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Mechanism of Action of Anti-C1-Inhibitor Autoantibodies: Prevention of the Formation of Stable C1s-C1-inh Complexes

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Abstract

Background: Acquired C1-inhibitor (C1-inh) deficiency is usually associated with the presence of circulating C1-inh autoantibodies. These autoantibodies have been shown previously to bind to two synthetic peptides corresponding to C1-inh amino acid residues 438–449 (peptide 2) and 448–459 (peptide 3) but not to peptide 1 (residues 428–440).

Materials and Methods: Affinity-purified C1-inh autoantibodies from two patients with acquired C1-inh deficiency were studied for their effects on the inhibition of C1s activity by C1-inh using SDS-PAGE and hydrolysis of a synthetic ester.

Results: Functional studies confirmed that the anti-C1-inh autoantibodies abrogated C1-inh activity, and their maximum effect was produced when the concentrations of C1-inh and autoantibody were approximately equimolar. The autoantibodies prevent the formation of the C1s-C1-inh complex, but they do not dissociate the preformed complex, suggesting that the autoantibodies act prior to the formation of the enzyme–inhibitor complex. In the presence of autoantibodies, C1s cleaves C1-inh, and a stable covalent bond between C1s and C1-inh does not form. Peptides 2 and 3, but not peptide 1 inhibited autoantibody activity, thus C1-inh inhibitory activity for C1s was expressed fully.

Conclusions: Our data indicate that the anti-C1-inh autoantibodies convert C1-inh to a substrate by preventing the formation of the stable covalent protease–serpin complex. The data also suggest a possible therapeutic use for peptides 2 and 3 or their derivatives in the management of patients with type II acquired angioedema (AAE).

Introduction

During activation of the serum complement system, complement component C1 binds to immune complexes or other complement activators. Binding of C1 to a target results in activation of the complement serine proteases C1r and C1s, which are subunits of the C1 complex. C1s subsequently cleaves complement components C4 and C2. The activity of C1r and C1s is controlled by the serpin C1-inhibitor (C1-inh)[3], which is the sole circulating inhibitor of C1 subcomponents C1r and C1s (1). C1-inh has a calculated polypeptide molecular weight of 52.84 kDa but is seen in SDS-PAGE as a glycosylated popypeptide of apparent *Mr* 105–115 kDa. Data from neutron-scattering studies showed that glycosylated C1-inh has *Mr* 71 kDa in nondenaturing conditions (2). C1-inh regulates C1 activity in two ways: it binds reversibly to precursor C1r and C1s to prevent autoactivation (3), and it binds to activated C1r and C1s to inhibit their enzymatic activity (4). During the latter reaction,

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C1r or C1s attacks C1-inh between residues R444 and T445 and appears to form a stable (covalent) enzyme–pseudosubstrate complex. The tetrametric C1-inh-C1r-C1s-C1-inh complex then dissociates from the C1 complex leaving C1q bound to the complement-activating particle (4). Like other serpins, C1-inh forms a stable complex with the proteases it inhibits. The serpin–protease complexes do not dissociate in SDS-PAGE analysis. The precise mode of interaction of serpins and proteases is not fully established (5), but it is assumed that the protease attacks the serpin as if it were a substrate. Secondary interactions then stabilize the enzyme–pseudosubstrate complex. The cleavage of the pseudosubstrate can be driven to completion in nonphysiological conditions, such as high pH.

C1-inh deficiency is manifest clinically as angioedema (AE) and may be inherited (HAE) or acquired (AAE). Two types of AAE have been described. Type I is associated with an anti-idiotypic response to plasma or membrane immunoglobulin and occurs in patients with multiple myeloma, chronic lymphocytic leukemia, or benign monoclonal gammopathy (6). The binding of the anti-idiotype to antibody appears to cause excessive C1 activation with consumption of C4 and C2 (6). Excessive catabolism of C1-inh results in low serum levels of this protein (6). In type II AAE, autoantibodies to C1-inh are present (7) and serum C1-inh levels as judged by immunoassays are normal or only slightly reduced, but it circulates as a cleaved, functionally inactive form (8) which has an apparent *Mr* of 96 kDa on SDS-PAGE. In our previous study (9), we described 6 patients with type II AAE. The autoantibodies were clonally restricted and recognized an epitope C-terminal to the reactive site residue (R444); the epitope was centered on the sequence QQPF (residues 452–455). The possibility that these antibodies recognized a second, structurally similar epitope (LLVF, residues 446–449) was also noted. In this report we studied the mechanism of action of two of these autoantibodies and show that both prevent the formation of a stable C1s-C1-inh complex, although they still permit cleavage of C1-inh by C1s at the reactive site (P1) residue.

Materials and Methods

Patients

Two patients (Patients 1 and 2; see Table 1 in ref. 9) with type II AAE were studied. Both patients had immunoglobulin (Ig)G kappa anti-C1-inh autoantibodies in their sera. The clinical details and the characteristics of the antibodies are described by He et al. (9). Both autoantibodies recognized the synthetic peptides corresponding to residues 438–449 of C1-inh (SAISVARTLLVF-peptide 2) and 448–459 (VFEVQQPFLFVL-peptide 3) but not a synthetic peptide corresponding to residues 428–440 (TETGVEAAAAASAI-peptide 1). Both antibodies bound better to peptide 3 and the use of substituted peptides and protein modeling suggested that the epitope comprised the tetrapeptide Q452, Q453, P454, F455 (9). It was concluded that binding to peptide 2 was due to the structural similarity of the tetrapeptide L446, L447, V448, F449 with Q452–F455 (9).

Synthetic Peptides

Three peptides (peptides 1, 2, and 3; see above) were obtained from Alta Biosciences (Department of Biochemistry, University of Birmingham, Birmingham, England).

Preparation of IgG from Serum by Protein G Affinity Chromatography

Normal and patients sera (10 ml) were incubated with 5 ml protein G Sepharose (Pharmacia, Milton Keynes, U.K.) at 4°C on a rotating stirrer. After 30 min, the mixture was poured into a 15-ml column and the nonadsorbed protein washed through with phosphate-buffered saline (PBS) (pH 7.4) until the absorbance at 280 nm was 0.002. The column was eluted using 100 mM glycine-HCl, pH 2.9, and fractions containing protein were adjusted to pH 7.4 by addition of 2 M Tris-HCl buffer (pH 8.0) prior to dialysis against PBS (pH 7.4).

Affinity Purification of Anti-C1-Inhibitor Autoantibodies

Human C1-inh (5 mg in 2 ml PBS) was mixed with 2 ml cyanogen bromide-activated Sepharose (Pharmacia) according to the manufacturer's instructions. The coupling efficiency was 83%. IgG (5.0 mg) purified from patient's serum was incubated with 1 ml of C1-inh-Sepharose at 4°C on a rotating stirrer for 30 min. The mixture was poured into a column, the gel washed extensively with PBS to remove nonspecifically bound IgG molecules, and then washed with 4 column volumes of water. The bound anti-C1-inh autoantibody was eluted with 3 M $MgCl_2$.

The eluted protein was dialyzed against PBS prior to use. The concentration of IgG was determined spectrophotometrically using an extinction coefficient (OD_{280} 1 mg/ml, 1 cm = 1.4) (10). Between 0.07 and 0.1 mg of anti-C1-inhibitor autoantibody could be purified from 5.0 mg total IgG. These antibody preparations contained only IgG as shown by SDS-PAGE, and they bound to solid-phase C1-inh but not to bovine serum albumin (BSA). Binding to solid-phase C1-inh was inhibited by fluid-phase C1-inh.

Purification of C1-Inhibitor and C1s and Radiolabeling

C1-inh was purified from fresh human plasma using the method of Pilatte et al. (11). Activated C1s was purified using the method of Sim (12). SDS-PAGE analysis using 7.5% gels run under nonreducing conditions showed that C1-inh had an apparent molecular weight of 115 kDa with a minor band at 96 kDa, whereas under reducing conditions, C1-inh appeared as a single band of 105 kDa. Under reducing conditions, C1s appeared as two bands of 58 kDa and 27 kDa, whereas under nonreducing conditions, it appeared as a single band of 85 kDa. Concentrations of C1-inh and C1s were determined using extinction coefficients (OD_{280}, 1 mg/ml, 1cm) of 0.36 for C1-inh (13) and 0.95 for C1s (14). For the calculation of molar concentrations of C1-inh and C1s, values of 105 kDa and 85 kDa, respectively, were used.

C1s was radiolabeled with $Na^{125}I$ (Amersham International, Little Chalfont, U.K.) using Iodobeads (Pierce and Warriner, Chester, Cheshire, U.K.) (15). Free iodide was separated from the labeled protein by gel filtration on a PD-10 (Sephadex G-25) column (Pharmacia). The specific activity of ^{125}I-labeled C1s was 127,000 cpm/μg.

SDS-PAGE, Autoradiography, and Western Blotting

Gels of 10% and 7.5% w/v acrylamide were used according to the method of Laemmli (16). Samples were boiled for 2 min in 1:1 (v/v) sample buffer (0.125 M Tris base, 4% (v/v) SDS, and 20% (v/v) glycerol pH6.8) in the absence or presence of 5% (v/v) 2-mercaptoethanol (Sigma). After electrophoresis, gels were stained with Coomassie Blue to visualize protein bands, dried prior to autoradiography, or used for Western blotting (17).

For autoradiography, gels were dried, wrapped in Clingfilm (Borden, North Baddesley, Southampton, U.K.) and exposed to X-ray film (Blue Sensitive Film, Genetic Research Instrumentation Ltd, Dunmow, England) in the dark at room temperature.

For Western blotting, proteins were transferred to nitrocellulose membranes (Hybond-Super; Amersham International) from polyacrylamide gels in 25 mM Tris/192 mM glycine buffer containing 20% (v/v) methanol and 0.1% (w/v) SDS at 4°C using a current of 200 mA. Transfer was completed in 6 hr. After blotting, the membrane was washed in 10 mM Tris-HCl (pH 8.0) containing 150 mM NaCl, 1 mM EDTA, and 0.1% (v/v) Triton ×100 (rinse buffer). Membranes were then incubated (6 hr at 4°C) in blocking buffer (rinse buffer containing 2% (w/v) BSA and 0.02% (w/v) sodium azide) to block nonspecific protein binding sites. C1-inh was detected by incubating (2 hr at 4°C) the membrane with blocking buffer containing 1/2,000 (v/v) goat anti-human C1-inh (Incstar, Wokingham, Berkshire, U.K.), washing the membrane (3 times, 15 min in 100 ml for each wash) with rinse buffer, incubating (2 hr at 4°C) with 1/3,000 (v/v) peroxidase-labeled rabbit anti-goat IgG (Incstar) in rinse buffer containing 2% (w/v) BSA, rewashing the membrane, and then developing the reaction using the ECL System (Amersham International) according to the manufacturer's instructions. The membrane was then exposed to high-performance luminescence detection film (Hyperfilm-ELC, Amersham International). Autoradiographs were scanned using a Molecular Dynamics Scanner (Molecular Dynamics, Inc. Chesham, Buckinghamshire, U.K.), to measure the area under the curve.

Effect of Anti-C1-inh Autoantibodies on Reaction between C1s and C1-inh

C1-inh, C1s, and autoantibodies were diluted in PBS prior to their addition to reaction mixtures. Reactions were carried out in PBS containing 1% (v/v) Triton ×100 at 37°C for 2 hr. Full details of each reaction are given in the figure legends.

Kinetic Assays

Inhibition of C1s activity by C1-inh was determined by the method of Sim and Reboul (19) with minor modifications. Briefly, C1s, C1-inh, and autoantibodies were dialyzed against 10 mM Tris-HCl, pH 8.0, containing 100 mM NaCl and 1 mM EDTA. The chromogen, propionyl L-lysyl

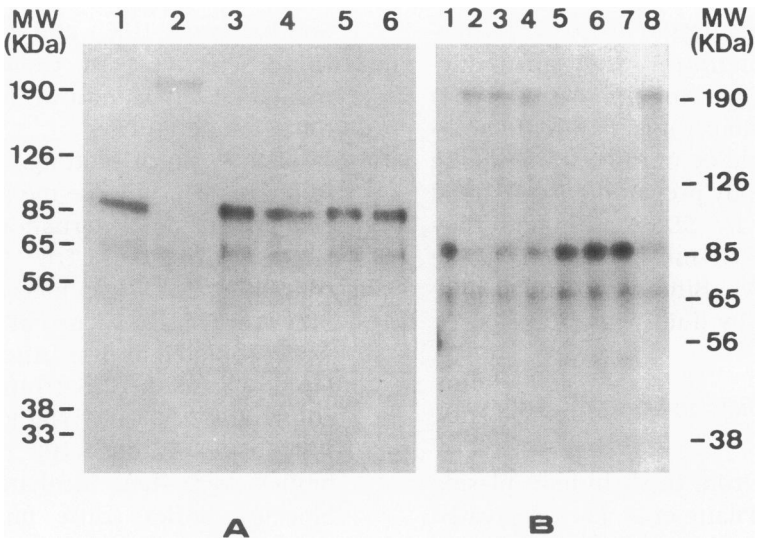

Fig. 1. Inhibition of C1s-C1-inh complex formation by autoantibodies. All protein reagents were diluted with PBS-1% Triton ×100 and the reaction volume was set to a total volume of 40 μl. (A) Complete inhibition of C1s-C1-inh complex formation by autoantibody. Fixed concentrations of both C1-inh (2.1 μM) and ^{125}I-labeled C1s (1.8 μM) were used in the reactions. Autoantibodies were preincubated with C1-inh for 2 hr at 37°C before addition of C1s. Incubation was continued for a further 2 hr. The reaction was stopped by addition of SDS-PAGE sample buffer and samples were analyzed by SDS-PAGE under nonreducing conditions. An autoradiograph of the dried gel is shown. The observed radioactive bands are: >190 kDa, C1s-C1-inh complex; 85 kDa, C1s; 65 kDa, a form of C1s in which part of the heavy chain is lost (28,29); 27 kDa, light chain of C1s dissociated from intact protein. Lane 1: ^{125}I-labeled C1s alone; lane 2: C1-inh and ^{125}I-labeled C1s; lane 3: Patient 1 autoantibody (0.9 μM), C1-inh, and ^{125}I-labeled C1s; lane 4: Patient 1 autoantibody (0.9 μM) and ^{125}I-labeled C1s; lane 5: Patient 2 autoantibody (0.9 μM), C1-inh, and ^{125}I-labeled C1s; lane 6: Patient 2 autoantibody (0.9 μM) and ^{125}I-labeled C1s. (B) Inhibition of C1-inh activity by different concentrations of patient 1 autoantibody. The concentrations of C1-inh and ^{125}I-labeled C1s used in this experiment were the same as in A. Lane 1: ^{125}I-labeled C1s alone; lane 2: C1-inh and ^{125}I-labeled C1s; lane 3: autoantibody (0.07 μM), C1-inh, and ^{125}I-labeled C1s; lane 4: autoantibody (0.17 μM), C1-inh, and ^{125}I-labeled C1s; lane 5: autoantibody (0.33 μM), C1-inh, and ^{125}I-labeled C1s; lane 6: autoantibody (0.75 μM), C1-inh, and ^{125}I-labeled C1s; lane 7: autoantibody (0.92 μM), C1-inh, and ^{125}I-labeled C1s; lane 8; normal IgG (1.0 μM), C1-inh, and ^{125}I-labeled C1s.

(ε-benzyloxycarbonyl) glycyl l-arginine p-nitroanilide hydroacetate (C_2H_5CO-Lys (ε-Cbo)-Gly-Arg-pNA) (Immuno Ltd, Heidelberg, Germany) was dissolved in 100 mM sodium phosphate, pH 8.0, 100 mM NaCl, and 15 mM EDTA to a concentration of 28 μM.

In order to determine the 50% inhibitory concentration of C1-inh, C1s was used at a fixed concentration (23.5 nM in a final reaction volume of 3 ml) with the concentration of C1-inh ranging from 0 to 30.5 nM. This series of reactions was incubated at 37°C for 30 min before adding the chromogen to a final concentration of 8.4 μM, after which the absorbance changes at 405 nm were recorded at 20-sec intervals for 6 min using a Perkin Elmer spectrophotomer. Residual C1s activity (%) was calculated using the equation: $[1 - (\{ x - y \}/x)] \times 100$, where x = slope of C1s alone and y = slope of C1s in the presence of C1-inh.

Results

Effect of Anti-C1-inh Autoantibodies on C1s-C1-inh Complex Formation

Incubation of unlabeled C1-inh with ^{125}I-labeled C1s resulted in the formation of the C1s-C1-inh complex (Fig. 1A). When anti-C1-inh autoantibody was preincubated with C1-inh prior to the addition of ^{125}I-labeled C1s, the C1s-C1-inh complex was not seen. As a control experiment, C1s was incubated with the two anti-C1-inh autoantibodies without C1-inh. This had no effect on the appearance of C1s in SDS-PAGE analysis. The effect of the autoantibodies on C1-inh-C1s

interaction was dose-dependent and for total inhibition of C1s-C1-inh complex formation, approximately equimolar concentrations of autoantibody antigen binding sites and C1-inh were required (Fig. 1B). Normal IgG did not influence the formation of the C1s-C1-inh complex (Fig. 1B).

Incubation of peptides 2 or 3 with the autoantibodies prior to their incubation with C1-inh blocked the effect of the autoantibodies as shown by the formation of the C1s-C1-inh complex. Peptide 1 had no effect on the activity of the autoantibodies (Fig. 2).

Effect of Anti-C1-inh Autoantibodies on Stability of C1s-C1-inh Complex

When anti-C1-inh autoantibody was added after the incubation (2 hr at 37°C) of ^{125}I-labeled C1s with C1-inh, the C1s-C1-inh complex was detected (Fig. 3). Prolonged incubation (up to 16 hr at 37°C) of the C1s-C1-inh complex with anti-C1-inh autoantibodies did not result in dissociation of the complex. During a 6-hr incubation at 37°C, the presence of a 10-fold molar excess of autoantibody reactive sites over C1-inh did not result in dissociation of the C1s-C1-inh complex (data not shown).

Effect of Anti-C1-inh Autoantibodies on Cleavage of C1-inh by C1s

A 50% inhibitory amount of autoantibody was incubated with C1-inh for 2 hr at 37°C. Following the addition of different concentrations of C1s, the tubes were incubated at 37°C for a further 3 hr. The reaction products were separated by SDS-PAGE and then blotted onto nitrocellulose, C1-inh was detected by sequential incubations with primary antibody, goat anti-human C1-inh, secondary antibody, and rabbit anti-goat IgG conjugated with horse-radish peroxidase, followed by detection using the ECL system. In the presence of either autoantibody, the intensity of the 96 kDa band (cleaved form) increased whereas the intensity of the 115 kDa band (uncleaved form) decreased with increasing concentration of C1s (Fig. 4A). A small proportion (approximately 10%) of the purified C1-inh was in the cleaved form. This may be due to cleavage by C1s or possibly another protease during purification. Scanning densitometry of the Western blot showed that as the C1s concentration increased, the intensity of the 96 kDa band increased (Fig. 4D), whereas the intensity of the 190 kDa band (C1s-C1-inh complex) only increased until the C1s concentration reached approximately 0.7 μM (Fig. 4A lane 6; Fig. 4D). At higher concentrations of C1s the intensity of this band remained constant. In this and other experiments we were never able to produce complete cleav-

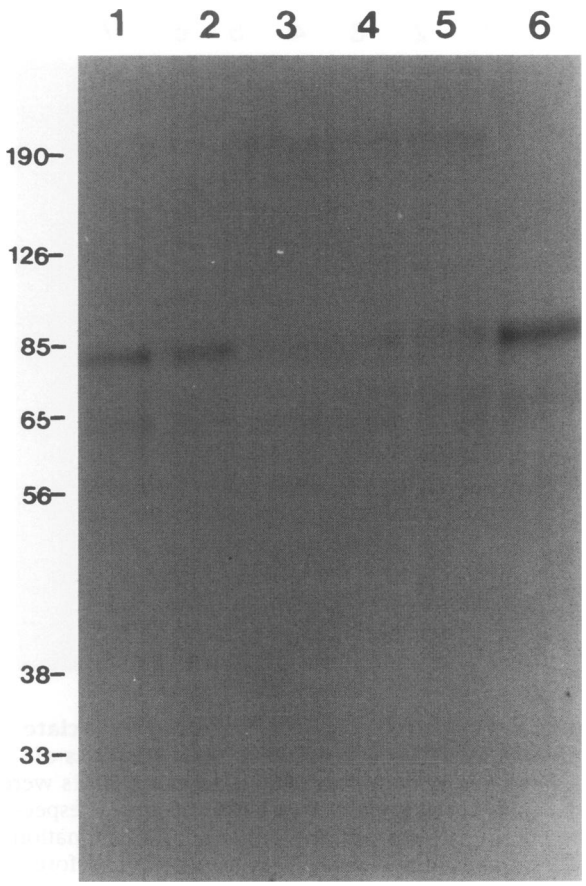

Fig. 2. The effect of synthetic peptides on anti-C1-inh autoantibodies. The concentrations of C1-inh, ^{125}I-labeled C1s, and autoantibodies used in the reaction were 2.1 μM, 1.8 μM (75,000 cpm), and 0.5 μM, respectively. The concentration of the autoantibody used was calculated to cause about 50% inhibition of complex formation (see Fig. 5A). All protein reagents were diluted with PBS-1% Triton ×100 and the reaction volume was set to a total volume of 40 μl. Lane 1: C1-inh, ^{125}I-labeled C1s, and Patient 1 autoantibody. Lane 2: peptide 1 (2.6 μM) was preincubated with Patient 1 autoantibody at 37°C for 2 hr and then with C1-inh for a further 2 hr before addition of ^{125}I-labeled C1s. Incubation was then continued for a further 3 hr. Lane 3: as in lane 2, except that peptide 2 (2.6 μM) was used instead of peptide 1. Lane 4: the same as in lane 2, except peptide 3 (2.6 μM) was used instead of peptide 1. Lane 5: C1-inh and ^{125}I-labeled C1s; lane 6: ^{125}I-labeled C1s alone. The same results were obtained with the autoantibody from Patient 2.

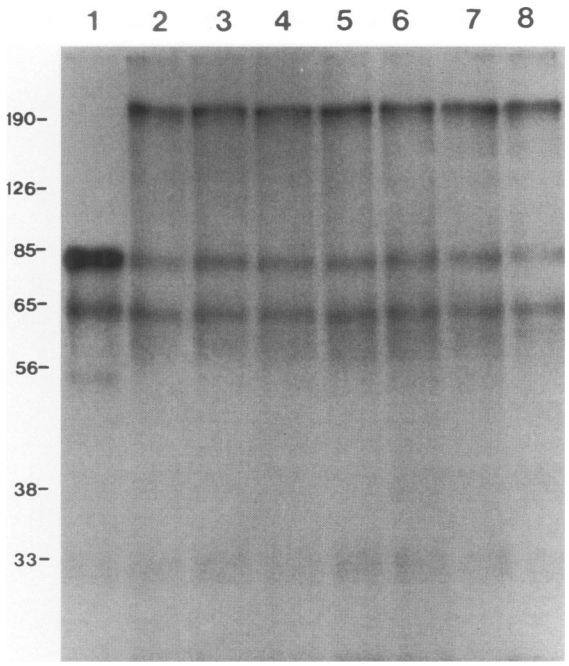

Fig. 3. Failure of autoantibodies to dissociate the C1-inh-C1s complex. The concentrations of C1-inh and autoantibodies and ^{125}I-labeled C1s were 2.1 μM, 0.9 μM, and 1.8 μM (75,000 cpm), respectively. In the case of C1s-C1-inh complex formation, C1-inh was preincubated with C1s for 2 hr before addition of autoantibody. The reaction volume was 40 μl in PBS-1% Triton ×100. Lane 1: ^{125}I-labeled C1s alone; lane 2: C1-inh and ^{125}I-labeled C1s incubated 2 hr, 37°C; lanes 3–8: C1-inh and ^{125}I-labeled C1s incubated 2 hr, 37°C, then autoantibody added and incubation continued for 1 hr (lane 3), 2 hr (lane 4), 4 hr (lane 5), 6 hr (lane 6), 8 hr (lane 7), and 16 hr (lane 8). Data shown are from Patient 1 autoantibody but an identical result was obtained with Patient 2 autoantibody.

age of C1-inh with a moderate (2-fold) molar excess of C1s and a 50% inhibitory concentration of autoantibody. Furthermore, we were never able to obtain more than 80% of C1-inh in SDS-stable C1s-C1-inh complexes with a 2-fold molar excess of C1s in the absence of autoantibody. In order to demonstrate that complete cleavage of C1-inh could be produced, we used approximately equimolar concentrations of C1-inh at 2.1 μM and autoantibody at 1.3 μM) (2.6 μM antigen binding sites) and an excess of C1s (8.8 μM). Under these conditions, we observed that the 115 kDa band was completely cleaved (Fig. 4B), although even with this concentration of C1s, in the absence of autoantibody, only approximately 80% of the C1-inh was incorporated into SDS-resistant C1s-C1-inh complexes. This observation suggests that a proportion of the purified C1-inh had been partially denatured, or altered in conformation such that it was not cleaved by C1s or that the complex formed was reversible on SDS-PAGE. The autoantibody may therefore induce the correct conformation for C1s cleavage or may simply drive forward the reaction towards cleavage and an irreversible state.

Under reducing conditions, uncleaved C1-inh (105 kDa) and cleaved C1-inh (96 kDa) could not be separated by SDS-PAGE on 7.5% gels; they appeared as a single, broad band along with two bands of 52 and 25 kDa (Fig. 4C). Other bands were not seen. These results were confirmed using two anti-C1-inh autoantibodies and the sizes of the fragments were confirmed using three sets of protein markers (Sigma C3187, C3312, and SDS-7). The 52 and 25 kDa bands were much fainter than the 96 kDa band, indicating that only a small proportion of 96 kDa band was further cleaved (Fig. 4C). This extra cleavage reaction could be C1s-mediated at R219/T222, which would generate polypeptides of the correct size and disulphide linkage, or it could be due to cleavage with a second protease contaminating one of the reagents. In this regard, there is no plasmin cleavage site that would generate fragments of this size (plasmin cleaves at KG [194–195] and KK [284–285]; 18). A secondary cleavage of C1-inh by C1s has not been documented in previous studies relying on SDS-PAGE with Coomassie Blue staining to detect the reaction products. However, immunoblotting is 10^2- to 10^3-fold more sensitive and therefore more likely to detect this secondary cleavage reaction. In contrast, C1-inh in plasma samples of the AAE patients studied displayed only a 96 kDa band under reducing and nonreducing conditions (data not shown), suggesting that the cleavage of the 96 kDa form was a feature of the in vitro system. Incubation of normal IgG with C1-inh and C1s in place of autoantibody did not result in C1-inh cleavage. Incubation of autoantibody with C1-inh in the absence of C1s did not result in C1-inh cleavage (Fig. 4C).

Stoichiometry of Autoantibody–C1-inh–C1s Interactions

Using a fixed concentration of C1s (23.5 nM in a final reaction volume of 3 ml) it was found that a concentration of 12.7 nM of C1-inh produced approximately 50% inhibition of C1s activity (48.3%, 49.7%, and 48.8% in three separate

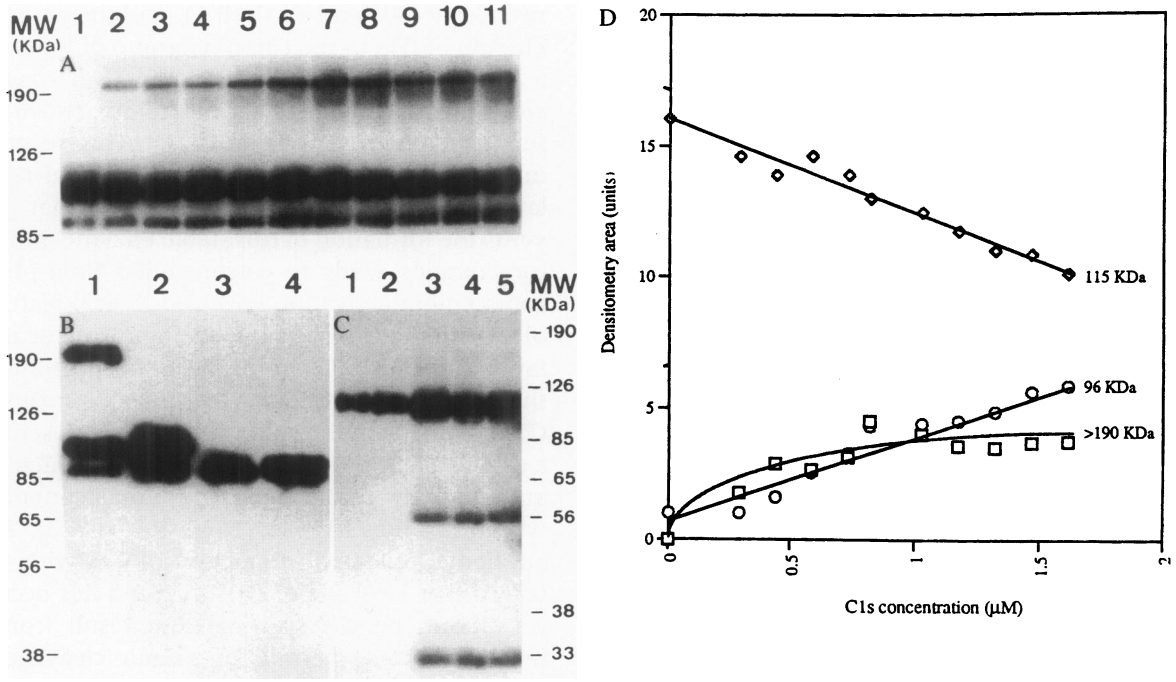

Fig. 4. Western blots of interactions of C1-inh, autoanti-C1-inh, and C1s. All protein reagents were in PBS and the reaction volumes were all 40 μl. (A). Western blot from nonreducing SDS-PAGE. The concentration of C1-inh was 1.4 μM while the autoantibody was used at the 50% inhibitory concentration (0.4 μM). The concentrations of C1s were varied. Reaction time of autoantibody with C1-inh was 2 hr and that of the autoantibody-C1-inh mixture with C1s was 3 hr at 37°C. The reactions were stopped by addition of sample buffer and samples were separated by SDS-PAGE (7.5% w/v polyacrylamide). Proteins were transferred to nitrocellulose by blotting and detected by goat anti-human C1-inh and rabbit anti-goat IgG conjugated with horse radish peroxidase. C1-inh was detected using ECL reagents and the result is shown. The observed bands are: >190 kDa, C1s-C1-inh complex; 115 kDa, intact C1-inh; 96 kDa, a cleaved form of C1-inh. Lane 1: C1-inh alone; lanes 2–11: C1-inh and Patient 1 autoantibody incubated for 2 hr, 37°C, which then continued for a further 3-hr incubation with different amounts of C1s: 0.3 μM (lane 2); 0.4 μM (lane 3); 0.6 μM (lane 4); 0.7 μM (lane 5); 0.8 μM (lane 6); 1.0 μM (lane 7); 1.2 μM (lane 8); 1.3 μM (lane 9); 1.5 μM (lane 10), and 1.6 μM (lane 11). (B) Western blot from nonreducing SDS-PAGE. The concentrations of C1-inh and autoantibody were 2.1 μM and 1.3 μM, respectively, and that of C1s was 8.8 μM in a total reaction volume of 40 μl. The reaction conditions were the same as in A, except that incubation of autoantibody-C1-inh mixture with C1s was for 6 hr at 37°C. Lane 1: C1-inh and C1s; lane 2: C1-inh alone; lane 3: Patient 1 autoantibody and C1-inh and then C1s; lane 4: Patient 2 autoantibody and C1-inh and then C1s. (C) Western blot from reducing SDS-PAGE. Reaction concentrations of C1-inh, autoantibody, and C1s were 2.1 μM, 1.3 μM, and 1.8 μM, respectively. Lane 1: C1-inh alone; lane 2: C1-inh and autoantibody; lanes 3–5: C1-inh and autoantibody were incubated for 2 hr at 37°C, then C1s was added and incubation continued for 1 hr (lane 3), 3 hr (lane 4), and 5 hr (lane 5). This gel does not resolve the 105 kDa and 96 kDa bands effectively, so they appear (lanes 3–5) as a single broad band. Data shown in this figure were obtained with Patient 1 autoantibody but a similar results were obtained with Patient 2 autoantibody. (D) Scanning densitometry of the gel shown in Fig. 4A. As the concentration of C1s increased, the intensity (area under the curve) of the 115 kDa band of native C1-inh decreased, while that of the 96 kDa band (cleaved C1-inh) continued to increase. The intensity of the 190 kDa band (C1s-C1-inh complex) increased to reach a plateau level when the C1s concentration was approximately 1 μM. The density of the 190 kDa band may be an underestimate of the amount of C1s-C1-inh complex formed because of the possibility of inefficient transfer of higher molecular weight species from gel to membrane.

experiments) (Fig. 5A). These ratios of C1s:C1-inh are consistent with a reaction in which 100% inhibition of C1s is produced by an equimolar concentration of C1-inh; i.e., 11.8 nM C1s is inhibited by 12.7 nM C1-inh. Using concentrations of C1s and C1-inh of 23.5 nM and 12.7 nM, respectively, we determined that the autoantibody concentration that resulted in total loss of C1-inh activity was 13 nM (26 nM antigen binding sites) in the reaction volume of 3 ml (Fig. 5B,

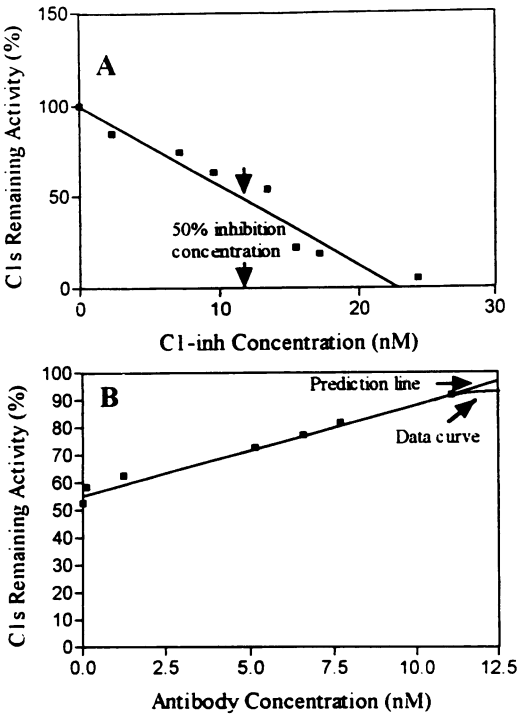

Fig. 5. Measurements of reaction stoichiometry of C1-inh, C1s, and autoantibody. (A) The effect of C1-inh on C1s. The concentration of C1s used in the assay was 23.5 nM and that of C1-inh ranging from 0 to 24.4 nM in a total reaction volume of 3 ml. The half-inhibitory concentration of C1-inh (12.6 nM) is indicated by an arrow. The vertical axis in the plot is the remaining C1s activity calculated according to the enzyme activity assay described in Materials and Methods. (B) The effect of autoantibody on C1-inh. The concentrations of C1s and C1-inh used in the assay were those for half-inhibitory conditions as seen in A and that of autoantibody ranged from 0 to 5 μg in a total reaction volume of 3 ml. The complete inhibitory concentration of autoantibody is .13 nm (arrows). Data shown were obtained with Patient 1 autoantibody but similar results were obtained with Patient 2 autoantibody.

arrows). Within the experimental limits of measurement of concentrations, this is likely to indicate a 1:1 stoichiometry for the C1-inh–autoantibody interaction. The higher autoantibody C1-inh molar ratio required to inhibit C1-inh activity in this experiment, compared with the dose–response study shown in Fig. 1b, may be related to the far lower concentration of C1-inh used in this study.

Discussion

The interaction of C1s with C1-inh results in cleavage of the latter between the reactive site residue (R444) and T445 (P'1), and the covalent binding of C1s to the carboxyl group of R444 via a transacylation reaction involving an oxygen atom of the active site serine residue (S195) of the light chain of C1s (19–21). This reaction inhibits C1s activity permanently and thus regulates C1 activity (2). Any mechanism that prevents the formation of this stable enzyme–inhibitor complex leads to uncontrolled fluid-phase complement activation with the clinical features of C1-inh deficiency (angioedema). Genetic mutations resulting in amino acid substitutions at the reactive site residue yield inactive forms of C1-inh (5). Mandle et al. (23) recently described a case of acquired C1-inh deficiency in which the autoantibody recognized an epitope contained within residues P15–P1 (T430–R444) (23). This antibody prevented interaction of C1s with C1-inh so that cleavage of C1-inh could not occur.

C1-inh deficiency could also result from a mechanism that permits enzymatic cleavage of C1-inh by C1s but prevents formation of the stable enzyme–inhibitor complex, thus converting C1-inh to a substrate. Mutations in the proximal hinge region of C1-inh, A434E (24), V432E, and A434T (25), convert C1-inh to a substrate for C1s. The mutant C1-inh species are cleaved to forms with a molecular weight of 96 kDa. The data presented here suggest that the C1-inh autoantibodies studies produce C1-inh deficiency by such a mechanism. However, these autoantibodies recognize the epitope QQPF and/or LLVF, both of which are C-terminal to the R444 reactive site residue (9).

The first set of experiments, using ^{125}I-labeled C1s, showed that although the autoantibodies prevented the formation of a stable enzyme–inhibitor complex, they were unable to dissociate the complex, even with a large (10-fold) molar excess of autoantibody. Thus C1-inh autoantibodies must act prior to the formation of the enzyme–inhibitor complex.

In the second set of experiments, incubation of C1-inh with autoantibodies prior to exposure to C1s resulted in cleavage of C1-inh, with the production of the 96 kDa form and the failure to form a C1s-C1-inh complex. These two sets of experiments show that in the presence of the autoantibodies, C1s is able to cleave C1-inh, probably between the reactive site residue (R444) and T445. This cleavage is not followed by the formation of a stable enzyme–inhibitor complex. Thus C1-inh inhibitory activity is lost and C1s maintains its active form after it dissociates from cleaved form C1-inh-C1s complex.

The effect of autoantibodies in "converting" C1-inhibitor from an inhibitor to a substrate is entirely consistent with previous findings (26). The site of interaction of autoantibody with C1-inh has now been clarified (9), permitting more detailed analysis of the mechanism.

The dose–response studies using ^{125}I-labeled C1s and the data from kinetic assays show that for complete abrogation of C1-inh activity, equimolar concentrations of autoantibody and C1-inh were required. Interestingly, the serum molar concentrations of the two autoantibodies studies were similar to those of C1-inh (9). There are at least three explanations as to why anti-C1-inh autoantibodies result in the production of cleaved inactive C1-inh. The possibility that the autoantibodies themselves have enzymatic activity was excluded by the demonstration that cleavage of C1-inh only occurred when C1s was present in addition to the autoantibody (Figs. 3 and 4). It is also possible that the binding of the anti-C1-inh autoantibody produces a conformational change in C1-inh, which makes it susceptible to proteolysis by enzymes other than those which it usually regulates. Although we have no direct evidence to show that this does not occur in vivo, the observations that the 96 kDa cleaved form of C1-inh circulates in vivo and is generated from the incubation of C1-inh, anti-C1-inh autoantibody, and C1s in vitro suggest that this is not the case. Our data support the view that the binding of anti-C1-inh autoantibody to C1-inh converts C1-inh from a pseudosubstrate to a substrate. As a result of the autoantibody being bound to C1-inh, the bond formed between S195 of C1s and R444 of C1-inh is destroyed, probably because the bond is attacked by reactive small molecules in the solvent, i.e., by hydrolysis. In other experiments (27), we have shown that C1s, like anti-C1-inh autoantibodies, binds to peptide 3, which suggests that the distal hinge region of C1-inh contains a secondary binding site for C1s. We hypothesize that the interaction of C1s with this secondary binding site is essential for the formation of a stable C1s-C1-inh complex. Binding of the autoantibody to C1-inh would prevent the interaction of C1s with the secondary binding site and expose the bonding atoms to the solvent, resulting in their hydrolysis so that a stable complex does not form.

The observation that even with a 4-fold molar excess of C1s over C1-inh, the latter was not completely incorporated into the SDS-stable enzyme–inhibitor complex, suggests that a proportion of the C1-inh preparation was in a conformationally altered form. However, complete cleavage of C1-inh occurred while C1-inh autoantibody was preincubated with C1-inh, which suggests that the autoantibody can also produce a conformational change in C1-inh, rendering it susceptible to proteolysis by C1s. An alternative explanation is that a proportion of the C1s-C1-inh complexes formed are reversible and that the autoantibody drives the reaction to the stage at which it is irreversible.

The observation that peptides 2 and 3, but not peptide 1, inhibited the effect of the autoantibody on C1s-C1-inh complex formation confirms our previous conclusion (8) that both peptides 2 and 3 contain an epitope recognized by the autoantibody. Furthermore, they suggest that both of these peptides might have therapeutic value in the management of patients with AAE with C1-inh autoantibodies of the specificity of those described in this study. Our original report described six patients; since then, we have diagnosed a further six, all of whom have autoantibodies that react strongly with peptide 3 and to a lesser extent, with peptide 2, suggesting that this specificity is common to type II AAE.

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