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Inactivation of C1̄ inhibitor by proteases: demonstration by a monoclonal antibody of a neodeterminant on inactivated, non-complexed C1̄ inhibitor

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SUMMARY

Monoclonal antibodies were raised against kallikrein–C1̄ inhibitor and factor XIIa–C1̄ inhibitor complexes. One of the monoclonal antibodies (KII) appeared to react predominantly with C1̄ inhibitor complexes in an ELISA. However, the apparent binding of KII to C1̄ inhibitor complexes was probably due to the presence of proteolytically inactivated C1̄ inhibitor in the complex mixture used for the coating:KII did not bind either kallikrein–C1̄ inhibitor or factor XIIa–C1̄ inhibitor complexes generated in plasma by dextran sulphate. SDS–PAGE analysis of C1̄ inhibitor incubated with proteases revealed that KII-reactive C1̄ inhibitor has a lower molecular weight than native C1̄ inhibitor. We propose that the determinant that reacts with KII is exposed after cleavage of C1̄ inhibitor in its reactive site. The monoclonal antibody KII will enable us to study the inactivation of C1̄ inhibitor in human inflammatory disease.

C1-esterase inhibitor (C1̄-Inh) is one of the serine protease inhibitors in plasma (Travis & Salvesen, 1983). It plays an important role in the regulation of activation of complement and the contact system of coagulation: C1̄-Inh is the only known inhibitor in plasma of activated C1r and C1s (Sim *et al.*, 1979), the major inhibitor of activated Hageman factor (factor XIIa) (De Agostini *et al.*, 1984), and a major inhibitor of kallikrein (van der Graaf, Koedam & Bouma, 1983). During inhibition, sodium dodecyl sulphate (SDS)–resistant equimolar complexes are formed between a protease and C1̄-Inh, as a result of the interaction of the active site serine of the protease and the reactive site of C1̄-Inh (Travis & Salvesen, 1983; Salvesen *et al.*, 1985). We decided to study the role of C1̄-Inh in various pathological conditions. For this purpose, monoclonal antibodies (mAbs) were raised against various forms of C1̄-Inh. Here we report a monoclonal antibody that recognizes an epitope exposed on inactivated, non-complexed C1̄-Inh. Kallikrein–C1̄-Inh and factor XIIa-C1̄-Inh complexes were purified from plasma that had been incubated with dextran sulphate (50 μg per ml) for 20 min at 37° (DXS plasma), by anion-exchange chromatography on a DEAE–Sephacel column (Pharmacia, Uppsala, Sweden), followed by affinity chromatography on Sepharose to which rabbit antibodies against human C1̄-Inh had been coupled. A BALB/c mouse was hyperimmunized by repeated intraperitoneal injections with purified kallikrein–C1̄-Inh and factor XIIa-C1̄-Inh complexes, either in complete Freund's adjuvant (first injection) or in incomplete Freund's adjuvant (booster injections: 3, 6 and 9 weeks after the first injection). Four days after the fourth injection, spleen cells were fused with Sp2/o-Ag14 myeloma cells. Fusion and hybridoma selection were performed according to standard procedures.

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Culture supernatants were initially screened for the presence of specific antibodies by an ELISA, in which C1̄-Inh complexes were used as antigen. After limiting dilutions, supernatants of cloned hybridoma cells were subsequently screened for specificity of antibodies by anti-C1̄-Inh, anti-kallikrein and anti-factor XII ELISAs. Coating of ELISA plates was performed by incubating the wells with 150 μl of the corresponding antigen in PBS (2–5 μg ml). The proteins used were obtained as follows: factor XII, purified according to the method of Griffin & Cochrane (1976), was kindly provided by Dr B. N. Bouma, Dept. of Haematology, Academic Hospital, University of Utrecht. Kallikrein was isolated from fresh plasma according to the method of Nagase & Barrett (1981) with minor modifications: the kallikrein-containing fractions, eluted from the soybean–trypsin inhibitor–Sepharose, were pooled, absorbed with protein A–Sepharose (Pharmacia), and further purified by fast protein liquid chromatography on a mono-Q column (Pharmacia). In non-reduced SDS–polyacrylamide gel electrophoresis (SDS–PAGE) the kallikrein preparation migrated as a single protein band of MW approximately 88,000. C1̄-Inh was isolated from a C1̄-Inh concentrate (this Institute) by affinity chromatography on Con A–Sepharose followed by anion-

exchange chromatography on a SI-17 column of the FPLC system (Pharmacia). The C1-Inh preparation migrated as a major protein band of MW ~110,000 and a minor band of MW ~98,000 in non-reduced SDS–PAGE. On screening by ELISA, five clones were identified that produced anti-C1-Inh antibodies, three clones that produced anti-kallikrein antibodies, and one clone (KII) that produced antibodies that appeared to react predominantly with C1-Inh complexes.

As KII appeared to react with a neodeterminant exposed on complexed C1-Inh, it might be a useful reagent for the detection of C1-Inh complexes in plasma. Therefore, KII was coupled to CNBr-activated Sepharose 4B (Pharmacia) and used in a radioimmunoasssay (RIA): KII–Sepharose suspension was incubated with serial dilutions of either fresh plasma or DXS plasma: binding of C1-Inh to the Sepharose was detected by a subsequent incubation with ^{125}I-anti-C1-Inh antibodies. Technical details of the RIA procedure as well as the preparation of ^{125}I-anti-C1-Inh antibodies are as described previously (Hack *et al.*, 1981). Results obtained with 10 μl plasma samples are shown in Table 1. From the dose–response curves it was calculated that DXS plasma contained eleven times more C1-Inh that bound to KII–Sepharose than fresh plasma. This was expected since DXS plasma contains high levels of kallikrein-C1-Inh and factor XIIa-C1-Inh complexes. However, when in a similar RIA procedure affinity-purified ^{125}I-anti-kallikrein antibodies were used instead of ^{125}I-anti-C1-Inh, no significant binding of kallikrein was detected (Table 1). This was not due to technical deficiencies: with Sepharose to which a mAb (RII) was coupled that binds all forms of C1-Inh, kallikrein-C1-Inh complexes were easily detected in DXS plasma. Similar results were obtained when ^{125}I-anti-factor XII-antibodies were used.

Since KII bound neither C1-Inh complexes nor native C1-Inh (the latter was already evident from the results obtained with the ELISAs, and this was further corroborated by absorption experiments with purified C1-Inh), we reasoned that KII-reactive C1-Inh in DXS plasma represented proteolytically inactivated C1-Inh, i.e. C1-Inh originated from dissociating kallikrein-C1-Inh and/or factor XIIa-C1-Inh complexes. In order to verify this hypothesis, purified C1-Inh labelled with ^{125}I by the Iodogen method was incubated with either C1s (purified from a Cohn-I fraction of human plasma by anion-exchange chromatography on a DEAE–Sephacel column), kallikrein, trypsin, or elastase (trypsin and elastase were obtained from Sigma Chemical Company, St Louis, MO). After incubation with the proteases, ^{125}I-C1-Inh was inactivated (it no longer bound to C1s-Sepharose). Mixtures of ^{125}I-C1-Inh and proteases, and KII-immunoprecipitates of the mixtures were analysed by SDS–PAGE followed by autoradiography (Fig. 1). The mixtures of ^{125}I-C1-Inh and either C1s or kallikrein (Lanes 2 and 3) both contained C1-Inh complexes, C1-Inh of normal MW (~110,000), and degradation products (MW ~98,000 and 91,000). The mixtures of ^{125}I-C1-Inh and either trypsin or elastase (Lanes 4 and 5) only contained degraded ^{125}I-C1-Inh. Analysis of the KII-immunoprecipitates (Lanes 6–9) revealed that KII only binds cleaved C1-Inh, and not complexed ^{125}I-C1-Inh. ^{125}I-C1-Inh not incubated with protease did not significantly bind to KII–Sepharose (i.e. less than 3%), and therefore immunoprecipitation with KII of untreated ^{125}I-C1-Inh was not performed.

When C1-Inh is proteolysed in its reactive site by proteases such as trypsin or elastase, a C-terminal peptide of MW ~12,000 is cleaved off (Salvesen *et al.*, 1985). The highest MW of the C1-Inh species in the mixtures with elastase or trypsin that bound to KII was approximately 12,000 less than that of native C1-Inh (Fig. 1). Therefore, we conclude that KII binds to a

Table 1. Binding of C1-Inh in plasma and DXS plasma to mAb KII

		Binding of:	
		^{125}I-anti-C1-Inh	^{125}I-anti-kall.
KII-Sepharose	Plasma	24%	4%
	DXS plasma	43%	4%
	PBS	4%	4%
RII-Sepharose	Plasma	40%	4%
	DXS plasma	42%	28%
	PBS	3%	3%

0·5 ml of PBS–Tween-20 (0·1 w/v) – EDTA (10 mM) – Polybrene (0·05% w/v), pH 7·4, containing 20 μg of mAb coupled to 2 mg of Sepharose, was incubated with 10 μl of either fresh plasma or DXS plasma for 5 h at room temperature. The Sepharose beads were washed and incubated with ^{125}I-antibodies against C1-Inh or kallikrein for 16 h at room temperature. After washing, the radioactivity bound to Sepharose was measured and expressed as a percentage of the input. RII is a mAb that reacts equally well with native, complexed and inactivated C1-Inh.

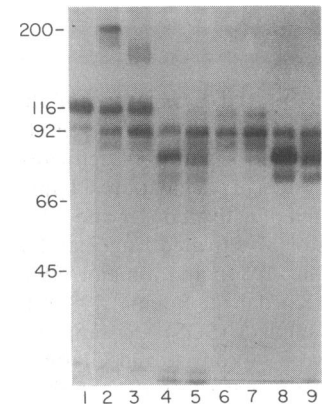

Figure 1. Autoradiography of non-reduced SDS–PAGE (7%) of (a) mixtures of ^{125}I-C1-Inh and either PBS (1), C1s (100 μg/ml) (2), kallikrein (100 μg/ml) (3), trypsin (25 μg/ml) (4), or elastase (25 μg/ml) (5), and (b) KII-immunoprecipitates of mixtures of ^{125}I-C1-Inh and either C1s (6), kallikrein (7), trypsin (8), or elastase (9). The samples were prepared as follows: (a) 40 μl of the mixtures that were incubated for 30 min at room temperature were added to 100 μl of non-reducing SDS sample buffer (6·25 mM Tris HCl, pH 6·8, containing 2% (w/v) SDS, 10% (w/v) glycerol, and 0·001% bromophenol blue); (b) 100μl of the mixtures were incubated for 5 hr with 0·5 ml of KII–Sepharose suspension. After a washing procedure, Sepharose-bound antigen was dissociated with 65 μl of non-reducing SDS sample buffer. Samples (a) and (b) were incubated for 30 min at room temperature, followed by heating in boiling water for 5 min, and centrifugation for 10 min at 2700 *g*. Samples of 50 μl (containing approximately 1500 c.p.m.) were electrophoresed. Left-hand numbers (MW × 10^{-3}) indicate the migration of the high molecular weight protein markers of BioRad (BioRad Laboratories, Richmond, CA).

neodeterminant that is exposed upon cleavage of C1̄-Inh in its reactive site. The apparent binding of KII to C1̄-Inh complexes in the ELISA (see above) was due to the presence of proteolytically inactivated C1̄-Inh in the complex mixture used for coating.

De Agostini *et al.* (1985) reported that C1̄-Inh in complex with kallikrein exposes a neodeterminant that is also present on proteolytically inactivated C1̄-Inh (modified C1̄-Inh). Our study demonstrates that proteolytic inactivation of C1̄-Inh coincides with exposure of another neodeterminant that is not present on either native or complexed C1̄-Inh. The biological relevance of these neodeterminants remains to be established.

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