Binding of α_2 -macroglobulin and limulin: regulation of the plasma haemolytic system of the American horseshoe crab, *Limulus*

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The mediator of haemolysis in the plasma of the horseshoe crab, *Limulus polyphemus*, is limulin, a sialic acid-binding lectin. The haemolytic activity of limulin is inhibited by thiol ester-reacted forms of *Limulus* α_2 -macroglobulin, the third-most abundant protein of the plasma. *Limulus* α_2 -macroglobulin that has experienced cleavage of its internal thiol ester bond, consequent to reaction with proteases, or with the small primary amine, methylamine, reduces the haemolytic activity of limulin when present at molar excesses that approximate the relative concentrations of these two proteins in the plasma. Native, unreacted *Limulus* α_2 -macroglobulin has no effect on the haemolytic activity of limulin. Limulin binds thiol ester-reacted forms of *Limulus* α_2 -

macroglobulin both in a solid-phase assay and in solution with an avidity 10–25 times higher than native, unreacted *Limulus* α_2 -macroglobulin. Protease-reacted α_2 -macroglobulin functions as a marker for the presence of foreign proteases in the blood of *Limulus*, and thus of pathogenic organisms that release proteases as facilitators of invasion and pathogenicity. Modulation of the haemolytic system represents a novel function for α_2 -macroglobulin.

Key words: cytolysis, innate immunity, lectin, pentraxin, proteinase clearance, thiol ester.

INTRODUCTION

The cytolytic destruction of invading pathogens by proteins of the plasma is an important immune defence strategy of higher animals. In mammals, the best studied cytolytic effector in the plasma is the complement system, a complex multi-component cascade of proteases and membrane-active effectors that generate the proteinaceous transmembrane pores that are responsible for the osmotic lysis of the targeted cells [1]. Although the proto-stomate invertebrates lack the complement system [2], many nevertheless possess cytolytic systems mediated by proteins of the blood cells or the plasma [3–9]. In the American horseshoe crab, *L. polyphemus*, the plasma-based cytolytic system is mediated by a single protein, the sialic acid-binding lectin, limulin [10]. Limulin (M_r 300 × 10³; [11]) is a member of the pentraxin protein family and is present in the plasma of the *L. polyphemus* at 30–50 nM [12]. In assays utilizing sheep red cells as the model foreign cell, purified limulin is haemolytic at 3–10 nM, and the removal of the limulin from plasma eliminates haemolytic activity [10]. Haemolysis depends on the sialic acid-binding activity of limulin, since sialylated glycoconjugates, such as fetuin and the sialic acids *N*-acetylneuraminic acid and colominic acid, inhibit haemolysis, and desialylation of the target cells renders them immune to cytolysis [10].

The third most abundant protein in *Limulus* plasma (after haemocyanin and C-reactive protein) is α_2 -macroglobulin (M_r 370; [13]), which is present at 1–5 μ M [6]. Like mammalian α_2 -macroglobulin, the *Limulus* homologue is a broad-spectrum protease-binding protein [14–16], which functions as the principal mediator of protease clearance from the plasma [17]. Most

members of the α_2 -macroglobulin protein family contain a unique internal β -cysteinyl- γ -glutamyl thiol ester bond [18–20], which links cysteine-999 with glutamine-1002 in *Limulus* α_2 -macroglobulin [21]. The reaction of α_2 -macroglobulin with proteases activates the cleavage of this internal thiol ester bond, exposing the thiol of cysteine and the γ -carbonyl of glutamine. Activation of the thiol ester initiates a substantial molecular compaction [13,22,23], exposing a previously cryptic domain that is recognized by cell-surface receptors (the receptor-binding domain; [24]). Recognition results in the binding, internalization, and degradation of *Limulus* α_2 -macroglobulin with its cargo of bound protease [17]. Small primary amines such as methylamine react with the thiol-esterified glutamyl residue [15], and induce a nearly identical conformational change in *Limulus* α_2 -macroglobulin, with a concomitant exposure of the free thiol of the thiol-esterified cysteinyl residue [13], and activation of receptor recognition [17]. Thiol ester-reacted, but not native, forms of *Limulus* α_2 -macroglobulin inhibit the haemolytic action of limulin [25]. Here we show that this inhibitory action is mediated by the preferential binding of thiol ester-reacted forms of α_2 -macroglobulin to limulin.

MATERIALS AND METHODS

Purification of α_2 -macroglobulin and limulin

Recently collected adult *L. polyphemus* were obtained from the Marine Resources Center of the Marine Biological Laboratory (Woods Hole, MA, U.S.A.). Blood was collected under sterile,

Abbreviations used: BS³, bis(sulfosuccinimidyl)suberate; HRP, horseradish peroxidase; PE-agarose, phosphoethanolamine-agarose; PEG, poly(ethylene glycol)-8000.

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lipopolysaccharide-free conditions by cardiac puncture from pre-chilled animals, and the blood cells were removed immediately after bleeding [26]. It is important to avoid degranulation of the blood cells, since this releases proteases [27] and active-site protease inhibitors [28–30] into the serum. Animals were released into the ocean unharmed after bleeding. Most of the haemocyanin was removed from the plasma by ultracentrifugation (141 000 *g* for 16 h), or by incubation with 3 % poly(ethylene glycol)-8000 (PEG) with centrifugation at 30 000 *g* for 30 min. PEG was added to a final concentration of 10 % (w/v), the preparation centrifuged as above, and the precipitate recovered and redissolved in 0.1 M citrate, pH 6.7. The remaining traces of the haemocyanin were precipitated by the addition of zinc acetate to a final concentration of 0.1 M. The supernatant was then precipitated with 10 % PEG and the precipitate dissolved in buffer A [0.15 M NaCl, 10 mM CaCl₂, 50 mM Tris (pH 7.4)]. This fraction (3–10 % PEG cut) was then depleted of Sepharose-binding proteins by passage over a column of Sepharose 4B (Pharmacia) equilibrated with buffer A (0.2 vol. resin/vol. of 3–10 % PEG cut). This was the starting material for purification of α_2 -macroglobulin and limulin.

The pentraxin proteins (*Limulus* C-reactive protein and limulin) were removed by incubation with phosphoethanolamine-agarose (PE-agarose) (Sigma), which binds the pentraxins (0.1 vol. of resin/vol. of plasma). The PE-agarose was washed with buffer A modified to contain 1 M NaCl and eluted with 0.1 M sodium citrate, pH 6.7 to recover the pentraxin fraction. Following dialysis with buffer A, the pentraxin fraction was further fractionated by passage over a column of fetuin-Sepharose equilibrated with buffer A. The breakthrough fraction from the fetuin-Sepharose column is *Limulus* C-reactive protein. The bound fraction is the lectin limulin. After washing the fetuin-Sepharose with buffer A containing 1 M NaCl, limulin was eluted with 0.1 M sodium citrate, pH 6.7. After dialysis with buffer A, and a second purification on fetuin-Sepharose, pure limulin free of contaminating proteins was recovered [10].

The unbound fraction from the incubation with PE-agarose was incubated again with PE-agarose to remove all pentraxin proteins, and was then further purified by chromatography on a 100 $\times$ 1.6 cm column of Sephacryl S-300HR gel-filtration resin to remove aggregated α_2 -macroglobulin, and was stored in 0.1 M citrate, pH 6.7 at 4 °C. The activity of α_2 -macroglobulin was determined by the protection of bound trypsin from the macromolecular active-site inhibitor, soybean trypsin inhibitor [31], using trypsin whose fractional activity had been determined by active-site titration with *p*-nitrophenyl *p*'-guanidinobenzoate hydrochloride [32].

The thiol ester of *Limulus* α_2 -macroglobulin was activated by reaction with methylamine as described previously [31], which resulted in a 96 % loss of the trypsin-binding activity. Unreacted methylamine was removed by dialysis. Proteolytically active trypsin was purified by affinity chromatography of commercially available trypsin (Sigma) on benzamidine-Sepharose [33]. To form the proteinase- α_2 -macroglobulin complex, a two-fold molar excess of trypsin was exposed to α_2 -macroglobulin in 50 mM Tris (pH 8.0) for 3 min at room temperature, and the protease- α_2 -macroglobulin complex was separated from the unbound trypsin by gel filtration on Sephadex G-50.

Solution-phase binding

Limulin was labelled with carrier-free Na¹²⁵I using the chloramine T method as described by Erlich et al. [34]. The specific activity of the labelled limulin was 1×10^7 c.p.m./ μ g of protein. For protein cross-linking, ¹²⁵I-limulin was incubated with

50 molar excess of *Limulus* α_2 -macroglobulin (native or methylamine-reacted), then 10 mM bis(sulfosuccinimidyl)suberate (BS³) in 50 mM phosphate buffer (pH 7.4) was added. After 30 min incubation at room temperature, unreacted BS³ was quenched with 0.17 vol. of 50 mM ethanolamine, pH 7.4 (method described in [35]). Samples were subjected to gel-filtration chromatography on a calibrated 95 $\times$ 1.6 cm column of Sephacryl S-300 HR. The radioactivity of the eluted fractions was determined by γ -counting (LKB-1275 Minigamma; LKB Instruments, Gaithersburg, MD, U.S.A.).

Ligand blot analysis

Limulin was biotinylated by reaction with *N*-hydroxysuccinimide long-chain biotin (NHS LC-biotin; Pierce, Rockford, IL, U.S.A.) in a carbonate/bicarbonate buffer (pH 9.1) for 4 h at 4 °C [36], and then separated from free biotin by dialysis against buffer A. Different forms of *Limulus* α_2 -macroglobulin (native, methylamine-treated and trypsin treated) were subjected to electrophoresis under non-denaturing conditions on a 6 % continuous polyacrylamide gel [37]. After electrophoresis, samples were transferred to nitrocellulose [38]. After blocking the non-specific protein-binding activity with 3 % BSA in PBS with 0.05 % Tween 20 (1 h at room temperature), the blot was incubated with 10 μ g/ml of biotinylated limulin for 16 h at 4 °C, and then with avidin-peroxidase (1:1000 dilution; Boehringer Mannheim) and the peroxidase was visualized with 4-chloro-1-naphthol.

Solid-phase binding assay

Limulin (4 μ g/ml and 100 μ l/well) was adsorbed to Reacti-bind maleic anhydride-activated polystyrene plates (Pierce) in PBS (pH 7.3) for 12 h at 4 °C. Under these conditions approx. 10 % of the limulin bound to the microtiter well, as measured by determining the binding of [¹²⁵I]limulin of known specific activity. Wells were washed with 0.1 % BSA in PBS, 0.05 % Tween 20 (PBS-Tween 20) to remove unbound limulin, then were blocked for 1 h at room temperature with 1 % BSA in PBS-Tween 20. Samples of *Limulus* α_2 -macroglobulin (native and methylamine-treated) were incubated in the limulin-coated wells for 2 h at room temperature in buffer A. Wells were washed three times with PBS-Tween 20 then incubated with affinity-purified rabbit anti-*Limulus* α_2 -macroglobulin (1:1000) followed by horseradish peroxidase (HRP)-conjugated goat-anti-rabbit immunoglobulin (1:10000). HRP was visualized with *o*-phenylenediamine (Sigma). The reaction was stopped with 3 M HCl, and the reaction product was evaluated with an ELISA reader (Bio-Rad) at 490 nm.

Haemolysis and haemagglutination

The haemolytic activity of the plasma was determined in duplicate or triplicate samples using sheep red blood cells [7,10]. Unactivated sheep erythrocytes in Alsevers solution were obtained from Cappel (West Chester, PA, U.S.A.). The buffer system was modified DGVB buffer (0.19 M NaCl, 0.18 mM CaCl₂, 0.5 mM MgCl₂, 2.5 % glucose, 0.1 % gelatin, 2.5 mM sodium barbital, pH 7.3). The reaction mixtures contained 3×10^7 washed sheep red cells, the sample to be tested, and the modified DGVB buffer to a final volume of 800 μ l. The reaction mixtures were incubated with shaking at 22–23 °C for 4 h, and the reaction was terminated by adding 2 ml of ice-cold PBS containing 5 mM EDTA, followed by centrifugation to remove the red cells. The extent of haemolysis was determined by monitoring released haemoglobin

in the supernatant by the optical absorbance at 412 nm and was compared with full haemolysis produced by hypotonic lysis of the red cells.

The haemagglutination assay was performed using sheep erythrocytes that had been washed and suspended at 2% (v/v) in buffer A. An array of serial two-fold dilutions of the test sample was prepared in 200 μ l round-bottom microtiter wells (Sigma), using buffer A as a diluent, and then mixed with equal volumes of the erythrocyte suspension (50 μ l final volume). The array was incubated for 45 min at room temperature, and scored for haemagglutination. Haemagglutinated erythrocytes formed a uniform mat covering the entire curved lower surface of the microtiter well ('umbrella formation'); non-agglutinated erythrocytes formed a compact pellet at the very bottom of the well ('button formation') [8]. The haemagglutination end point was the highest dilution of sample that produced visible agglutination.

RESULTS AND DISCUSSION

Cytolysis of foreign cells by components of the plasma or serum is an important immune defence strategy for a variety of animals. The cytolytic action of the human complement system, and the cytolytic systems of many invertebrates, have conventionally been studied by the ability to lyse mammalian erythrocytes (haemolysis). In this regard, the erythrocyte, although hardly a pathogen, is a model foreign cell whose lysis is easily quantified. In contrast to the complex, multi-component vertebrate complement system, the cytolytic system of *Limulus* plasma depends on the action of a single protein, the sialic acid-binding lectin limulin, which can haemolyse red blood cells as the purified protein and whose removal eliminates the haemolytic action of the plasma [10].

The haemolytic action of purified limulin is sensitive to the ionic environment and shows a broad activity-maximum at NaCl concentrations between 0.2 M and 0.35 M (Figure 1A). Although these salt concentrations would be non-physiological for an immune effector from a vertebrate, it is important to note that the blood of the horseshoe crab has the ionic composition of sea water (0.5 M NaCl). At increasing concentrations toward the activity maximum, this dependence on ionic strength correlates inversely with the haemagglutinating action of limulin (Figure 1B). The curve showing the Ca^{2+} dependence for haemolysis is sigmoidal, with a surprisingly sharp rise from zero activity at 0.65 mM to maximal activity at 0.85 mM (Figure 2A). This may reflect the Ca^{2+} dependency of binding of limulin to the red cell, since haemagglutination shows a similar dose-dependence on the concentration of Ca^{2+} (Figure 2B). Under optimized conditions, haemolysis is dependent upon the concentration of limulin (M_r 300×10^3) between 2 and 8 nM (Figure 3).

Haemolysis at the level of the individual red cell appears to be an all-or-nothing condition, because the fractional haemolysis, as measured by the appearance of haemoglobin in solution, is paralleled by the decrease in the number of red cells present, as determined by haemocytometer counts (results not shown). Partial haemolysis is not the consequence of all cells releasing some of their haemoglobin, but is the result of a few cells haemolysing completely and liberating all of their haemoglobin. The macromolecular osmolytes dextran-8 (M_r $8\text{--}12 \times 10^3$) and, to a lesser extent, dextran-4 (M_r $4\text{--}6 \times 10^3$) block haemolysis (Figure 4). Protection is reversible because red cells treated with limulin plus dextran show partial lysis when washed into limulin- and dextran-free buffer (results not shown). This suggests that limulin causes haemolysis by insertion into the plasma membrane to generate hydrophilic channels that allow water to flow into the

Figure 1 Dependence of limulin-mediated haemolysis and haemagglutination on ionic strength

Haemolysis and haemagglutination were determined as described in the Materials and methods section. The haemagglutination titer (**B**) is expressed as the minimum concentration of limulin in a two-fold dilution series that showed positive haemagglutination. Haemolysis and haemagglutination of sheep erythrocytes showed an inverse correlation between 0.07 and 0.2 M NaCl, with haemolysis (**A**) increasing and haemagglutination (**B**) decreasing over this range of ionic strengths. The assays were conducted with 0.85 mM CaCl_2 , 10 mM Tris and at different concentrations of NaCl.

cell in response to the high internal concentration of macromolecular osmolytes, principally the protein haemoglobin. Protection is imagined to result from the presence of osmolytes (e.g. the dextrans) larger than the channel pore size in the external milieu at concentrations sufficient to balance the osmotic pressure of haemoglobin in the cell, thereby protecting the cell from osmotic rupture [39]. Dextran-8 fails to block haemagglutination at low Ca^{2+} concentrations (results not shown) and exerts the same protective effect against haemolysis at standard (0.85 mM) and high (10 mM) Ca^{2+} concentrations (Figure 4). The activity of dextran, therefore, does not involve Ca^{2+} sequestration. The

Figure 2 Dependence of limulin-mediated haemolysis and haemagglutination on Ca²⁺

The haemagglutination titer (B) again is expressed as the minimum concentration of limulin that showed positive haemagglutination. Haemolysis (A) and haemagglutination (B) showed a dependence on the concentration of Ca²⁺ between 0.6 and 0.85 mM. A similar precipitous rise in haemolysis, this time between 0.43 and 0.49 mM CaCl₂, was found using a 0.17 M NaCl, 2.3 mM barbital buffer system (results not shown).

molecular size of dextran-4 is approx. 1.7 nm [40], indicating an effective pore size for membrane-associated limulin approximating this value.

Proteinases are essential virulence factors for a broad range of microbial and multicellular parasites [41–44], and an important function of the proteinase inhibitors of potential hosts for these parasites is to inactivate and clear these foreign proteinases as a strategy of immune defence. The multifunctional proteinase inhibitor, α_2 -macroglobulin, is found in a diverse array of higher animals and is the third most abundant protein of the plasma of *Limulus* [45]. Its ability to bind and mediate the clearance of proteinases of every catalytic mechanism makes it a key element in the clearance of foreign proteinases, and its presence in the

Figure 3 Haemolysis by limulin at optimized salts

Haemolysis by purified limulin conducted at 0.25 M NaCl, 10 mM Tris, 0.85 mM CaCl₂, showed a concentration dependence between 2–8 nM limulin (*M_r* 300).

Figure 4 Protection by high-molecular-mass osmolites

Haemolysis was conducted under optimal conditions (7 nM limulin, 0.25 M NaCl, 0.85 mM CaCl₂) in the presence of 30 mM carbohydrate osmolites of different molecular weights as indicated. The high-molecular-mass osmolites dextran-8 and dextran-4 protected the limulin-treated red cell against haemolysis. Smaller osmolites exert a reduced protecting effect. Protection is not due to sequestration of Ca²⁺ by the dextrans since protection occurs at high concentrations of Ca²⁺ (sample 6).

Figure 5 Inhibition of limulin-mediated haemolysis by thiol ester-reacted α_2 -macroglobulin

Haemolysis of sheep red cells was achieved in all cases with 10 nM limulin. Native, unreacted *Limulus* α_2 -macroglobulin has no effect on limulin-mediated haemolysis (sample 2), whereas methylamine (MA)-reacted α_2 -macroglobulin (sample 3) and trypsin-reacted α_2 -macroglobulin (M_r 370; sample 4) are inhibitory. The molar excess of α_2 -macroglobulin (780 nM) over limulin (10 nM) approximates the relative concentrations of the two proteins in the plasma [12]. Trypsin, itself inactivated by PMSF (sample 5), has no effect on limulin-mediated haemolysis.

proteinase-reacted form is a potential indicator of parasitic invasion [19]. Thiol ester-reacted forms of *Limulus* α_2 -macroglobulin, generated by reaction with proteinases or methylamine, inhibited the haemolytic activity of purified limulin (Figure 5). Inhibition of limulin-mediated cytolysis was specific for the thiol ester-reacted form of α_2 -macroglobulin; native, unreacted α_2 -macroglobulin had no effect on limulin-mediated haemolysis (Figure 5).

Cleavage of the thiol ester bond of *Limulus* α_2 -macroglobulin generates a new free thiol at cysteine-999 [21]. This is the only free thiol of the protein [20,46] and appears to be important for the ability of thiol ester-reacted α_2 -macroglobulin to inhibit limulin-mediated haemolysis. Treatment of methylamine-reacted *Limulus* α_2 -macroglobulin with the thiol alkylating agent iodoacetamide abolished the ability of α_2 -macroglobulin to inhibit haemolysis (Figure 6). Alkylation of the thiol of methylamine-reacted α_2 -macroglobulin also abolished its ability to reduce the haemagglutinating activity of limulin (results not shown).

The inhibition of haemolysis correlates with a direct interaction of limulin preferentially with the thiol ester-reacted form of α_2 -macroglobulin. Solution-phase binding of limulin to thiol ester-reacted α_2 -macroglobulin could be demonstrated by gel-filtration chromatography. Purified 125 I-limulin eluted at an apparent molecular mass of 300 kDa (Figure 7A), consistent with it being a duodecamer of 25–30 kDa subunits [10,11]. A high-molecular-mass peak of [125 I]limulin appeared during chromatography on

Figure 6 Role of the free thiol in the ability of α_2 -macroglobulin to inhibit limulin-mediated haemolysis

Methylamine (MA)-reacted *Limulus* α_2 -macroglobulin was either left untreated or was treated with 0.1 M iodoacetic acid in 1 M Tris (pH 8.0) for 30 min, and the sample was then dialysed into haemolysis buffer. Haemolysis was conducted under standard conditions, with all samples containing 7 nM limulin in a haemolysis buffer of 0.19 M NaCl, 0.85 mM CaCl₂, 0.5 mM MgCl₂, 0.14 M dextrose, 2.5 mM barbitol, pH 7.3. Results shown are the average of 5 separate experiments. Alkylation by iodoacetic acid of the new free thiol exposed at cysteine-999, following activation of the thiol ester bond by methylamine, eliminates the inhibitory action of the thiol ester-activated form of *Limulus* α_2 -macroglobulin.

Sephacryl S-300 HR resin when limulin and methylamine-reacted α_2 -macroglobulin were co-chromatogrammed (Figure 7B). This peak was absent when 125 I-limulin was chromatographed alone (Figure 7A), and was much reduced in the presence of native α_2 -macroglobulin (Figure 7B). The shoulder of 125 I-limulin, displaced to a high molecular mass in the presence of native α_2 -macroglobulin, is probably due to a minor contamination of this preparation with thiol ester-reacted α_2 -macroglobulin, estimated by the trypsin binding assay [31] to be approx. 10%. The binding in solution appears to be of relatively low affinity because the limulin- α_2 -macroglobulin complex required stabilization with the protein cross-linker BS³ (Pierce). The position of the peak was consistent with a size larger than that of thyroglobulin (M_r 660 $\times$ 10³), and equal or smaller than that of proteins excluded from the internal compartment of the resin (M_r = 1.5 $\times$ 10⁶).

In ligand blot studies, limulin bound to thiol ester-reacted *Limulus* α_2 -macroglobulin electroblotted from a pore-limit electrophorogram to nitrocellulose (Figure 8), but failed to bind to native α_2 -macroglobulin. Thiol ester-reacted *Limulus* α_2 -macroglobulin also bound to immobilized limulin in a solid-phase assay. The binding of limulin to thiol ester-reacted α_2 -macroglobulin showed a 10–25 times greater avidity than to native α_2 -macroglobulin in the solid-phase assay (Table 1). Unlike the solution-phase binding assay, chemical cross-linking was not

Figure 7 Solution-phase binding of limulin with methylamine-reacted *Limulus* α_2 -macroglobulin

^{125}I -Labelled limulin was subjected to size-exclusion chromatography using Sephacryl S-300HR resin in the presence or absence of *Limulus* α_2 -macroglobulin. In the absence of α_2 -macroglobulin, limulin showed a single symmetrical elution peak consistent with a molecular mass of approx. 300 kDa (A). A second high-molecular-mass peak was present in the presence of methylamine (MA)-reacted *Limulus* α_2 -macroglobulin (B). This peak was reduced in the presence of native *Limulus* α_2 -macroglobulin (B). The appearance of a small quantity of high-molecular-mass complex in the presence of native α_2 -macroglobulin (B) may have been due to the existence of small quantities of thiol ester-reacted α_2 -macroglobulin in the sample (estimated at 10% using the trypsin-binding α_2 -macroglobulin activity assay [31]) and/or to a low level of binding of limulin to native α_2 -macroglobulin.

required to detect binding in the solid-phase assay. This may reflect an increase in binding avidity resulting from the association of single α_2 -macroglobulin molecules to multiple limulin molecules immobilized on the solid surface.

α_2 -Macroglobulin was initially identified in *Limulus* and vertebrates as a protease inhibitor [15,16], and as the carrier molecule responsible for the clearance of proteases from the blood [17]. In mammals, α_2 -macroglobulin also functions as the principal carrier for peptide growth factors in the plasma [47]. Here we report a novel function for this protein. Inhibition of limulin-mediated cytotoxicity is restricted to the thiol ester-reacted form of *Limulus* α_2 -macroglobulin. The selective binding of limulin to thiol ester-reacted forms of *Limulus* α_2 -macroglobulin suggests that direct binding of the two proteins is responsible for the modulation of haemolysis (Figure 5) by thiol ester-reacted *Limulus* α_2 -macroglobulin. The role of this binding in immunity is not clear from these studies, but might function in the potentiation of the proteinase clearance functions of α_2 -macroglobulin [17].

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Figure 8 Ligand blot detection of binding of biotinylated limulin to immobilized *Limulus* α_2 -macroglobulin

Limulus α_2 -macroglobulin was subjected to non-denaturing PAGE [6% polyacrylamide gel, Tris/glycine buffer (pH 8.3)] and was then electro-transferred to nitrocellulose membranes. The nitrocellulose blots were incubated with biotinylated limulin and then with HRP-conjugated avidin, and stained with 4-chloro-1-naphthol in the presence of H_2O_2 . Left panel, Ponceau S-stained blot; right panel, ligand blot probed with biotinylated limulin, then HRP-conjugated avidin. In both panels: lane 1, native *Limulus* α_2 -macroglobulin; lane 2, methylamine-treated *Limulus* α_2 -macroglobulin; lane 3, trypsin-treated *Limulus* α_2 -macroglobulin. A $1 \mu\text{g}$ sample was loaded into each lane. Methylamine-treated α_2 -macroglobulin does not migrate as fast as trypsin-treated α_2 -macroglobulin in this gel system, but migrates faster than native α_2 -macroglobulin.

Table 1 Solid-phase binding of native *Limulus* α_2 -macroglobulin ($\alpha_2\text{M}$) and methylamine-treated *Limulus* $\alpha_2\text{M}$ (MA- $\alpha_2\text{M}$) to substratum-immobilized limulin

Limulin-coated microtiter wells were probed first with native or methylamine-reacted α_2 -macroglobulin and then reacted with affinity-purified anti-*Limulus* α_2 -macroglobulin antibody followed by HRP-conjugated goat anti-rabbit IgG. HRP was visualized with *o*-phenylenediamine and the reaction product measured spectrophotometrically at 490 nm.

$\alpha_2\text{M}$ concentration ($\mu\text{g}/\text{ml}$)	A_{490}		Binding ratio: MA- $\alpha_2\text{M}$ /Native $\alpha_2\text{M}$
	Native $\alpha_2\text{M}$	MA- $\alpha_2\text{M}$	
0.0	0.0	0.0	—
0.25	0.005	0.131	26
0.5	0.016	0.157	10
1.0	0.020	0.251	13
2.0	0.024	0.446	19

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