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Link to publication

Heavy chains of inter alpha inhibitor (IαI) inhibit the human complement system at early stages of the cascade *

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* Running title: IαI inhibits the complement system

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Background: Inter alpha inhibitor (IαI) contains a protease inhibitor bikunin and two heavy chains. Complement activation is enhanced in mice lacking IαI.
Results: Human IαI/its heavy chains added to serum inhibited all complement pathways at early stages.
Conclusions: Human IαI inhibits complement when enriched relative to complement components.
Significance: Inhibition of complement by IαI may be particularly important locally in tissues.

SUMMARY

Inter alpha inhibitor (IαI) is an abundant serum protein consisting of three polypeptides: two heavy chains (HC1 and HC2) and bikunin, a broad-specificity Kunitz-type proteinase inhibitor. The complex is covalently held together by chondroitin sulphate but during inflammation IαI may interact with TNF-stimulated gene 6 protein (TSG-6), which supports transesterification of heavy chains to hyaluronan. Recently, IαI was shown to inhibit mouse complement *in vivo* and to protect from complement-mediated lung injury but the mechanism of such activity was not elucidated. Using human serum depleted from IαI, we found that IαI is not an essential human complement inhibitor as reported for mice and that such serum has unaltered hemolytic activity. However, purified human IαI inhibited classical, lectin and alternative complement pathways *in vitro* when added in excess to human serum. The inhibitory activity was dependent on heavy chains but not bikunin and detected at the level of initiating molecules (MBL, properdin) in the lectin/ alternative pathways or C4b in the classical pathway. Furthermore, IαI affected formation and assembly of C1 complex and prevented assembly of the classical pathway C3-convertase. Presence and putative interactions with TSG-6 did not affect the ability of IαI to inhibit complement thus implicating IαI as a potentially important complement inhibitor once enriched onto hyaluronan moieties in the course of local inflammatory processes. In support of this, we found a correlation between IαI/HC-containing proteins and hemolytic activity of synovial fluid from patients suffering from rheumatoid arthritis.

Inter alpha inhibitor (IαI) consists of three different polypeptides: heavy chain 1 (HC1), heavy chain 2 (HC2) and bikunin, which are covalently linked together by ester bonds to chondroitin sulphate chain (1). This complex with a molecular mass of 225 kDa is produced mainly by hepatocytes and circulates in blood reaching 0.5 mg/ml (2). Originally, IαI was recognized as a proteinase inhibitor because of its bikunin moiety,

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which belongs to a group of Kunitz-type serine proteinase inhibitors (3) and can be also found in blood in a free form (4). Even though the serum concentration of IαI is relatively high, it has low specific activity and can be considered as a minor protease inhibitor under physiological conditions (5). Therefore, alternative functions of IαI have been sought and investigated. Thus, bikunin and IαI are implicated in such processes as oocyte maturation and ovulation (6), protection from sepsis (7), stabilization of hyaluronan coat on cell surfaces (8) and protection of hyaluronan-containing extracellular matrix components from degradation in arthritic synovial fluid (9). Concerning the interaction with hyaluronan, TNF-stimulated gene 6 protein (TSG-6) plays an essential role as it supports two subsequent transesterification reactions: first it binds covalently HC1 or HC2 of IαI and then in such a complex transfers them to hyaluronan moieties, where heavy chains become conjugated and free TSG-6 released (10,11). Elevated levels of covalent HC-hyaluronan complexes were found in sera (12) as well as synovial fluids (13) of rheumatoid arthritis (RA) patients.

Since IαI is an abundant serum protein, it was reasonable to investigate its interactions with other blood constituents such as proteins of the complement system, which forms the first line of defense from invading pathogens (14). Additionally, complement guides adaptive immunity and plays a role in removal of immune complexes and dying cells. Complement activation can be triggered by three distinct pathways initiated by binding of the C1 complex to antibodies (classical pathway), mannose-binding lectin (MBL) or ficolins to carbohydrate moieties (lectin pathway) or spontaneous tick-over of C3 (alternative pathway, which may also be initiated by properdin)(15). C1 complex is composed of recognition molecule C1q and two proteinases C1r and C1s while MBL and ficolins form complexes with MBL-associated proteinases MASP-1 and MASP-2 (16). Thus triggered enzymatic cascades converge at the level of C3 convertases, which are built up from C4b2a in the classical and the lectin pathways or C3bBb in the alternative pathway, respectively. These enzymatic complexes process the C3 molecule to surface-bondable C3b and small anaphylatoxin C3a. By addition of further

C3b molecules, convertases gain affinity to C5, which is similarly cleaved into C5b and anaphylatoxin C5a. Immobilized C5b initiates formation of the pore-forming membrane attack complex (MAC) consisting of subsequently added C6, C7, C8 and several C9 molecules. The proteolytic cascade of complement must be tightly regulated, since excessive activation contributes to pathology of such diseases as rheumatoid arthritis, ischemia injury, glomerulonephritis, systemic lupus erythematosus, Alzheimer's (17,18). Self cells and tissues are protected by a panel of soluble and membrane-bound complement inhibitors.

Recently, knockout mice lacking bikunin and thus IαI were used to show that IαI inhibits mouse complement *in vivo* (19). *In vitro* experiments employing IαI -deficient and sufficient mouse plasma revealed that the former produced significantly more C5a following both classical and alternative pathway activation. Moreover, addition of IαI to normal mouse serum resulted in inhibition of complement activation whereas addition of bikunin alone had no effect, implying that complement inhibitory activity was localized in the heavy chains. Importantly, the complement inhibitory activity of IαI was manifested *in vivo* in a complement-dependent lung injury model (19).

Herein, we investigated if IαI also acts as an inhibitor of the human complement system *in vitro*. We investigated which complement pathways are affected by IαI and at which point of the cascade interference took place. Finally, we analyzed how interaction with TSG-6 influences complement inhibitory activity of IαI.

EXPERIMENTAL PROCEDURES
Proteins - All complement proteins were bought from Complement Technologies. For C1 complex formation/dissociation experiments, we used C1q purified in-house as described in (20). Bikunin was from ProSpec-Tany Technogene. IαI was purified from human plasma as follows: 500 ml of frozen plasma was thawed and centrifuged for 30 min at 6.000 x G, then filtered thorough 0.45 nm filter and applied onto DEAE-Sephadex A50 column (GE Healthcare). Elution was carried out step-wise in 10 mM Na-citrate buffer pH 7.0 containing increasing, non-linear NaCl

concentrations of 0.23 M, 0.5 M and 1M, respectively. Fraction eluted by 0.5 M NaCl was diluted four fold with distilled water to 290 mOsm/l, supplemented with 2.5 g/l of L-lysine and applied onto DEAE-Sepharose column (GE Healthcare) equilibrated with 5 mM Na-citrate, 5 mM sodium phosphate buffer pH 6.0. Then, elution was continued with the same buffer containing 0.16, 0.23, 0.28 and 0.35 M NaCl, respectively. Fraction eluted with 0.28 M NaCl was diluted with distilled water seven fold to 100 Osm/l, pH was set at 7.45 and the sample was applied onto a heparin column (GE Healthcare) equilibrated with 20 mM Na-citrate buffer pH 7.45. Elution was carried out in 40 mM Na-citrate buffer pH 7.45 containing 0.05 or 0.2 M NaCl, respectively. Fractions eluted with 0.2 M NaCl containing pure IαI were pooled, dialysed against PBS buffer, concentrated to at least 1.5 mg/ml and stored at -80 ºC until use. The yield of I⟨I purification was within the range of 10-40 mg of pure protein from liter of plasma. HC1 and HC2 were prepared by alkaline hydrolysis of IαI, during which NaOH treatment of ester bonds linking its protein components to chondroitin sulphate moiety releases free heavy chains and bikunin (21,22). Purified IαI was subjected to 0.05 M NaOH for 15 min at room temperature after which Tris pH 8.0 was added to the final concentration of 0.25 M for rapid neutralization. Subsequently, the preparation was dialysed against 20 mM phosphate buffer pH 7.6, loaded onto MonoQ column (GE Healthcare) and eluted with linear gradient of 0-0.7 NaCl in the same buffer. Fractions containing H1 and H2 chain were pooled and stored at -80 ºC until use. TSG-6 protein was purified as described previously (11).

Preparation of IαI *– deficient and sufficient sera -* Blood from thirteen healthy volunteers was collected according to approval issued by local ethical committee. After collection, blood was kept at room temperature for 30 minutes to coagulate, then on ice for another 60 minutes followed by centrifugation for 7 minutes at 700 x G. Normal human serum (NHS) was collected, centrifuged again to remove residual erythrocytes, aliquoted and stored at -80 ºC. To deplete IαI, fibronectin or to obtain serum treated with irrelevant antibody, 200 µl of serum was pre-

cleared with 100 µl of protein G Sepharose (GE Healthcare) for 1 h at 4º C, then centrifuged for 5 min at 800 x G and added to another 50 µl of protein G-sepharose previously saturated with 200 µg of anti- IαI rabbit polyclonal antibody or anti-fibronectin rabbit polyclonal antibody or rabbit antibody (anti-goat polyclonal antibody (all from Dako Cytomation). After 1 h incubation with shaking at 4º C samples were centrifuged and serum collected. To assess the degree of spontaneous complement activation during the depletion process, we also compared the master stock of original serum with depleted sera in hemolytic assays. Efficiency of depletion of IαI was examined by western blotting, where 1 µl of both IαI -depleted and control sera were separated by electrophoresis under reducing conditions, transferred to PVDF membrane and probed with anti- IαI rabbit polyclonal antibody (Dako Cytomation) diluted 1:500 followed by anti-rabbit HRP-conjugated antibody (Dako Cytomation). The intensity of IαI band in each sample was evaluated with MultiGauge 2.0 software (Fuji Film).

Synovial fluid samples - Synovial fluid was taken from 20 consecutive patients fulfilling the American College of Rheumatology criteria for RA and seeking care at the Department of Rheumatology at Lund University hospital due to a synovitis in one knee joint. The collection of synovial fluids upon informed consent was approved by the Regional Ethical Review Board in Lund, Sweden. Material was frozen at -80 ºC until being used in experiments. Albumin content in synovial fluid was analyzed by western blotting, in which mouse anti-human albumin antibody (Abcam 1b.731, diluted 1:10000) followed by anti-mouse HRP (Dako, 1:1000) were used for detection. We also assessed content of IαI using western blotting with polyclonal rabbit anti-IαI antibody (Dako Cytomation, as described above) and complement activity using the hemolytic assay described below. Intensity of protein bands in western blots was evaluated with MultiGauge 2.0 software.

Labeling of IαI *and C1s with I-125 -* Thirty µg of purified IαI or C1s in PBS was mixed with two iodobeads (Pierce), 18 MBq of ^{125}I in a total

volume of 500 μl and incubated for 15 minutes with gentle stirring. Afterwards, the reaction mixture was applied onto a PD-10 column (Pierce) and eluted with PBS. Fractions containing iodinated proteins were collected and glycerol (VWR Prolabo) added to 50% v/v followed by storage at -20 ºC.

Complement assays - Proteins and sera were tested for activity of classical or alternative complement pathways by hemolytic assays performed as described (23). Briefly, the classical complement pathway was activated on antibody-sensitized sheep erythrocytes and performed in DGVB[++] buffer (2.5 mM veronal buffer, pH 7.35, 72 mM NaCl, 140 mM glucose, 0.1% gelatin, 1 mM MgCl$_2$ and 0.15 mM CaCl$_2$) at 0.2% NHS whereas the alternative pathway was activated on rabbit erythrocytes in Mg-EGTA buffer permissive for this pathway only (2.5 mM veronal buffer, pH 7.3, 70 mM NaCl, 140 mM glucose, 0.1% gelatin, 7 mM MgCl$_2$, and 10 mM EGTA) at 2% NHS. Additional assays, which evaluated the efficiency of 2 μM of IαI or factor H (FH) were performed at 3.5% serum in order to minimalize buffer and mass load effects. Hemolytic assays testing complement activity of synovial fluid were performed using sensitized sheep erythrocytes in DGVB[++] buffer at 1% synovial fluid.

Deposition of particular complement components in the presence of analyzed proteins was performed and analyzed on ELISA plate (Maxisorp, Nunc). Individual complement pathways were initiated by coating the surface with 5 μg/ml of aggregated IgG (classical pathway, Biosite Immuno), 20 μg/ml of zymosan (alternative pathway, Sigma) or 100 μg/ml of mannan (lectin pathway, Sigma), respectively. Then the protein to be studied was mixed with NHS and deposited complement components were detected with specific antibodies as described (24). Additionally, for detection of properdin, polyclonal goat anti human properdin antibody A239 (Complement Technologies) was used and for detection of C5 we employed mouse monoclonal anti human C5 antibody A217 (Quidel).

Formation of C1 complex was studied using Maxisorp plates coated with 5 μg/ml C1q overnight at 4 ºC in carbonate buffer pH 9.6.

Plates were washed with 1% BSA (Sigma) in PBS. Then 4 μg/ml C1r and trace amounts of radiolabelled C1s mixed with the protein to be studied were incubated for 1 h at 37 ºC, then washed and residual radioactivity was measured in gamma counter Gamamaster 1277 (LKB Wallac). Dissociation of C1 complex was studied using plates coated with aggregated IgG at 4 μg/ml overnight at 4 ºC. Then 10 μg/ml C1q, 4 μg/ml C1r and trace amounts of radiolabelled C1s were added and incubated for 90 minutes at room temperature. Wells were washed and analyzed proteins were added and incubated for another 1 h at 37 ºC following washing step and measurement of the residual radioactivity.

Cleavage of factor B, C2 and C4 was examined by western blotting. To induce cleavage of factor B, 25 μg/ml zymosan (Sigma), 15 μg/ml of properdin, C3 and +/- 1.5 μg/ml factor D and proteins to be studied were mixed in Mg-EGTA buffer and incubated for 1 h at 37 ºC, then loaded onto 10% SDS-PAGE under reducing conditions and blotted onto PVDF membrane. After blotting, the membrane was probed for factor B with polyconal goat anti factor B antibody A235 (Complement Technologies) followed by rabbit anti goat-HRP conjugated antibody (Dako Cytomation). Intensities of unprocessed factor B bands were assessed by MultiGauge software. Cleavage of C2 and C4 was induced by mixing 2.5 μg/ml of aggregated IgG, 2.5 μg/ml of C2, C4 +/- 0.5 μg/ml C1 with 20 μl of protein G Sepharose beads and proteins to be studied. Samples were incubated for 1 h at 37 ºC, run onto 10% SDS-PAGE under reducing conditions and blotted. Membrane was probed with goat anti human C2 (Calbiochem/EMD Millipore) followed by rabbit anti goat-HRP conjugated antibody (Dako Cytomation) or rabbit anti-human C4c followed by swine –anti rabbit HRP (both from Dako Cytomation), respectively.

Formation of classical C3 convertase was assessed by flow cytometry. First, 10^6 sheep erythrocytes (Håtunalab Ab) were sensitized with anti-sheep erythrocytes antibody (Amboceptor, Boegringer) diluted 1:2000 in 1 ml of DGVB++ buffer for 20 min at 37 ºC, then washed twice and incubated for another 30 minutes with 2% C3-depleted serum (Quidel) +/- proteins to be studied. This step should allow formation of C3 classical

convertase (C4b2a) upon interference of added compounds but not further steps of the complement cascade. Afterwards, the number of C4 molecules incorporated to the cell membrane was detected with rabbit anti human C4c polyclonal antibody followed by FITC-conjugated anti rabbit antibody (both from Dako Cytomation). The samples were analyzed using Partec CyFlow space flow cytometer and (Partec) and FlowJo software (Tri Star).

Binding of ^{125}I-labelled IαI *to complement proteins* - Break-apart 96-well microtiter plates (Nunc) were coated with 10 μg/ml of MBL, factor B, factor D, properdin, C1, C2, C3, C3b, C4, C4b, C5, C6, C7, C8 and C9 at 37 ºC for 1 h in carbonate buffer pH 9.6. The plates were washed and blocked with blocking buffer (1% BSA in PBS with 1 mM Ca^{2+}/Mg^{2+}) for 1 h. Then, trace amounts of ^{125} I-labeled IαI in blocking buffer were added and incubated for 1 h at 37 ºC, washed four times PBS buffer with 1 mM Ca^{2+}/Mg^2 and the radioactivity associated with wells was measured with gamma counter.

Modeling of IαI-dependent complement consumption at a structural surface – Ten microliters of protein A-Dynabeads (Dynal AS, Norway) slurry per experimental point were washed twice with $DGVB^{++}$ buffer. Beads were then incubated with 35 μg of anti-IαI polyclonal antibody for 10 minutes at 37ºC, thereafter washed twice and incubated again with 6.5 μg of IαI in total volume of 50 μl $DGVB^{++}$ for another 10 minutes. After washing, beads were incubated with 2.5% NHS in the same buffer for 5 minutes. Serum was collected and used in hemolytic assay on sensitized sheep erythrocytes at a final concentration of 1%.

Statistical analyses- Statistical analyses of the results were performed using Prism software (Graph Pad Software). One-way ANOVA or two-way ANOVA tests were used to determine the level of significance.

RESULTS
Human IαI *inhibitor is not as essential serum complement inhibitor as it was observed in mice* - Experimental complement activation *in vitro*

revealed that mouse serum sufficient in IαI generates approximately 20% or 70% less C5a than IαI deficient serum via alternative and classical complement pathways, respectively (19). In order to assess if this is true in human serum, IαI was depleted using specific antibodies. The procedure of absorption of specific serum protein introduces a risk of spontaneous complement activation by antibodies bound to protein G beads leading to complement consumption. Therefore, three independent batches of serum were prepared: one treated with specific IαI antibodies attached to beads and two other treated in a similar manner but with irrelevant antibodies of the same isotype (anti-fibronectin and anti-goat antibodies). Furthermore, we compared the hemolytic activity of depleted sera with a sample of the same batch of NHS taken pre-depletion, in order to assess the degree of spontaneous complement activation during the protein absorption process. Depletion of IαI was confirmed by Western blotting and densitometric analyses revealed that 85-90% of total IαI was retained on the beads (Fig. 1a). We found no significant differences in complement-mediated hemolysis between IαI-depleted and control sera at any tested serum concentration, neither in the classical nor the alternative pathways (Fig. 1b-c). Spontaneous complement activation during serum treatment was noticeable at low concentrations but it was not excessive, as revealed by readout obtained for the original batch of serum. Thus, removal of the majority of IαI from human serum did not appear to affect complement activation. However, one cannot exclude a significant role of IαI in controlling complement activation when its concentration is elevated locally. Therefore we tested whether IαI purified from plasma can inhibit human complement when added in excess to NHS.

Purified IαI, HC1 and HC2, but not bikunin inhibit human complement - Purity of IαI, isolated heavy chains and bikunin were evaluated by SDS-PAGE followed by silver staining (Fig. 2a). IαI and the heavy chains were largely free of contaminations and migrated as sharp bands while bikunin appeared as a diffused band around 40 kDa, as reported by others (25). When intact IαI and its components were tested in hemolytic assays, statistically significant complement inhibition by

IαI, HC1, HC2 but not by bikunin was detected in both classical and alternative pathways (Fig. 2b-c). Inhibition was more pronounced in the classical pathway assay compared with the alternative one, but the latter uses higher serum concentrations and therefore a direct comparison cannot be made. To eliminate possible buffer and protein mass effects, we increased the serum concentration to 3.5% and further tested 2 μM IαI and FH for alternative pathway inhibition. These experiments resulted in almost complete inhibition of hemolysis (1.27% +/- 1,16 and 2.25 % +/- 2.04 hemolytic activity remaining for IαI and FH, respectively). Notably, purified heavy chains were very sensitive to inactivation and showed negligible activity unless stored at -80 ºC in small aliquots (data not shown).

IαI *inhibits all complement pathways at early stages* - Knowing that purified IαI is able to inhibit complement, we performed a more detailed analysis to determine the exact points of the complement cascade, which are affected by IαI in each particular pathway. This approach enabled us to investigate the effect of IαI on the lectin pathway, which was not possible to study in hemolytic assays. We chose to study critical stages of all complement cascades, which determine pathway initiation (C1q, properdin, MBL), formation of convertases (C3b, C4b), and proceeding to formation of MAC (C5 and C9). For the classical pathway, the binding of C1 complex to IgGs was not affected by IαI (Fig. 3a). However, there was a drastic decrease in deposition of C4b and the following complement factors C3b, C5 and C9 thus leading to the conclusion that inhibition of the classical pathway by IαI takes place before the stage of classical convertase (C4b2a) formation. The alternative pathway was affected already at the level of initiation (properdin and C3b) but with a more pronounced effect at the level of deposition of MAC (Fig. 3b). Furthermore, IαI inhibited binding of MBL to mannan and entirely abolished deposition of C4b and the following complement factors (Fig. 3c)

Binding of IαI *to purified complement components* - Since complement inhibitory activity of IαI can be expected to rely on protein-protein interactions, we tested if radiolabeled IαI interacts directly with any of the complement proteins immobilized on microtiter plates. Of the complement proteins tested, radiolabeled IαI bound strongest to proteins of the alternative pathway: properdin and factor D, to proteins of classical pathway: C1q, C2 and C4 but also to MAC components such as C5, C6 and C8 (Fig. 4). Binding to these proteins was competed by non-labeled IαI but not by irrelevant protein (data not shown). However, we were not able to saturate the binding within the range of 1-100 nM, therefore we assume that the K_D value of this interaction lies above 10^{-7} M and may take place *in vivo* only at high concentration of ligands.

IαI *prevents formation and enhances dissociation of the C1 complex* - As we detected both a decrease in C4b deposition and also binding of radiolabeled IαI to C1q protein, we tested if IαI interferes with C1 complex formation and dissociation and thus inhibits enzymatic activity of the C1 complex necessary for further processing of downstream complement components. Both formation and dissociation of the C1 complex were affected by IαI, but the effect was more pronounced in the assay measuring formation of the complex (Fig. 5a, b). However, it appeared that the ability of IαI to affect C1 complex formation and stability could not be solely responsible for the drastic effect of IαI on the classical pathway observed in the hemolytic assay where much lower concentrations of IαI had a significant effect (Fig. 2). Therefore we assumed that the effect of IαI was cumulative through the proceeding stages of the classical pathway and we continued investigating its involvement downstream from C1q.

IαI *does not inhibit C1s but prevents formation of the classical convertase* - Binding of IαI to C1q may affect not only formation and dissociation of C1 complex but also the enzymatic activity of the C1s molecule, which cleaves C2 and C4. We examined whether addition of IαI to a reaction mixture containing C1, C2, C4 and aggregated IgG bound to the solid surface of Sepharose beads resulted in a decreased amount of products of cleavage by C1s: C2a/C2b and C4a/C4b. The cleavage rate of C2 was not different between IαI and control protein (BSA) treated sample (data not shown). Similarly, there was the same intensity of

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the band corresponding to α′ chain of C4 molecule, which is formed upon its cleavage to C4a and C4b (data not shown). Since we did not detect changes in the enzymatic activity of C1s by measuring cleavage of C2 and C4 while IαI interacted with both C2 and C4, we investigated the effect of IαI on the next stage of the cascade, classical convertase formation. Using a flow cytometry -based assay we detected a significant effect of IαI on the formation of the classical convertase, which was comparable to the effect of the main inhibitor of the classical pathway C4b-binding protein (C4BP) (Fig. 5c-d). However, IαI had no effect on acceleration of C3 convertase decay, in contrast to C4BP used as positive control (data not shown).

IαI inhibits activation of factor B - IαI significantly decreases deposition of early components of alternative complement pathway. Looking for a primary reason of such result, we considered binding to properdin, which can act as independent initiator of alternative pathway (15). Additional explanation can be offered by binding of IαI to factor D, which cleaves factor B to Ba and Bb. Impairment of this process disables formation of alternative C3 convertase (C3bBb). To verify this hypothesis, we mixed zymosan particles with properdin, C3, factor B and factor D in a buffer permissive for activation of alternative complement pathway and monitored the cleavage of factor B. Densitometric analysis of the 98 kDa band corresponding to intact factor B showed that IαI, but not BSA caused statistically-significant dose-dependent accumulation of intact factor B (Fig. 6).

Interaction with TSG-6 does not affect complement inhibition by IαI - TSG-6 was reported to covalently bind IαI at an equimolar ratio and transfer its heavy chains to hyaluronan moieties (10). As complement inhibitory activity of IαI is localized to the heavy chains, we tested if intramolecular rearrangements caused by TSG-6 impair the ability of IαI to downregulate complement. To this end, we used hemolytic assays to trace complement inhibition by IαI alone, but now we added IαI and TSG-6 simultaneously. We chose concentrations of IαI, which resulted in moderate complement inhibition

(0.1 μM for classical pathway and 1 μM for alternative pathway, respectively) to be able to detect potential changes upon TSG-6 addition. Surprisingly, we observed dose-dependent inhibition of the classical complement pathway by TSG-6 alone and an additive effect for inhibition by IαI and TSG-6, when introduced together (Fig. 7a). TSG-6 had no effect on the IαI -mediated inhibition of the alternative pathway (Fig. 7b).

IαI and HC in synovial fluid of RA correlate with hemolytic activity – Knowing that HC of IαI are enriched in arthritic joints with ongoing inflammation, we wanted to check the role of these proteins in synovial fluid. First, we found that synovial fluid pooled from several RA patients, unless heat-inactivated, supported hemolysis of sensitized sheep erythrocytes already at 1% concentration in DGVB^{++} buffer. Furthermore, 1 μM purified IαI inhibited the hemolytic activity of the synovial fluid completely (Fig. 8a). Then, we performed western blotting to visualize levels of IαI and HC–containing proteins in individual patients (Fig. 8b) and correlated these with the hemolytic activity of individual synovial fluids. Notably, all patients positive for IαI showed increased levels of HC-containing proteins of a molecular mass around 120 kDa, which could be HC-bikunin or TSG-6/HC complexes, as previously described (22,26), compared to serum. That would confirm an active HC enrichment in synovial fluid. Content of IαI and 120 kDa bands correlated positively with the hemolytic activity of synovial fluid (Fig. 8c,d). To exclude increased synovial membrane permeability as a simple explanation of higher complement content in samples, we also measured the content of serum albumin but we did not find any correlation between its concentration and the level of hemolysis (Fig. 8e). Depletion of IαI from synovial fluid using antibodies did not affect their hemolytic activity (data not shown) implying that physiologically attainable fluid phase concentration of IαI or HC is still too low to affect complement in synovial fluid. Nonetheless, in order to explain the positive correlation between the concentration of complement inhibitor IαI and hemolytic activity, we hypothesized that the concentration of free IαI and HC in the synovial fluid reflects the amount of these proteins, which

are immobilized within the structure of the arthritic joint, for example on the surface of cartilage. Bound fraction may inhibit complement activation on permissive surfaces, thus resulting in lower complement consumption *in vivo*. In this case the residual complement activity would be what we indeed measured in our hemolytic assay. To evaluate this hypothesis we used protein A Dynabeads, which simultaneously served as a complement activation surface and platform for IαI attachment. We found conditions at which beads alone or beads coated with a saturating amount of IαI-specific antibody consumed a comparable, substantial amount of complement activity. However, addition of a low amount of purified plasma IαI (antibody at 15-fold molar excess), which became immobilised to the beads, before addition of NHS, resulted in significantly higher hemolytic activity retained in serum (Fig. 8f).

DISCUSSION

Extrapolation of results obtained in mouse models to human pathophysiology is potentially riskable in the case of the complement system due to significant differences in how the system works in these two species. For example, the mouse complement system contains unique inhibitors such as Crry (27) while other inhibitors such as C4BP lack some functionally important domains compared to human homologues (28). Moreover, it is known that the mouse classical complement pathway exists mainly at an initiation level because of critical changes in mouse C4, which diminish further classical C5 convertase (C4b2a3b) activity (29). Therefore, we investigated if the abundant serum protein IαI reported as a relevant inhibitor of mouse complement (19) holds the same function in the human system. Lack of IαI in the mouse serum led to significantly higher levels of complement activation but we found that this was not the case in human serum when approximately 90% of total IαI was depleted. Nevertheless, purified human IαI acted as a complement inhibitor *in vitro*, when it was added in excess to NHS. Especially for the classical pathway, the activity of IαI was comparable to the effect of the acknowledged soluble inhibitor of the classical and lectin pathways, C4BP. Also in mouse model,

complement inhibition by IαI was pronounced more in classical than in the alternative pathway. The difference in IαI importance between the two species may lie in the fact that human complement inhibitors, especially these of classical complement pathway, are more redundant than mouse counterparts. Depletion of C4BP, which is the main fluid phase inhibitor of the classical and lectin pathways in man, changes the serum hemolytic activity only marginally (30,31). The classical complement pathway is actively initiated while the alternative pathway is continuously activated at low level and its further propagation depends on the ratio of activating to inhibiting factors. Therefore shortage of alternative pathway inhibitors leads to massive complement activation and ensuing complement depletion, as found in patients with FH deficiency (32). Although we found that IαI may also inhibit the alternative complement pathway in humans, it appears to be much less potent than FH as revealed at 1 and 0.5 μM concentrations. Therefore, depletion of IαI does not affect overall level of complement activation as long as functional FH is present in serum.

Deposition assays showed that IαI inhibited the three complement pathways early either at the level of initiation (alternative and lectin pathways) or before the formation of C3-convertase (classical pathway). In the course of classical pathway activation, there was no evidence of decreased C1q deposition but a drastic decrease in the amount of deposited C4b. Additionally, radiolabeled IαI bound C4 but also C2 and C1. We hypothesized that inhibition of the classical pathway may stem from additive effects on subsequent steps preceding classical C3-convertase formation such as C1 complex formation, proteolytic activity of C1s/C1r or association of C2 and C4 with activated C1 complex. Indeed, we found that IαI interferes with formation of the C1 complex and to a lower degree with its stability, but it also decreases formation of the C3-convertase (C4bC2a) on the cell surface, whereas it had no effect on C1s enzymatic activity. Furthermore, we detected decreased deposition of MBL in the lectin pathway activation upon IαI addition. As a possible explanation we consider the existence of many structural and functional similarities

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between C1q and MBL (33). It is likely that IαI interacts not only with C1and C1q, which is readily detectable using purified proteins, but also with MBL, although with much lower affinity, which made it impossible to detect the interaction under current conditions. The interaction is apparently strong enough to disturb binding of MBL to mannan and we did not detect any interaction between IαI and mannan itself. Some important interactions between proteins of the complement system occur at high micromolar concentrations and cannot be detected at normal ionic strength (e.g. C4BP and C3b or C4b unless these are immobilized allowing for avidity binding by polymeric C4BP containing multiple binding sites) (34). Blockade of the alternative pathway may be attributed to the interaction between IαI and properdin as well as decreased ability to generate cleavage products of factor B by factor D in the presence of IαI. However, it cannot be ruled out that a lower cleavage rate of factor B was also caused by the interaction between IαI and C3, as the latter binds factor B and exposes it for further cleavage during alternative convertase (C3bBb) formation. Interestingly, IαI or its heavy chains pulled down mouse C3 from serum (19) and we detected interaction of IαI with human C3.

We found that TSG-6 did not diminish the ability of IαI to inhibit complement. Surprisingly, we found that TSG-6 alone was able to inhibit the classical complement pathway. Although this protein was previously considered to be an anti-inflammatory mediator (35), there were no reports about its direct effect on complement. The only apparent relation between TSG-6 and complement is the presence of a CUB module in its structure, which shows high homology with the CUB domains of the complement proteinases C1s and C1r of the classical and the MASPs of the lectin pathway (36). The effect observed in the hemolytic assay was possibly due to competition based on structural similarities. Nonetheless, concentrations of TSG-6, at which we observed an apparent influence on complement, are not known to occur *in vivo* (37). Thus, complement inhibition by TSG-6 might not be physiologically relevant but importantly, transesterification reactions supported by TSG-6 preserve ability of IαI's heavy chains to inhibit complement even if TSG-6 is introduced in large excess. Human blood

contains up to 500 μg/ml or 2 μM of IαI and therefore this compound is potentially available at even higher concentrations than used in our study.

We also attempted to show the importance of IαI and HC-containing proteins in controlling complement activation at sites of local inflammation. The ideal model appears to be RA (reviewed in (17)) with synovial fluid as a clinical material since IαI is present in synovial fluid of RA patients (38) and HC complexes are actively accumulated (13,26). Importantly, part of these complexes are covalently linked to hyaluronan moieties within the joint matrix. We found a positive correlation between IαI/HC-containing proteins in synovial fluid and hemolytic activity. As an explanation, we showed that fluid phase IαI at physiological concentrations cannot influence complement activation in arthritic joints but once immobilized on a complement-activating surface, it significantly reduces complement consumption, therefore maintaining a higher potential for complement activity in synovial fluid.

Systemic complement inhibition by IαI in mice appears to be in agreement with its reported protection of neonatal mice against sepsis (7). However, there are reports stating that a high level of IαI is a powerful diagnostic marker for neonatal sepsis in humans (39), whereas systemic complement inhibition is herein questioned. So far, the mechanism of the sepsis-protective effect of IαI is not known and may be connected to the anti-proteolytic activity of IαI enhanced by factors released upon inflammation (40). Serum levels of IαI were also studied in breast cancer patients receiving chemotherapy (41). Proteomic analyses revealed upregulation of C3, C4 and IαI-related fragments but nephelometric analyses of total C3 and C4 showed decreased levels of these proteins after chemotherapy. Further analysis of C3 fragments in responders and non-responders suggested that complement activation is a single parameter distinguishing these two groups. Unfortunately, there were no available data concerning the correlation between IαI levels and C3 processing, so the significance for our studies is not clear.

Taken together, our results show that IαI is not an essential inhibitor controlling the overall activation of the human complement system, as it was shown in mice, but its activity could be highly

relevant at sites of local inflammation, where IαI could be enriched disproportionately to other complement proteins and where the classical pathway could be of importance.

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FOOTNOTES

[2] The abbreviations used are: IαI: inter-alpha inhibitor, TSG-6: TNF –stimulated gene 6, HC1: heavy chain 1, HC2: heavy chain 2, MBL: mannose-binding lectin, C4BP: C4b-binding protein, NHS: normal human serum, MAC: membrane attack complex, FH: complement factor H, RA: rheumatoid arthritis.

FIGURE LEGENDS

FIGURE 1. Complement activity of human IαI -depleted and control sera. A) Western blot of IαI-depleted serum (ΔIαI), fibronectin-depleted serum (ΔFBN), serum treated with irrelevant antibody (RbαGt) and original batch of NHS used for preparation of depleted sera. Lanes in the right panel were

purposely overloaded to visualize residual IαI and related proteins. Numbers below the lanes gives percentage of IαI band intensity referred to NHS as 100%. Complement activity in IαI -deficient and control sera was analyzed using hemolytic assays employing antibody sensitized sheep erythrocytes (B, classical pathway) or rabbit erythrocytes (C, alternative pathway). Data were collected from three independent experiments and are shown as means +/- standard deviations (SD). No statistically significant differences between ΔIαI, ΔFBN and RbαGt sera were detected as estimated by two-way ANOVA.

FIGURE 2. Complement inhibition by purified IαI and its components. The purity of used proteins was evaluated by SDS-PAGE and subsequent silver staining (A). Complement inhibitory activity was tested in hemolytic assays under conditions permissive for the classical pathway (B) or the alternative pathway (C). The background readout (obtained for heat-inactivated serum) was subtracted from all results and then the value of 100% was set for readout when no protein was added to the sample (positive control). Particular concentrations of given proteins were compared to positive control and symbols *, ** and *** stand for levels of significance $p < 0.05$, $p < 0.01$ and $p < 0.001$ according to two-way ANOVA with Bonferroni post-test. Data were collected from at least three independent experiments and are shown as means +/- SD.

FIGURE 3. Deposition of complement components upon IαI addition. The three complement pathways were initiated in their permissive buffers by IgG (classical pathway, A), zymosan (alternative pathway, B) or mannan (lectin pathway, C) and depositions of consecutive complement proteins were detected with specific antibodies. The value of 100% was set for NHS when no protein was added to the sample and the readout obtained for heat-inactivated serum was subtracted from all values. Background bar indicates the unspecific complement activation on BSA-coated wells. Level of significance of differences between samples treated with IαI and BSA at the same concentration (negative control) is represented by symbols ** or *** standing for $p < 0.01$ and $p < 0.001$ according to one-way ANOVA with Bonferroni post-test. Data were collected from at least three independent experiments and are shown as means +/- SD.

FIGURE 4. Binding of [125]-I labeled IαI to complement proteins. (A) Microtiter plates were coated with purified complement proteins, blocked and incubated with [125]-I labeled IαI. After washing, remaining radioactivity was measured with γ-counter and calculated as % of total radioactivity applied. Data were collected from three independent experiments and are shown as means +/- SD. Symbol *** states for the level of significance $p < 0.001$ calculated by one-way ANOVA with Bonferroni post-test, when the readout of given complement protein was compared to blank (no complement protein coated and blocked).

FIGURE 5. IαI affects the initial stages of classical complement pathway. C1 complex was assembled on the surface of microtiter plate from purified C1q, C1r and radiolabelled C1s and the effect of IαI was tested during (A) or after formation (B) by measuring retained radioactivity. C1 inhibitor, which is known to disrupt C1 complex at both stages was used as a positive control, whereas alpha 1 microglobulin (α1M) was used as control protein. The readout obtained for sample without any protein added was considered as 100% and compared to all samples. Levels of significance of these differences are represented by symbols * or *** standing for $p < 0.05$ and $p < 0.001$ according to one-way ANOVA with Bonferroni post-test. Data were collected from at least three independent experiments and are shown as means +/- SD. To test the effect of IαI on classical convertase formation, sheep erythrocytes were sensitized and subjected to C3-depleted serum capable to develop complement activation up to C4b2a level. The amount of C4 inserted to cell membrane was measured by flow cytometry. Presented histograms (C) are chosen from duplicates of four independent experiments. Negative control – no serum added, positive control – no protein added, BSA control – the same weight concentration of BSA as for IαI D) Quantification of mean fluorescence intensity (MFI), where the readout obtained for positive

control was set as 100% and compared to readouts obtained for given concentrations of studied proteins. Symbol *** indicated $p<0.001$ according to one-way ANOVA with Bonferroni post-test.

FIGURE 6. Influence of IαI on cleavage rate factor B. A) Permissive conditions for cleavage and factor B were set up and then addition of IαI or BSA was analyzed with respect to substrate elimination. Results were monitored by western blotting. B) Quantification of remaining factor B band calculated from 3 independent experiments. Data are shown as means +/- SD, symbol *** states for $p<0.001$ according to one-way ANOVA with Bonferroni post-test calculated when the readouts for IαI and BSA were compared.

FIGURE 7. Influence of TSG-6 on complement inhibitory activity of IαI. Sensitized sheep erythrocytes (classical pathway, A) or rabbit erythrocytes (alternative pathway, B) were applied to measure complement-mediated hemolysis when IαI at concentration of 0.25 μM (A) or 1 μM (B) was introduced together with variable concentrations of TSG-6. The effect of IαI alone was compared to the effect of IαI with given concentration of TSG-6. Symbols * and ** stand for $p<0.05$ or $p<0.01$ according to one-way ANOVA with Bonferroni post-test. Data were collected from at least three independent experiments and are shown as means +/- SD.

FIGURE 8. Importance of IαI and HC in controlling complement activity in synovial fluid from RA patients. A) Sensitized sheep erythrocytes were used to measure the effect of purified IαI or BSA on complement activation in synovial fluid. Symbol *** stands for $p<0.001$ according to one-way ANOVA. B) Western blotting of NHS and twenty samples of synovial fluid from RA patients was use to assess IαI and HC content. Then, Pearson correlation between hemolytic activity of individual synovial fluid sample and IαI, HC and albumin band intensities were plotted in panels C, D and E respectively. F) Residual hemolytic activity of serum treated with protein A Dynabeads +/- immobilized IαI. Symbol * stands for $p<0.05$ according to one-way ANOVA. All hemolytic assay data were collected from 3 independent experiments and western blotting was repeated twice to ensure equal sample loading.
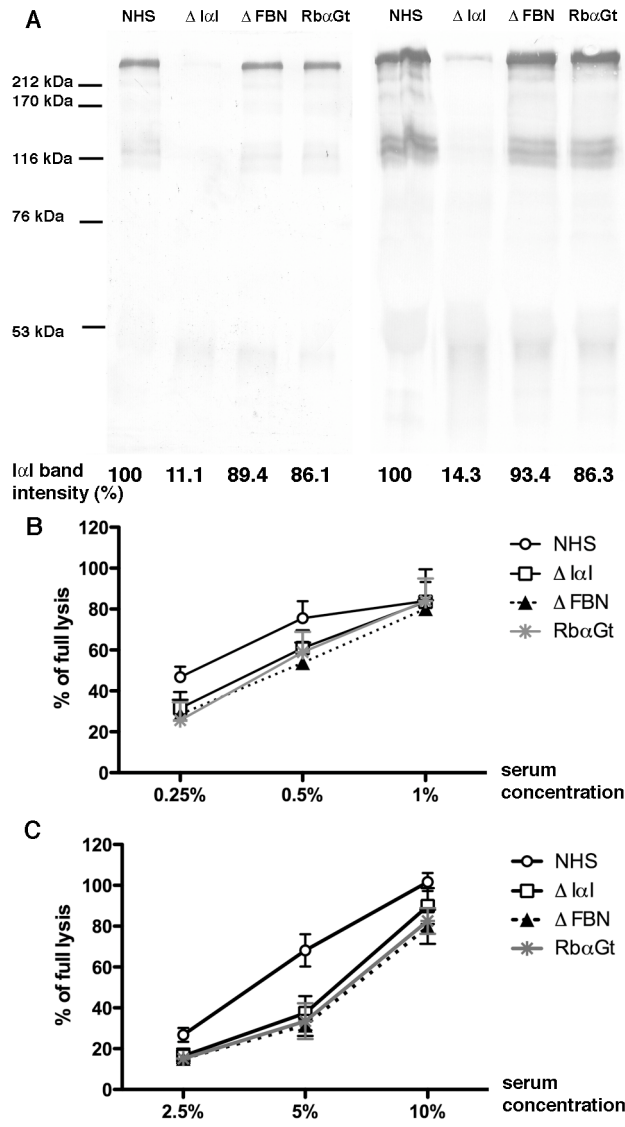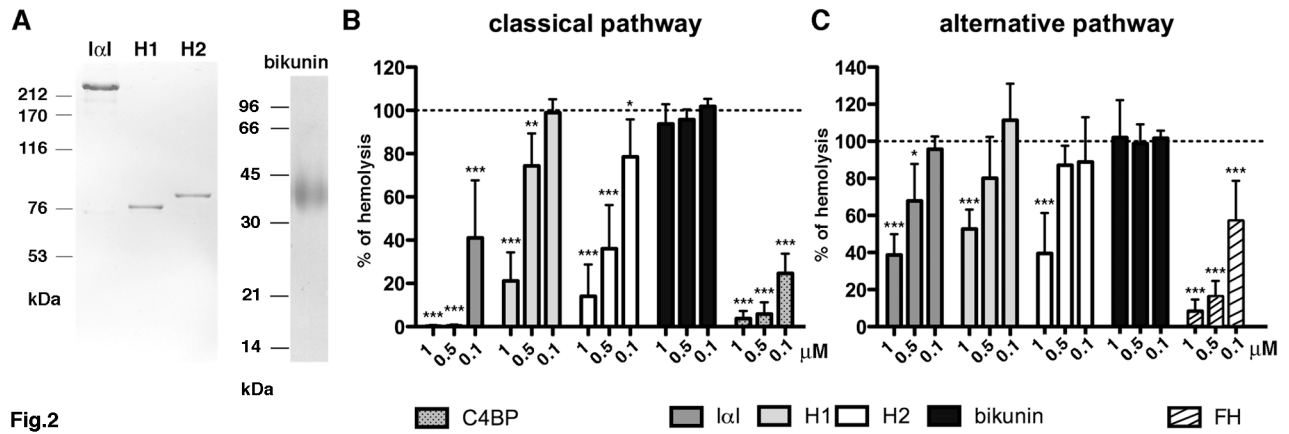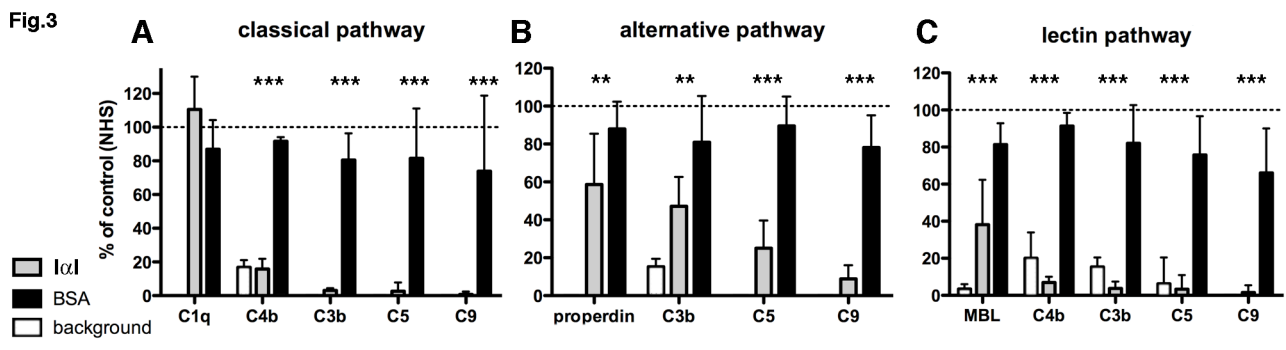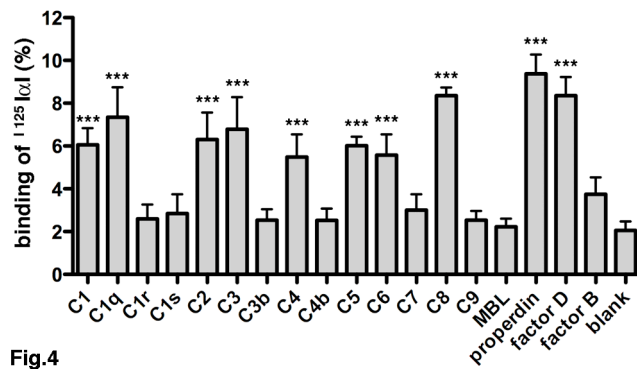
Fig. 1

Fig.2

Fig.3

A   classical pathway

B   alternative pathway

C   lectin pathway

% of control (NHS)

Iαl
BSA
background

Fig.4

A C1 complex formation

B C1 complex dissociation

C

D

Iαl   C1 inh.   α1 M

Fig.5

A

| | no D | no protein | Iαl 1μM | Iαl 0.5 μM | Iαl 0.25 μM | BSA 1 | BSA 0.5 | BSA 0.25 |

97 kDa —   ← B
66 kDa —   ← Bb
45 kDa —
      ← **Ba**
30 kDa —

B

***

Iαl
BSA

% of intact factor B

no factor D   no protein   1 μM   0.5 μM   0.25 μM

Fig.6

A classical pathway

B alternative pathway

Fig. 7

□ IαI +TSG-6   ▨ TSG-6

Fig. 8