# THE COMPLEMENT REGULATORS C1 INHIBITOR AND SOLUBLE COMPLEMENT RECEPTOR 1 ATTENUATE ACUTE LUNG INJURY IN RABBITS

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ABSTRACT—Because activation of the complement system plays a major role in the pathogenesis of acute lung injury, the availability of new specific complement inhibitors represents a promising therapeutic approach. In the present study we investigated pulmonary edema formation and pulmonary artery pressure (PAP) in acute complement-induced lung injury for possible therapeutic impact of the complement regulators C1 inhibitor and soluble complement receptor 1. Eighteen isolated and ventilated rabbit lungs were perfused with pooled normal human serum (NHS, final concentration 35%) in Krebs-Henseleit buffer in a recirculating system. Lung weight gain and PAP were continuously recorded. Complement activation was blocked by the addition of C1 inhibitor (1.0 U/mL, n = 6) or sCR 1 (2.0 μg/mL, n = 6). Lungs that received NHS without inhibitors served as controls (n = 6). This study was performed according to the Helsinki Declaration and approved by the local government. Application of NHS resulted in an increase of PAP within 20 min from 8 ± 2 to 42 ± 6 mmHg, which was significantly ($P < 0.05$) decreased by C1-Inh (25 ± 5 mmHg) and sCR1 (20 ± 3 mmHg). Moreover, pulmonary edema formation after NHS, as assessed by overall weight gain, was reduced by both C1-Inh and sCR1, compared with controls. These findings were paralleled with significantly decreased thromboxane release rates and reduced tissue deposition of C3c and C5b-9. C1 inhibitor and sCR1 attenuate the complement-induced pulmonary capillary leakage and PAP increase, indicating the protective effect of complement inhibition in isolated perfused rabbit lungs.

KEYWORDS—complement, sCR1, C1 inhibitor, ARDS, C5b-9

## INTRODUCTION

Severe injury induces massive release of proinflammatory mediators predisposing the host to an increased susceptibility to infection, which often leads to acute respiratory disease syndrome (ARDS) or multiple organ failure (1). Activation of humoral and cellular cascade systems can be achieved by a variety of stimuli. Because inflammatory cascade systems are functionally closely related, induction of one system activates further inflammatory cascades, thereby perpetuating inflammation (2). The value of complement activation products as a parameter for the prediction of ARDS and sepsis development and for clinical outcome has been demonstrated in several clinical trials (3, 4). Moreover, depletion of complement with cobra venom factor or selective inhibition of the complement system by C1 inhibitor (C1-Inh) or soluble complement receptor 1 (sCR1) can preserve endothelial function and limit the degree of tissue injury in states of myocardial (5) or traumatic tissue injury (6). C1-Inh, a 105-kD α-2-neuraminoglycoprotein, is a member of the serine protease inhibitor superfamily (serpins) (7). C1-Inh is the only inhibitor of activated C1s and C1r of the classical pathway of complement (8) but also serves as a major regulator of both factor XIIa (9) and kallikrein of the contact system (10). In septic state, reduced levels of functional C1-Inh have been reported, pointing toward C1-Inh consumption after activation of coagulation-, complement- or contact systems (11). Complement receptor 1 (CR1, CD35), a single-chain membrane-bound glycoprotein with a dual function serves as potent regulator of C3/C5 convertases of both the classical and the alternate activation pathway (12) and mediates phagocytosis of C3b-opsonized targets (13). The extramembranous part of CR1 found in plasma was shown to retain full regulatory activity (5).

Until now few data exist concerning the effects of complement inhibition on the development of respiratory distress. Activation of the complement system plays a major role in the pathogenesis of acute respiratory distress and down-regulation of the complement cascade has demonstrated beneficial effects in a variety of human (14) and animal studies (15). Because the availability of new specific complement inhibitors could represent a promising therapeutic approach (for review, see 16), we hypothesized that blockage of the complement cascade with C1-Inh or sCR1 at an early step of the complement activation process might influence the course of complement-induced respiratory distress. Complement-dependent pulmonary vascular effects have been observed without significant participation of leukocytes in animals (17) and in humans (18). Thus, we in-

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vestigated the impact of complement inhibition on nongranulocyte-dependent complement effects in isolated blood-free perfused rabbit lungs (19, 20). Although rabbit cell surfaces spontaneously activate the human complement system, predominately via the alternate pathway (21), the investigation of the differential therapeutic effects of sCR-1 (both pathways) and C1-Inh (classical pathway) appeared to be of particular interest.

## MATERIALS AND METHODS

### Isolated perfused rabbit lung

The techniques of preparing and perfusing isolated rabbit lungs have been previously described in detail (19). Female chinchilla rabbits (orticolagus caniculus) weighing 2100 ± 196 g (mean ± SD) were anesthetized with ketamine (50 mg/kg) and xylazine (4 mg/kg) and anticoagulated with heparin-sodium (1000 U/kg) injected in the ear vein. After placement of a tracheostomy tube, the rabbits were mechanically ventilated with room air by means of a respirator (Servo Ventilator 900D; Siemens, Elema, Sweden). The thorax was opened via the diaphragm and after a median sternotomy, a catheter was inserted into the pulmonary artery. The lung organ preparation was isolated and suspended from a weight transducer (Hottinger & Baldwin Meβtechnik Type U1; Darmstadt, Germany) in a temperature-controlled (37°C) and humidified chamber. After the cannulation procedure, the lungs were perfused with 200 mL of Krebs-Henseleit hydroxy-ethyl-starch buffer solution (KHHB) by a roller pump (Masterflex 7566-10; Cole Palmer Instruments Co., Chicago, IL) at a constant volume inflow of 150 mL/min in a recirculating system. The lungs were ventilated with 4% $CO_2$ in air (frequency 25/min, tidal volume 30 mL, positive end-expiratory pressure [PEEP] 0.5–1.0 mbar) and, to avoid atelectasis formation, intermittently flushed by increasing the expiratory pressure up to 3 mbar for 3 inspirations. The pulmonary arterial (PAP) and airway pressures (AP) were continuously recorded via Statham strain gauge transducers. Because of a constant perfusion flow, alterations of perfusion pressure directly reflect alterations of pulmonary vascular resistance. Intermittently, samples of perfusate were taken for measurements of pH, $pO_2$, $pCO_2$, $O_2$ saturation (blood gas analysis system 288; Ciba Corning, Fernwald, Germany), oncotic pressure (Onkometer BMT 921; Dr. Karl Thomae GmbH, Germany) and for determination of thromboxane (TX) $B_2$ and C5b-9 concentrations. Initially, the lungs were perfused with KHHB solution by using low-flow rates in the opened circulatory system to remove remaining blood from the vascular bed. The perfusion fluid was then exchanged for fresh buffer via two separate perfusion circuits 2 min after the beginning of the extracorporeal circulation and 15 min later, after the flow was increased to 150 mL/min. After another 30-min steady-state period, these lungs had a constant mean PAP of 7.8–9.1 mmHg (zero-referenced at the hilum). The only lungs selected for the study were those that showed a homogenous white appearance with no signs of hemostasis or edema formation and that were completely isogravimetric during the steady-state period. The weight gain of the lungs expressed in g was continuously monitored. In previous experiments, the perfusion with KHHB was documented to maintain integrity of the microcirculation for more than 5 h in our model, which was assessed by measurements of PAP and weight gain, by biochemical analysis (lactate dehydrogenase [LDH], AA metabolites, histamine) and by ultrastructural studies. During the observation period, neither significant alterations in LDH, eicosanoids, and histamine release nor structural abnormalities (e.g., destruction of endothelial or epithelial cells) were found.

### Materials

A cell- and plasma-free perfusion medium was used in the present study to avoid the complex interactions with different circulating cells, which may mask direct effects on vascular tone and mediator release. The perfusate consisted of a Krebs-Henseleit buffer solution with a colloid oncotic pressure of 23–25 mmHg, yielding in final concentrations of $Na^+$: 138 mmol/L; $K^+$: 4.5 mmol/L; $Mg^{++}$: 1.33 mmol/L; $Cl^-$: 135 mmol/L; $Ca^{++}$: 2.38 mmol/L; glucose: 12 mmol/L; $HCO_3^-$: 12 mmol/L. The osmolality was approximately 330 mosm/kg (Mikro-Osmometer; Roebling Meβtechnik, Berlin, Germany). The pH of the buffer solution was adjusted to 7.4 with 1 M $NaHCO_3$. Effects due to possible endotoxin contamination of the plasma-free perfusate can be excluded in our model, as assessed in previous experiments. No hemodynamic reactions, thromboxane generation, or histamine release were observed after endotoxin addition to the perfusate in the absence of plasma complement components.

C1-Inh (Berinert HS) Lot 0736211 was a generous gift from Dr. Henkel, Centeon Pharma GmbH, Marburg, Germany. Human rsCR-1 was kindly provided by Dr. Levin, T-Cell Sciences Inc., Needham, Massachusetts. Doses were chosen according to previous studies (22, 23) and on the basis of our own dose-response experiments. Normal human serum (NHS) was prepared from blood of healthy blood donors.

### Complement activation

In situ complement activation was achieved by admixture of normal human serum to the perfusate of isolated rabbit lungs (final concentration 35%). Preliminary dose-response studies demonstrated dose-dependent pulmonary hypertension and lung edema formation (24). Because no differences have been reported between the responses to human serum and human plasma (25) human serum was used in our protocol.

### Experimental protocol

Eighteen lung preparations were randomly assigned to 3 groups. After a 30-min equilibration period, the first perfusate sample was drawn for measurements of baseline values. Thereafter, NHS was applied in a final concentration of 35% in the perfusion fluid. Subsequently, samples of perfusate were taken at 1, 5, 10, and 30 min after NHS injection to investigate mediator release. Six preparations in which NHS was given in absence of any inhibitor served as controls. The same protocol was performed in experiments, in which either the C1-Inh (1.0 U/mL) or sCR-1 (2.0 μg/mL) were added to the NHS before injection into the perfusion circuit. The doses of the inhibition compounds were chosen according to previous publications (26) and preceding dose-response studies in our model (24).

### Measurement of $TXB_2$

Thromboxane $B_2$ ($TXB_2$) was assayed from 100 μL of recirculating KHHB as stable hydrolysis product of thromboxane $A_2$ by an enzyme immunoassay (TiterZyme; $TXB_2$ EIA, PerSeptive Diagnostics Inc., Framingham, MA), according to the manufacturers' specifications. The cross-reactivity of $TXB_2$-antiserum with 2,3-Dinor-$TXB_2$ was 55.8%, 1.5% with 11-Dehydro $TXB_2$, 1.0% with prostaglandin $B_2$ ($PGB_2$) and 0.5% with $PGD_2$. Other eicosanoids exert cross-reactivity of <0.1%. Because a recirculating perfusion circuit was used in the present experimental setup, inactive $TXB_2$ accumulates during the observation period. Thus, release rates of $TXB_2$ were calculated from subsequent $TXB_2$ values in the perfusion buffer.

### Analysis of complement activation in the perfusate

For complement analysis, ethylenediaminetetraacetic acid (EDTA)-perfusate samples were stored deep frozen (−80°C) until measurement. C5b-9 was determined by an enzyme-linked immunosorbent assay, as previously described by Kotnik and coworkers (27).

### Immunohistoiogic analysis of C3c and C5b-9 tissue binding

After the observation period, tissue samples were randomly taken from the lungs. The lung specimens were fixed in 4% paraformaldehyde, 3.8% Na-tetraborate (pH 9.5) for 30 min at room temperature (RT) and thereafter in 4% paraformaldehyde, 3.8% Na-tetraborate, 10% sucrose (pH 9.5) for 12 h at 4°C. The tissue was embedded in Tissue Tec nitro-gel (Miles Inc., Elkhard, IN), shock frozen in liquid $N_2$ and stored at −80°C until immunostaining.

Briefly, after pretreatment with microwave (750 W) in citrate buffer solution (pH 6.0) for 2 × 10 min, lung tissue slices were incubated with primary rabbit anti-C3c IgG (Dako, Hamburg, Germany) at a dilution of 1:300 for 20 min at RT. Secondary antibodies (peroxidase labeled goat anti-rabbit IgG 1:400, Dako) were subsequently incubated at RT for 1 h and developed with diaminobenzidine (Sigma, Deisenhofen, Germany). For detection of the membrane attack complex lung tissues were incubated for 30 min at RT with monoclonal mouse anti C5b-9 (Dako, Hamburg, Germany; 1:100) followed by catalyzed signal amplification (CSA; Dako).

Semiquantitative evaluation was conducted by an experienced pathologist

(M.H.) who was unaware of the experimental protocol. Observed immunostaining was considered as strong (+++), clear (++), or slightly over background (+).

### Statistical analysis and data presentation

Data are presented as means ± standard error of means (SEM). Differences between groups were tested by one-way analysis of variance (ANOVA) followed by a Student Newman-Keuls multiple comparison procedure. Significance was accepted at $P < 0.05$.

This study was approved by the Animal Subject Protection Committee of the local government. The care and handling of animals were in accordance with the principles expressed in the Helsinki Declaration.

## RESULTS

### Pulmonary artery pressure

Baseline values of PAP (7–9 mmHg) and airway pressure between 10 and 12 mbar were similar in all groups and in agreement with previous studies reported by our group (19, 20). NHS administration induced an acute pressure increase up to 42 ± 6 mmHg within 20 min (Fig. 1). The pressure increase in the lungs was significantly ($P < 0.05$) reduced after pretreatment with both sCR1 (20 ± 3 mmHg) and C1-Inh (25 ± 5 mmHg). Control lungs showed massive lung edema and pulmonary artery pressure increase within 20 min, associated with elevated airway pressures up to 35 mbar, finally resulting in acute barotrauma of the lungs. In the C1-Inh group after an initial increase, PAP remained almost constant for 90 min before alveolar edema occurred. After primary PAP elevations, sCR1 was more potent in reducing pulmonary artery pressure until the end of the observation period. Levels of pulmonary artery pressure regained approximately baseline values (12 ± 1 mmHg).

### Lung edema formation

In parallel to the observed increase in pulmonary artery pressure, edema formation occurred in control lungs. From baseline values of 30–40 g control lungs showed a weight gain of 30 ± 7 g within 20 min. Both C1-Inh and sCR1 significantly reduced edema formation to 11 ± 4 ($P < 0.05$) and 2 ± 1 g ($P < 0.01$), respectively (Fig. 2). Lungs treated with C1-Inh showed a final weight gain of 12 ± 4 g after 90 min. SCR1-treated lungs had a delayed onset of lung edema formation with a final weight gain of 12 ± 4 g at the end of the observation period (120 min).

### Circulating C5b-9

C5b-9 levels at time point 0 min ranged between 4.1 and 5.9 μg/mL. Baseline values were set to 100%, and relative values were calculated (Fig. 3). In control lungs, C5b-9 levels increased to 873 ± 233% at 20 min, whereas C1-Inh (414 ± 104%) and sCR1 (258 ± 58%; $P < 0.05$) reduced terminal complement complex formation.

### Thromboxane $B_2$ measurement

Concentrations of circulating $TXB_2$ ranged between 6.8 and 10.0 nM, and additional release of $TXB_2$ was not detectable. Immediately after complement activation, $TXB_2$ release rates increased to 3000–3700 pmol/min. Thirty min later no further $TXB_2$ release was detectable. C1-Inh and sCR1 significantly suppressed thromboxane $B_2$ release rates 5 min after serum administration ($P < 0.001$; Fig. 4) and sCR1 additionally after 10 min ($P < 0.01$).

### Tissue deposition of C3c and C5b-9

To correlate the described functional findings in the lungs with pulmonary morphology, immunostaining was performed. Compared with controls, lungs pretreated with either C1-Inh or sCR1 showed significantly decreased C3c and C5b-9 precipitation in the pulmonary vasculature (Fig. 5).

## DISCUSSION

Inappropriate or excessive activation of the complement system can cause harmful, potentially life-threatening consequences due to severe inflammatory tissue damage (28, 29),

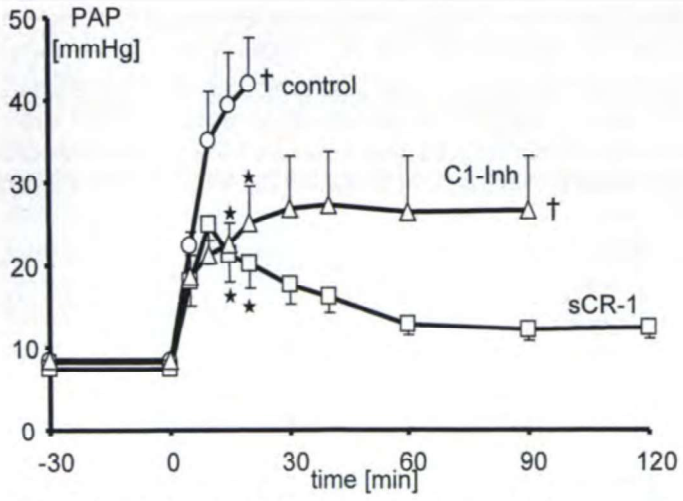


FIG. 1. **Time course of mean pulmonary arterial pressure in control lungs (n = 6) after administration of human serum (35%) and on pretreatment with C1-Inh (1 U/mL; n = 6) or with sCR-1 (2 μg/ml; n = 6).** The acute pressure increase as observed in controls was significantly ($P < 0.05$) reduced by both C1-Inh and sCR1. *$P < 0.05$; vs. control. †Severe pulmonary failure in two thirds or more experiments of the respective series.

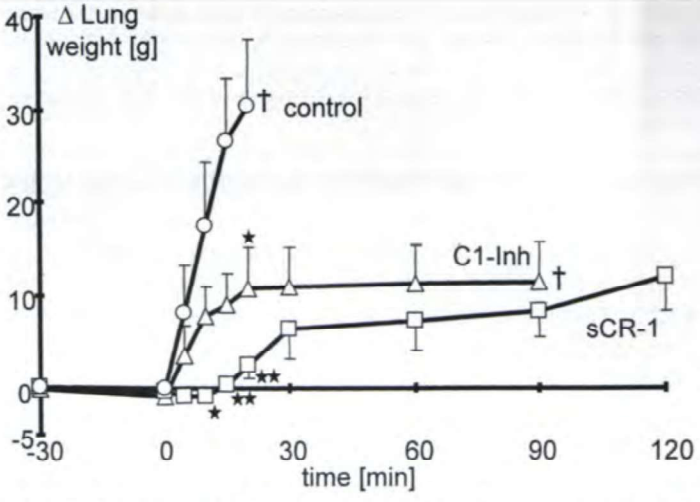


FIG. 2. **Time course of pulmonary edema formation as assessed by lung weight gain after administration of human serum (35%) in control lungs (n = 6) and under pretreatment with C1-Inh (1 U/mL; n = 6) or with sCR-1 (2 μg/mL; n = 6).** Severe lung edema (controls) was reduced by both C1-Inh ($P < 0.05$) and sCR1 ($P < 0.01$). *$P < 0.05$; **$P < 0.01$ vs. control. †Severe pulmonary failure in two thirds or more experiments of the respective series.

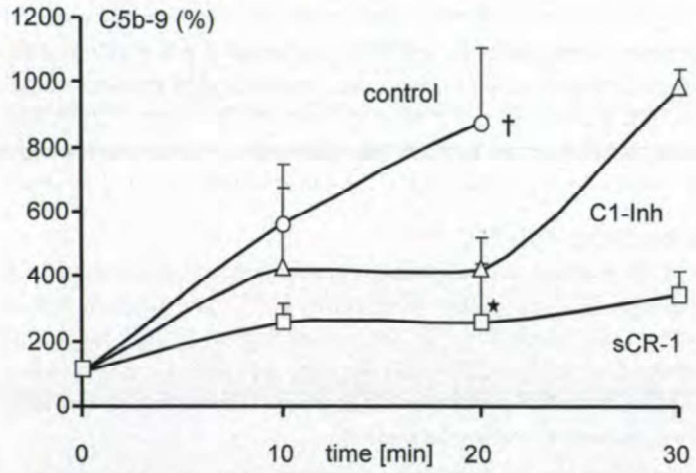


FIG. 3. **Generation of C5b-9 in the perfusate of the lungs after application of human serum (NHS; 35%) (0 min = 100%) in control lungs (n = 6), and on pretreatment with C1-Inh (1 U/mL; n = 6) or with sCR-1 (2 µg/mL; n = 6).** C5b-9 generation was markedly reduced in the presence of sCR1 and to a lesser extent by C1-Inh. $^{*}P < 0.05$ vs. control. †Severe pulmonary failure in two thirds of the control experiments.

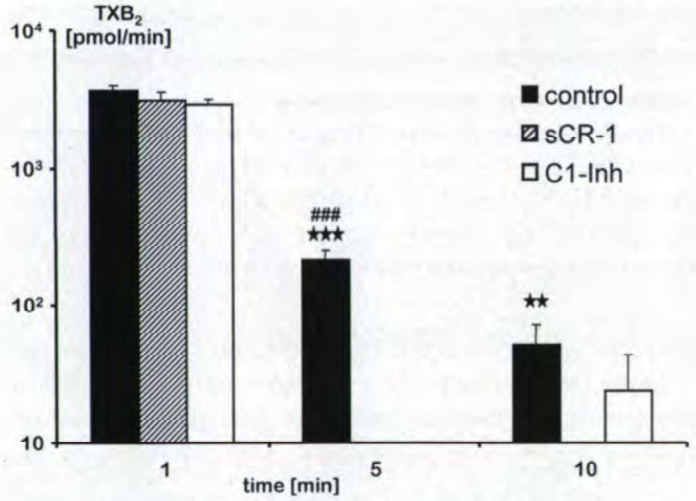


FIG. 4. **Release rates of thromboxane (TX) $B_2$ into the perfusate of the lungs.** Human serum induced the generation of $TXB_2$ in the control lungs (n = 6), which was significantly reduced by sCR1 (n = 6) and C1-Inh (n = 6). $^{***}P < 0.001$; $^{**}P < 0.01$ vs. sCR1; $^{###}P < 0.001$ vs. C1-Inh.

that are clinically manifested in various disorders, including aspiration-induced pneumonia (30), sepsis/systemic inflammatory response syndrome (SIRS) (31), and hyperacute graft rejection (23). Systemic activation of the complement system with generation of anaphylatoxins and pulmonary granulocytosis has been suggested as a general concept for the development of acute respiratory distress in patients suffering from bacterial sepsis or multiple trauma (3, 4, 32). Modulation of the complement cascade reduced tissue injury in a number of animal models of severe complement-dependent inflammation (11, 16). Therefore, it is believed that therapeutic down-regulation of complement is likely to arrest the process of certain diseases. Attempts to efficiently inhibit complement include the application of endogenous complement inhibitors, the administration of antibodies either blocking key proteins of the cascade reaction (e.g., C3, C5), neutralizing the action of

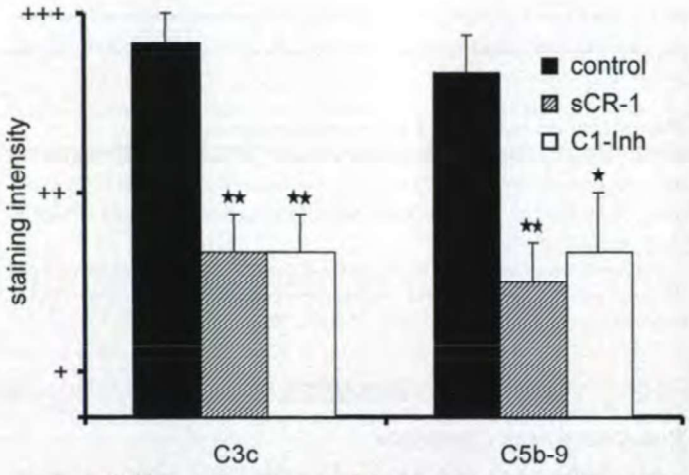


FIG. 5. **Staining intensity of microscopic slices after human serum-induced pulmonary edema in control lungs (n = 6) or after pretreatment with C1-Inh (1 U/mL; n = 6) or with sCR-1 (2 µg/mL; n = 6) treatment.** Tissue expression of C3c and C5b-9 was reduced after C1-Inh and sCR1. Mean staining intensity: +++, strong; ++, clear; +, slightly over background.

the complement-derived anaphylatoxin C5a, or interfering with complement receptor 3 (CR3, CD18/11b)-mediated adhesion of inflammatory cells to the vascular endothelium (16).

In the current study we investigated the possible protective effect of complement inhibition by C1-Inh and sCR1 in states of massive complement system activation. To analyze the pulmonary vascular response and mediator release involved in such complex reactions, experiments were chosen to be performed in isolated rabbit lungs. The used system is artificial but offers the essential advantage that effective *in situ* complement activation is induced in a controllable fashion and that its effects may be studied in detail in the absence of blood cells. According to results of Seeger and coworkers (25), experiments conducted in this study revealed a vasoconstrictive potential of human serum. In previous dose-finding studies serum concentrations of <25% induced reproducible pulmonary hypertension but did not cause pulmonary edema. To test the efficacy of C1-Inh and sCR1 to suppress both vascular resistance and edema formation, a concentration of 35% NHS was chosen. C3a and C5b-9 concentrations measured during those preliminary studies were closely correlated and increased with the amount of serum in the perfusion fluid. In the current study the complement inhibitors C1-Inh and sCR1 significantly decreased the pressure response to NHS (Fig. 1) and reduced pulmonary edema formation (Fig. 2). The reduced vasoconstriction and edema formation was paralleled by decreased levels of both soluble terminal complement complex (C5b-9; Fig. 3) in the circulating perfusate and tissue-bound complement C3c and C5b-9 (Fig. 5). Correspondingly, $TXB_2$ release rates into the perfusion fluid of the isolated rabbit lungs were reduced (Fig. 4).

In the present experiments, local complement activation was initiated in the pulmonary circulation at the surface of pulmonary cells rather than systemically and was monitored by the appearance of C3c and C5b-9 on the surface of pulmonary vascular endothelium and by increased levels of C5b-9 in the recirculating fluid. Under these conditions, acute alterations in

the pulmonary circulation, including $TXB_2$-mediated pressure response and vascular leakage, were evoked, which may be caused by *in situ* generation of membrane bound C5b-9 complexes rather than by anaphylatoxins (33). Those effects were reported to be absent on infusion of autologous rabbit plasma, heat-inactivated human serum, and inulin-activated serum but were dependent on C8 supply (25). Moreover, large quantities of circulating C5b-9 were ineffective, indicating that the membrane-bound and not the soluble membrane attack complexes were the active agents (25). The formation of C5b-9 complexes obviously triggers prostanoid generation in lung tissue, because C8 requirement has been reported for $TXB_2$ and $PGI_2$ generation (34, 35). In isolated rabbit lungs, $TXB_2$ release by endothelial cells was found to be the crucial mechanism of complement-induced pulmonary injury (25). In this connection, $TXB_2$ is likely to be induced by calcium influx into cells after stimulation with sublytic concentrations of C5b-9 (36).

Complement receptor 1 is a potent regulator of C3 and C5 activation (13). Thus, sCR1 develops its inhibitory activity at the common sequence of the complement cascade, regardless of which pathway caused activation (12). In the present study, sCR1 attenuated C5b-9 generation and protected lungs from subsequent tissue damage as assessed by edema formation and vasoconstriction. Because C1-Inh is an inhibitor of activated C1s and C1r (8), it modulates the activation sequence at an early step of the classical pathway but does not interfere with activation via the alternate pathway. Although the current study uses a pathomechanism that predominantly triggers the alternate pathway, a somewhat lower capability of C1-Inh to reduce tissue injury may have been expected, compared with sCR1. The protective effects of C1-Inh (for review, see 11), however, may be due to inhibitory effects on other mediator-generating cascade systems. Fisher et al. (37) has attributed improved survival after endotoxin shock in C3- and C4-deficient mice due to C1-Inh administration to the regulatory function of C1-Inh on kinin-generating systems. Second, activation of the classical pathways by proinflammatory mediators, which are released in response to sublytic doses of C5b-9 (36), may account for the impact of early classical pathway blockade by C1-Inh as demonstrated in the current study. Third, potential additional activation of the classical pathway, caused by anti-rabbit antibodies in the used NHS cannot be excluded, which would also explain the beneficial effects of C1-Inh in our experimental setup.

Summarizing the results obtained from the isolated lung, admixture of 35% human serum to the perfusion fluid provokes immediate activation of the complement cascade with production of C5b-9, subsequent tissue damage, and release of $TXA_2$. Within 20 min overwhelming pulmonary artery pressures and pulmonary edema occurred in the control group. The complement regulators C1-Inh and sCR1 significantly reduced complement-induced lung edema and vasoconstriction.

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