phagocytosis revealed by a novel lepirudin-based human whole blood model of inflammation. *Blood* 100, 1869–1877.
- Nielsen, E.W., Johansen, H.T., Gaudesen, Ø., Osterud, B., Olsen, J.O., Hogasen, K., Hack, C.E., Mollnes, T.E., 1995. C3 is activated in hereditary angioedema, and C1/C1-inhibitor complexes rise during physical stress in untreated patients. *Scand. J. Immunol.* 42, 679–685.
- Nielsen, E.W., Johansen, H.T., Hogasen, K., Willemin, W.A., Hack, C.E., Mollnes, T.E., 1996. Activation of the complement, coagulation, fibrinolytic and kallikrein-kinin systems during attacks of hereditary angioedema. *Scand. J. Immunol.* 44, 185–192.
- Pensky, J., Levy, L.R., Lepow, I.H., 1961. Partial purification of a serum inhibitor of C'1-esterase. *J. Biol. Chem.* 236, 1674–1679.
- Petersen, S.V., Thiel, S., Jensen, L., Steffensen, R., Jensenius, J.C., 2001. An assay for the mannan-binding lectin pathway of complement activation. *J. Immunol. Methods* 257, 107–116.
- Petersen, S.V., Thiel, S., Jensen, L., Vorup-Jensen, T., Koch, C., Jensenius, J.C., 2000. Control of the classical and the MBL pathway of complement activation. *Mol. Immunol.* 37, 803–811.
- Presanis, J.S., Hajela, K., Ambrus, G., Gal, P., Sim, R.B., 2004. Differential substrate and inhibitor profiles for human MASP-1 and MASP-2. *Mol. Immunol.* 40, 921–929.
- Sahu, A., Kay, B.K., Lambris, J.D., 1996. Inhibition of human complement by a C3-binding peptide isolated from a phage-displayed random peptide library. *J. Immunol.* 157, 884–891.
- Schoenmakers, S.H., Reitsma, P.H., Spek, C.A., 2005. Blood coagulation factors as inflammatory mediators. *Blood Cells Mol. Dis.* 34, 30–37.
- Seelen, M.A., Roos, A., Wieslander, J., Mollnes, T.E., Sjöholm, A.G., Wurzner, R., Loos, M., Tedesco, F., Sim, R.B., Garred, P., Alexopoulos, E., Turner, M.W., Daha, M.R., 2005. Functional analysis of the classical, alternative, and MBL pathways of the complement system: standardization and validation of a simple ELISA. *J. Immunol. Methods* 296, 187–198.
- Souza, D.G., Pinho, V., Pesquero, J.L., Lomez, E.S., Poole, S., Juliano, L., Correa Jr., A., De, A.C., Teixeira, M.M., 2003. Role of the bradykinin B2 receptor for the local and systemic inflammatory response that follows severe reperfusion injury. *Br. J. Pharmacol.* 139, 129.
- Stahl, G.L., Xu, Y., Hao, L., Miller, M., Buras, J.A., Fung, M., Zhao, H., 2003. Role for the alternative complement pathway in ischemia/reperfusion injury. *Am. J. Pathol.* 162, 449–455.
- Terai, I., Kobayashi, K., Matsushita, M., Fujita, T., Matsuno, K., 1995. Alpha 2-Macroglobulin binds to and inhibits mannose-binding protein-associated serine protease. *Int. Immunol.* 7, 1579–1584.
- Thiel, S., Vorup-Jensen, T., Stover, C.M., Schwaeble, W., Laursen, S.B., Poulsen, K., Willis, A.C., Eggleton, P., Hansen, S., Holmskov, U., Reid, K.B., Jensenius, J.C., 1997. A second serine protease associated with mannan-binding lectin that activates complement. *Nature* 386, 506–510.
- Weiser, M.R., Williams, J.P., Moore Jr., F.D., Kobzik, L., Ma, M., Hechtman, H.B., Carroll, M.C., 1996. Reperfusion injury of ischemic skeletal muscle is mediated by natural antibody and complement. *J. Exp. Med.* 183, 2343–2348.
- Williams, J.P., Pechet, T.T., Weiser, M.R., Reid, R., Kobzik, L., Moore, F.D.J., Carroll, M.C., Hechtman, H.B., 1999. Intestinal reperfusion injury is mediated by IgM and complement. *J. Appl. Physiol.* 86, 938–942.
- Zhang, M., Austen Jr., W.G., Chiu, I., Alicot, E.M., Hung, R., Ma, M., Verna, N., Xu, M., Hechtman, H.B., Moore Jr., F.D., Carroll, M.C., 2004. Identification of a specific self-reactive IgM antibody that initiates intestinal ischemia/reperfusion injury. *PNAS* 101, 3886–3891.
- Ziccardi, R.J., 1982. A new role for C-1-inhibitor in homeostasis: control of activation of the first component of human complement. *J. Immunol.* 128, 2505–2508.