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COMPLEMENT COMPONENT C1r MEDIATED CLEAVAGE OF THE HEAVY CHAIN OF THE MAJOR HISTOCOMPATIBILITY CLASS I ANTIGENS

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Apart from cleaving C1s, we demonstrate for the first time that: 1) at concentrations found in serum, the activated forms of the complement components C1r in addition to C1s can cleave the heavy chain of MHC class I antigens, 2) the cleavage by C1r and C1s is seemingly dependent upon a native configuration of the MHC class I antigen, since heat denaturation of the HLA antigens reduce the cleavage. The proteolytic fragments following C1 cleavage were characterized by precipitation with Con A-Sepharose, anti-MHC class I and anti-β_2-microglobulin antibodies. The proteolysis of the α-chain of MHC class I was shown to take place between the α_2 - and α_3 - domains as estimated by the Con A-Sepharose precipitation pattern on SDS-PAGE. The α_1 / α_2 fragment was still shown to interact with β_2-microglobulin as shown by immunoprecipitation.

Major histocompatibility class I (MHC class I) antigens are polymorphic cell surface molecules known to be of great importance in the generation of restricted immune responses. MHC class I antigens consist of a heavy transmembrane α-chain carrying the allotypic determinants, to which the light chain β_2-microglobulin is associated non-covalently. The three dimensional structure of human MHC class I antigen elucidated by crystallography revealed a groove on its surface between two long α– helices (α_1 and α_2) which lay across an eight stranded β-pleated sheet (1). An unidentified electron density, probably a mixture of peptides, was seen in the groove (1,2). Recently, binding of peptide fragments to MHC class I antigen has been shown to take part in the assembly of class I molecules (3) and peptides stabilize the conformation of the MHC class I antigen (4).

MHC class I antigens bind and present peptide fragments by the α_1- and α_2-domains, which are critical for interaction with the T cell receptor (2,5). In efforts to study the functional interaction of MHC class I molecules with the T cell receptor, peptides from the α_1- and α_2-domains and soluble forms of MHC molecules have been derived (6,7,8,9,10). The binding of soluble MHC class I antigens or peptides thereof to the T cell receptor is also evident by inhibition of allogenic T lymphocytes or allospecific T cell hybridoma mediated lysis of target cells (6,7,8,9,10). These data indicate that soluble forms of the MHC class I antigens can interact with the T-cell receptor and modulate an allogene induced immune response.

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Recently it was demonstrated that the C1s complement component cleaves β_2-microglobulin within the disulphide loop on the C-terminal side of Lysine-58 in the protein sequence (11) and the MHC class I antigen between the α_2- and α_3-domains of the heavy chain (12).
Our aim was to study: 1) whether the C1r complement component was able to cleave the heavy chain of MHC class I antigen, 2) to investigate the effect of C1 mediated proteolysis on heat denatured MHC class I antigen (i.e., if the proteolysis was dependent upon a native conformation of the MHC class I molecule), 3) to identify the glycosylated fragment(s) of the heavy chain generated after cleavage by C1r and C1s and 4) to characterize the fragments generated by immunoprecipitation analysis with antibodies against common and haplotype MHC class I specific determinants.

Materials and Methods

Purification of human MHC class I antigen (HLA)

HLA antigens from the human cell lines U937 (13) were purified by affinity chromatography on PA 2.6-Sepharose and HLA A:2 from the cell line CALOGERO (14) using PA 2.1-Sepharose (12). Eluted antigens were stored at -20°C.

Iodination of MHC class I antigen

HLA purfied from the cell line U937 was labelled with $Na^{125}I$ (IMS 30, Amersham,U.K.) according to the chloramine T method (15). Briefly, 100 µl of the MHC class I antigen preparation was added to $Na^{125}I$ (9.25 MBq, 0.25 mCi). Twenty µl chloramine T (2 mg/ml in PBS) was added under magnetic stirring and after 15 sec. at 20°C the iodination was stopped by adding 100 µl sodium metabisulfite (1 mg/ml).
HLA A:2 purified from the cell line CALOGERO was iodinated (IMS 30, Amersham,U.K.) with Iodogen (16). Briefly, 100 µl of the HLA A:2 preparation and $Na^{125}I$ (14.8 MBq, 0.4 mCi) were added to a test tube coated with 10 µg Iodogen. After 45 sec. at 20°C the reaction was stopped by gel filtration on a PD-10 column.
After both labelling methods unreacted iodine was separated from the iodinated protein by gel filtration on PD-10 columns (Pharmacia, Uppsala, Sweden), equilibrated and eluted with PBS 0.05 % Triton X-100. The fractions eluted in the void volume were collected and stored at 4°C.

Complement components

C1r and C1s (purity greater than 98% by SDS-PAGE) were both obtained from Calbiochem, San Diego, CA.,USA, or purified as previously described (12). C1-esterase inhibitor (C1-Inh) was purified as previously described by Sim and Reboul (17).

Incubation of C1 complement components and HLA antigens

^{125}I-labelled HLA antigens (3-5 x 10^6 cpm/ml) were incubated both with and without 0.5 µM C1r , 1 µM C1s in 20 mM Tris-HCL pH 7.4, 150 mM NaCl, 4 µM bovine serum albumin (BSA) at 37°C.
Incubation with C1-Inh was performed by a 15 minute pre-incubation of 4 µM C1-Inh and the C1 complement components at 37°C before addition of the ^{125}I-labelled HLA antigens. In experiments with Trasylol (Bayer AG, Leverkusen, FRG) 200 KIE/ml were added to the incubation mixture.

Pre-activation of C1s

Unactivated C1s 1 µM (12) was incubated 3 h at 37°C with 0.05 µM C1r (Calbiochem, San Diego,CA.,USA) in 20 mM Tris-HCL pH 7.4, 150 mM NaCl, 4 µM BSA. After pre-activation ^{125}I-labelled HLA antigens were added and the incubation was continued at 37°C. Samples were withdrawn after 0, 2 and 4 hours of incubation and applied on SDS-PAGE under reducing conditions. The bands were identified by autoradiograghy, excised and counted in a γ- counter.
As control 1 µM C1s and 0.05 µM C1r were incubated in separate samples for 3 h at 37°C before addition of ^{125}I-labelled HLA antigens.

Heat denaturation of ^{125}I-labelled HLA antigens

^{125}I-labelled HLA antigens were incubated 1 h at 60°C in 20 mM Tris-HCL pH 7.4, 150 mM NaCl, 4 μM BSA and cooled to 20°C before addition of C1 complement components and further incubated at 37°C. Samples were withdrawn after 0, 2 and 4 hours and applied on SDS-PAGE as earlier described. Reduction of the HLA cleavage was determined by comparing the degradation of ^{125}I-labelled HLA antigens incubated 1 h at 4°C before addition of C1 complement components.

Polyacrylamide gel electrophoresis, SDS-PAGE

Samples were mixed with sample buffer and applied on a 12% SDS-PAGE (18) under reducing conditions

Precipitation with Con A-Sepharose

^{125}I-HLA antigens were incubated 24 h at 37°C with 0.5 μM C1r and 1 μM C1s as described and further incubated 2 h at 4°C with Con A -Sepharose (Pharmacia, Uppsala, Sweden) in the absence or presence of 0.1 M α-methylmannoside. Finally the Con A-Sepharose was washed 3 times with 1ml PBS 0.1 % Triton X-100 and the samples were run on a 12 % SDS-PAGE under reducing conditions.

Precipitation with monoclonal antibodies

HLA A:2 labelled with iodine was incubated with 0.5 μM C1r and 1 μM C1s. After 12 hours at 37°C, tissue culture supernatants containing the monoclonal antibodies: HB 54 and HB 82 against HLA A:2, HB 149 against β_2-microglobulin, HB 95 (w6/32) and HB 118 (PA2.6) against monomorphic determinants on HLA and as a control HB 122 against HLA A:3 were added to the incubation mixture. Hybridomamas producing the antibodies were all obtained from the American Type Culture Collection, ATCC, Rockville, MD, USA. The incubation was continued for 16 hours at 4°C before precipitation with purified mouse IgG and rabbit anti mouse IgG antibodies (Z 259 DAKOPATTS, Copenhagen, Denmark). The precipitates were collected by centrifugation 13,000 x g for 15 min. at 4°C, solubilized in 100 μl SDS-PAGE sample buffer and 60 μl of the solubilized samples were applied on a 12 % SDS-PAGE under reducing conditions.

Results

Recently we have reported a proteolytic activity of activated C1s complement component on the major histocompatibility class I antigens (12). The initial incubations in this study were done with preparations of activated C1r and C1s obtained from Calbiochem, as shown by their apparent molecular weights under reducing conditions on SDS-PAGE which were 50,000, 35,000, 61,000, and 32,000, respectively. Incubation of the HLA antigens purified from the cell line U937 with the C1r or C1s preparations resulted in the formation of two fragments with an apparent molecular weight of 24,000 and 22,000 (Fig. 1 and 2). However, incubation with the purified HLA A:2 preparation and the complement components resulted in the formation of two fragments of 24,500 and 17,500 (Fig. 3).

Cleavage of the heavy chain of the HLA antigens by C1r or C1s was observed from the autoradiographs after incubation with 63 nM and 250 nM of C1r and C1s respectively. The cleavage of the HLA antigens was fast and observed within the first hours of incubation at 37°C (Fig. 1 and 2).

In order to further investigate the proteolytic activity of C1s, we purified it in its zymogen form with approximately >90% unactivated C1s (12). After pre-activation of zymogen C1s by the C1r preparation all of the C1s was activated as evident by the change in the apparent molecular weight from 87,000 to a 61,000 and a 32,000 heavy and light chain on SDS-PAGE under reducing conditions. As seen in Table I, the proteolytic processing of the heavy chain after pre-activation of zymogen C1s by C1r was far more effective than the additive cleavage of C1r and zymogen C1s.

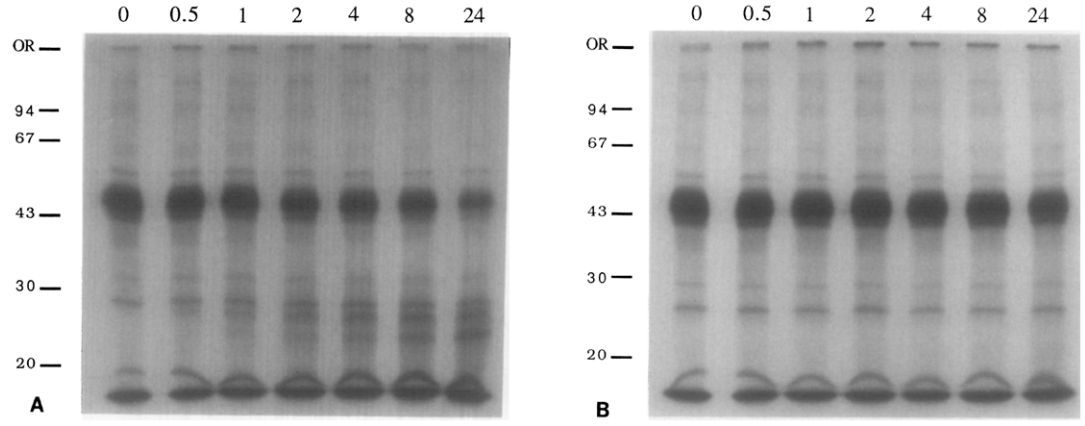


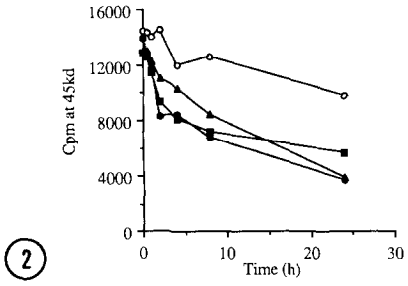
Fig. 1. Cleavage of affinity chromatography purified HLA antigens from U937 cells by C1 complement components.

A. ^{125}I-labelled HLA incubated with 0.5 μM C1r and 1 μM C1s in 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 4 μM BSA at 37°C.

B. ^{125}I-labelled HLA incubated in 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 4 μM BSA at 37°C.

Samples were withdrawn after 0, 0.5, 1, 2, 4, 8 and 24 hours, applied on a 12% SDS-PAGE under reducing conditions and submitted to autoradiography on Kodak X-Omat film.

The proteolytic activity of the C1 complement components against the heavy chain of the HLA antigens could be inhibited by pre-incubation 15 minutes at 37°C with 4 μM C1-esterase inhibitor. Furthermore no reduction of the proteolytic activity was observed after addition of Trasylol to a final concentration of 200 KIE / ml in the incubation mixture.



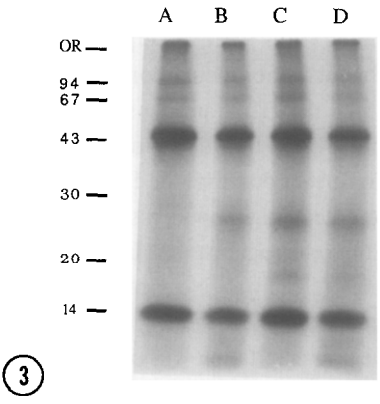


Fig. 2. Proteolytic cleavage of ^{125}I-labelled HLA by C1 complement components.

Affinity chromatography purified and ^{125}I-labelled HLA from U937 cells in 20 mM Tris - HCl (pH 7.4), 150 mM NaCl, 4 μM BSA was incubated at 37°C with; 0.5 μM C1r and 1μM C1s (●), 0.5 μM C1r (■), 1 μM C1s (▲) and without addition of complement components (○) Samples were withdrawn after 0, 0.5, 1, 2, 4, 8 and 24 hours, applied on a 12% SDS-PAGE under reducing conditions. The band at 45,000 was identified by autoradiography, excised and counted in a γ-counter.

Fig. 3. Cleavage of affinity chromatography purified HLA A:2 antigen by C1 complement components.

Affinity chromatography purified and ^{125}I-labelled HLA A:2 in 20 mM Tris -HCl (pH 7.4), 150 mM NaCl, 4 μM BSA was incubated for 12 hours at 37°C without addition of complement components (Lane A), 1 μM C1s (Lane B), 0.5 μM C1r (Lane C) and 0.5 μM C1r and 1μM C1s (Lane D). Samples were applied on a 12% SDS-PAGE under reducing conditions and submitted to autoradiography on Kodak X-Omat film.

Table I

Proteolytic activity of C1 against the heavy chain of human histocompatibility class I antigens purified from U937 cells

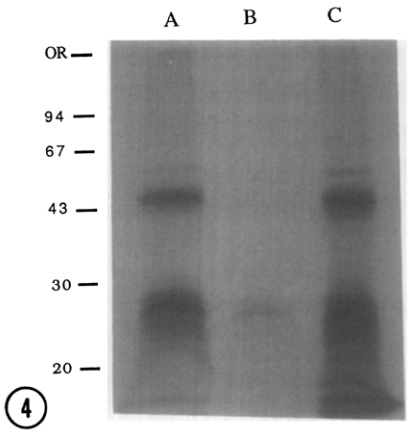
Incubation with	Cpm found at 45,000 Time at 37°C (h)			Cpm found at 24,000 Time at 37°C (h)			Cpm found at 22,000 Time at 37°C (h)		
	0	2	4	0	2	4	0	2	4
C1s 1 μM pre-activated with 0.05 μM C1r	9,516	7,046	5,852	1,009	1,465	1,895	871	1,213	1,582
C1s 1 μM not pre-activated	9,579	9,023	8,737	994	1,220	1,394	901	1,081	1,247
Activated C1r 0.05 μM	9,441	8,977	8,680	909	970	1,133	963	1,002	988

During our studies we observed that after high specific labelling of the MHC class I antigens, the susceptibility for cleavage by C1 was destroyed (data not shown), indicating that the proteolytic processing might be dependent upon a native configuration of the HLA antigen. In order to study this, the HLA-preparation was subjected to heat denaturation by pre-incubation for 1 h at 60°C. The C1 complement mediated proteolysis was reduced by 60 - 70 % after pre-incubation and this strongly supports that the cleavage observed must be dependent on a native configuration of the HLA antigens.

In order to identify the glycosylated fragments of the heavy chain, the reaction mixtures after incubation with the complement components were subjected to precipitation by Concanavalin A-Sepharose. Both the 24,000 and the 22,000 fragments induced from the HLA preparation from the U937 cells (Fig. 3) as well as the 24,500 fragment obtained from the HLA A:2 preparation were precipitated. No precipitation was observed of the 17,500 fragment obtained after incubation of the HLA A:2 preparation and the C1 complement components. Since the HLA antigens are glycosylated only at the α_1-domain of the heavy chain, the precipitated fragments must correspond to the two outer domains.
To further describe the fragment after incubation between the HLA A:2 antigen and the C1 complement components, precipitations with monoclonal antibodies were performed (Fig.5). Antibodies against the HLA A:2 antigen showed only precipitation of the heavy chain and β_2-microglobulin. No precipitation of the 24,500 fragment was observed. However, precipitation of the fragment was seen using an antibody against β_2-microglobulin and a weak precipitation was observed with the two antibodies against monomorphic determinants on the HLA antigen.

Discussion

In the classical pathway of the complement cascade, C1r autoactivates in a complex with antibodies, Ca^{2+}, C1q and C1s. Activated C1r then activates C1s which cleaves the complement components C4 and C2. This process is regarded as very specific due to a high specificity of the proteolytic forms of C1r and C1s.
Autoactivation of C1r occurs in the absence of Ca^{2+} (19) and both activated C1r and C1s show proteolytic activity against the heavy chain of human major histocompatibility complex (MHC) class I antigens (Fig. 1, 2 and 3).



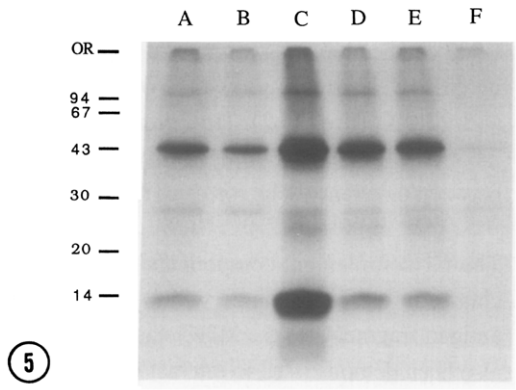


Fig. 4. Con A-Sepharose precipitation of C1 induced HLA fragments.
^{125}I-labelled HLA antigens from U937 cells were incubated with 0.5 μM C1r, 1 μM C1s and Con A-Sepharose as described in methods. C1 induced HLA fragments bound to Con A-Sepharose (Lane A), C1 induced HLA fragments bound to Con A-Sepharose in the presence of 0.1 M α-methylmannoside (Lane B), C1 induced HLA fragments (Lane C).

Fig. 5. Precipitation of C1 induced HLA A:2 fragment with monoclonal antibodies.
Affinity chromatography and ^{125}I-labelled HLA A:2 was incubated with C1 complement components and precipitated with monoclonal antibodies as decribed in the Materials and Methods section. Precipitation with antibodies against: HLA A:2, HB 54 (Lane A), HLA A:2, HB 82 (Lane B), β_2-microglobulin, HB 149 (Lane C), monomorphic HLA, HB 95 (Lane D), monomorphic HLA, HB 118 (Lane E), HLA A:3, HB 122 (Lane F).

The heavy (12) and the light chain, β_2-microglobulin (11) have earlier been shown to be substrates of activated C1s. The cleavage of the alpha chain of the MHC class I is the first demonstration of a substrate of the complement component C1 other than the complement system.

The 24,500, 24,000 and 22,000 fragments were all precipitated with Con A - Sepharose and consist of the α_1- and α_2-domains (Fig. 4). However, a fragment of approximately 20,000 containing the α_3-domain, transmembrane and cytoplasmatic part of the α-chain must be generated at the same time. This fragment of 17,500 was seen after incubation of the HLA A:2 preparation with C1r. Incubation with C1s alone did not show any 17,500 fragment, indicating a further degradation of this fragment by the C1s molecule not complexed bound to C1r since incubation with both the C1r and C1s in the same reaction vessel also showed the 17,500 fragment (Fig.3).

Immunoprecipitation of the fragments generated was not possible with antibodies against polymorphic determinants of the heavy chain of HLA A:2 due to low affinity of the antibodies for the fragments or a low concentration of the fragments. However the α_1 and α_2 fragment was precipitated with anti-β_2-microglobulin antibodies (Fig. 5), demonstrating that the α_1- and α_2- domain still is associated with β_2-microglobulin. Since high affinity binding of peptides to the antigenic grove is known to be dependent upon the association of β_2-microglobulin to the heavy chain (4). The association might therefore indicate that the ability of binding peptides of the α_1 / α_2 fragment generated is retained.

The cell line U937 is serologically-defined as HLA A3/w19; B5/18; Cw1/w3 (13) and CALOGERO is definded as HLA A2; Bw6/61; Cw2 (14). The HLA preparation from the U937 cells contained a mixture of the HLA antigens and proteolysis of the HLA preparation by either C1r or C1s showed two fragments containing the α_1- and α_2-domains of 24,000 and 22,000 (Fig. 1, 2). However, the HLA A:2 preparation showed only one band of 24,500 (Fig. 3) containing the two domains, indicating a slight different cleavage site between different allelic forms of the HLA antigen.

The C1 complement components generated soluble forms of the two outer domains of the class I antigens associated with β_2-microglobulin which all are involved in the binding of antigen fragments to the MHC class I molecule (2,4). This part of the class I molecules have also been demonstrated to interact with the T-cell receptor (5) and cleavage of the MHC class I antigen by the C1 complement components could well be of importance in the regulation of the immune response. However, further studies are needed to elucidate this, including purification of the fragments and analysis of the presence of the fragments in serum and lymph.

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