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The *Limulus* Blood Cell Secretes α_2-Macroglobulin When Activated

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Abstract. Alpha$_2$-macroglobulin, a protease-binding protein that is reactive with almost all endopeptidases, is present in high concentrations in the plasma of the horseshoe crab, *Limulus*. Alpha$_2$-macroglobulin was demonstrated by its ability to protect the active site of trypsin from inactivation by the macromolecular active site inhibitor, soybean trypsin inhibitor, and by reaction with an antiserum prepared against purified *Limulus* α_2-macroglobulin. The blood cells also contain α_2-macroglobulin in a form that is released when washed cells are stimulated to undergo exocytosis by treatment with the ionophore, A23187. Alpha$_2$-macroglobulin is detected in the materials released from the cells during degranulation both by activity in the soybean trypsin inhibitor-protection assay and by immunochemical staining of Western blots. The subunit molecular weight of the cell-associated form of α_2-macroglobulin, 185 kDa, is identical to that of the plasma form. The amount of α_2-macroglobulin contained within the cells of a given volume of blood is 0.5–2% of the quantity in solution in that volume of plasma. The distilled water lysates of N-ethylmaleimide-stabilized amebocytes used to detect endotoxin (*e.g., Limulus* amebocyte lysate or LAL) contain relatively large quantities of active α_2-macroglobulin. These preparations are essentially free of the principal plasma protein, hemocyanin, indicating that the cells had been well washed prior to lysis.

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Introduction

Higher animals deploy a variety of defense systems to cope with invading pathogens that are based on components in solution in the blood or associated with the blood cells. These systems operate to restrict the growth and invasion of pathogens and to disable their toxic products. The protein, α_2-macroglobulin, is an element in the system of humoral defenses that binds proteases of all of the major classes and from diverse sources, including proteases of microbes (Barrett, 1981; Feinman, 1983; Sottrup-Jensen, 1987, 1989). This breadth of reactivity contrasts with the relatively narrow range of proteases recognized by individual active-site protease inhibitors (Laskowski and Kato, 1980; Travis and Salvesen, 1983). Proteases bound to α_2-macroglobulin are rendered incapable of hydrolyzing protein substrates and, at least in mammals, are removed from the circulation when the α_2-macroglobulin-protease complex is internalized into secondary lysosomes following receptor-mediated endocytosis (Van Leuven, 1984). Alpha$_2$-macroglobulin is apparently of considerable evolutionary antiquity, because it has been demonstrated in vertebrates (Starkey and Barrett, 1982), arthropods (Armstrong and Quigley, in prep.; Quigley and Armstrong, 1983, 1985; Armstrong *et al.*, 1985; Hergenhahn and Söderhäll, 1985; Spycher *et al.*, 1987; Hergenhahn *et al.*, 1988), and molluscs (Armstrong, unpub. data)—forms whose evolutionary lineages diverged approximately 0.5–0.6 billion years ago. In the arthropod, *Limulus* (the American horseshoe crab), α_2-macroglobulin is present in the plasma at concentrations of approximately 0.3–3 μM (unpub. data), which is similar to the levels reported for humans (3.5 μM: Coan and Roberts, 1989; Harpel, 1987).

The blood of *Limulus* contains a single type of cellular element, the granular amebocyte (Armstrong, 1985a),

which functions as a thrombocyte. Blood clotting involves formation of a cellular plug of adherent amebocytes at sites of injury (Loeb, 1920; Bursey, 1977) and the release of the extracellular clotting system from exocytotic vesicles contained within the amebocyte (Bang, 1979; Mürer *et al.*, 1975; Armstrong and Rickles, 1982). This latter system consists of the structural protein of the clot and a system of proteases that act on the apo form of the clottable protein to convert it into the form that polymerizes into the fibrils of the extracellular clot (Levin, 1985). The present report documents the presence of α_2-macroglobulin in the granular amebocyte in a form that is released from the cell during exocytosis.

Materials and Methods

Limulus *blood cells*

One hundred ml of blood obtained under sterile, endotoxin-free conditions from pre-chilled animals by cardiac puncture (Armstrong, 1985b) was collected into sterile chilled 50 ml plastic centrifuge tubes (Falcon Plastics, Lincoln Park, New Jersey) and centrifuged at 150 $\times$ *g*. The plasma was discarded and the cells were resuspended in 20 ml of ice-cold anti-coagulant buffer [0.5 *M* sterile, endotoxin-free NaCl (Travenol Laboratories, Deerfield, Illinois), 0.01 *M* ethylenediaminetetraacetate, 0.1 *M* glucose, 0.056 *M* citrate buffer, final pH 4.6 (Söderhäll and Smith, 1983)]. The amount of cells in a preparation is presented as the volume of the cell pellet present at this stage. The cells were then washed 3 times with 20 ml/wash ice-cold endotoxin-free 0.5 *M* NaCl and resuspended in 15 ml endotoxin-free 0.5 *M* NaCl, 0.01 *M* $CaCl_2$. Exocytosis was initiated by adding the ionophore, A23187 (Sigma Chemicals, St. Louis, Missouri), to a final concentration of 10 μM. The preparation was incubated at 22°C for 3 h to allow the blood cells to degranulate, aggregate, and for the cell aggregate to contract. Approximately 13–14 ml of cell-free fluid was collected. This fluid contains the contents of the exocytotic granules, apparently uncontaminated by cytoplasmic constituents, because the cytoplasmic marker enzyme (lactate dehydrogenase) is present in the cells and can be released by detergent extraction of the cell pellet, but is absent from the materials released by the A23187-treated cells (Armstrong and Quigley, 1985).

SDS-polyacrylamide gel electrophoresis

Standard techniques were used for SDS-polyacrylamide gel electrophoresis (Laemmli, 1970). Soluble samples were dissolved in reducing sample buffer and were not boiled, to prevent heat fragmentation of the α_2-macroglobulin (Armstrong and Quigley, 1987), and electrophoresed at constant current on 7.5% polyacrylamide gels. We have found the coagulin clot of *Limulus* to dissolve only sparingly in reducing sample buffer (Rickles, unpub. data); not surprisingly, live blood cells and the cell clot produced following A23187-stimulated degranulation of blood cells failed to dissolve completely. Coagulin is the most abundant protein of both preparations. However, soluble proteins from the cells, including α_2-macroglobulin, are dissolved under these conditions.

Anti-α_2-macroglobulin antiserum

A purified preparation of the plasma form of *Limulus* α_2-macroglobulin (Quigley and Armstrong, 1985) was subjected to SDS-polyacrylamide gel electrophoresis (Laemmli, 1970; reducing conditions, 12% polyacrylamide gel) and transferred to nitrocellulose paper by electrophoretic blotting (Towbin *et al.*, 1979). The position of the protein was determined by Ponceau S staining. The band at 185 kDa was cut out, and the paper minced with scissors, suspended in water, and then fragmented by ultrasonication. Approximately 200 μg of nitrocellulose-bound α_2-macroglobulin was injected with Freund's complete adjuvant subcutaneously in multiple sites into female New Zealand white rabbits. A booster dose of 200 μg was given in incomplete adjuvant after 4 weeks, and antiserum was collected at 2-week intervals thereafter (Daino *et al.*, 1987).

Assay for α_2-macroglobulin activity

The functional assay for α_2-macroglobulin is essentially that of Armstrong *et al.* (1985). Briefly, bovine pancreatic trypsin (Sigma) was prepared as a stock solution at 1 mg/ml in 1 m*M* HCl and stored frozen until used. Protease activity was measured by the hydrolysis of the low molecular mass amide substrate, Nα-benzoyl-DL-arginine p-nitroanilide (BAPNA) (Sigma). The assay for α_2-macroglobulin activity depends on the ability of α_2-macroglobulin to bind trypsin without inactivating the active site of the enzyme. Alpha$_2$-macroglobulin-bound trypsin can hydrolyze BAPNA with equal efficiency to that of free trypsin (Quigley and Armstrong, 1983, Fig. 1). The ability of α_2-macroglobulin to suppress the proteolytic activity of proteases apparently depends on its ability to form a molecular cage around the protease molecule that establishes a steric barrier that prevents contact between protease and target proteins. This molecular cage also prevents the inactivation of the active site of the protease by high molecular mass active site protease inhibitors. Specifically, trypsin bound to *Limulus* α_2-macroglobulin is protected from inactivation by the active site inhibitor, soybean trypsin inhibitor (M_r = 21,000). This property is unique to the α_2-macroglobulin family of protease inhibitors and is the basis for an assay for α_2-macroglobulin activity (Ganrot, 1966) that

has been used to detect α_2-macroglobulin in the plasma of molluscs (Armstrong, unpub. data), *Limulus,* and crustaceans (Armstrong *et al.,* 1985). The sample suspected of containing α_2-macroglobulin is incubated with trypsin, and then saturating amounts of soybean trypsin inhibitor are added to inactivate all unbound trypsin. The determination of the rate of hydrolysis of BAPNA allows quantitation of the fraction of trypsin that is protected from inactivation by virtue of its binding to the α_2-macroglobulin in the sample. As far as we know, the assay is specific for the α_2-macroglobulin family of protease inhibitors.

Preparation of Limulus *amebocyte lysate (LAL)*

Lysates of *Limulus* amebocytes were prepared by hypotonic disruption of washed amebocytes at room temperature with sterile, pyrogen-free distilled water, essentially as described by Levin and Bang (1968). All glassware was siliconized, sterilized, and then rendered endotoxin-free by heating at 180°C in a dry oven. Adult female horseshoe crabs were bled directly into an equal volume of warm (40°C) 0.5 *M* NaCl, 0.005 m*M* N-ethyl maleimide (NEM, Sigma). Following sedimentation, the blood cells were resuspended in warm, NEM-containing saline, and then washed twice in warm saline without NEM. Hypotonic lysis of the cell button was accomplished by incubation in pyrogen-free distilled water (3/1 v/v of cells) at room temperature. The cell suspension was vortexed daily for 2 days and the resultant lysate (primary extract) was collected following centrifugation. The pellet was re-extracted by further incubation with distilled water (secondary extract). Lysate was stored at 4°C. Reactivity was determined by incubation with a standard preparation of endotoxin (*E. coli,* 026:B6, Difco Laboratories, Detroit, Michigan). In general, *Limulus* amebocyte lysate prepared in this manner has a protein concentration of 1.5–3.0 mg/ml and forms a gel in the presence of 10–100 pg/ml of endotoxin (Rickles *et al.,* 1979).

Results

Anti-α_2-macroglobulin antiserum

The antiserum recognized specifically the 185 kDa band of α_2-macroglobulin on immunoblots of purified α_2-macroglobulin (Fig. 1, lanes A.2 and B.1), whole *Limulus* plasma (Fig. 1, lanes A.3 and B.2), and *Limulus* plasma depleted of hemocyanin by ultracentrifugation (not shown). The antiserum did not cross react with α_2-macroglobulin from *Homarus* (the American lobster) or with human α_2-macroglobulin (not shown).

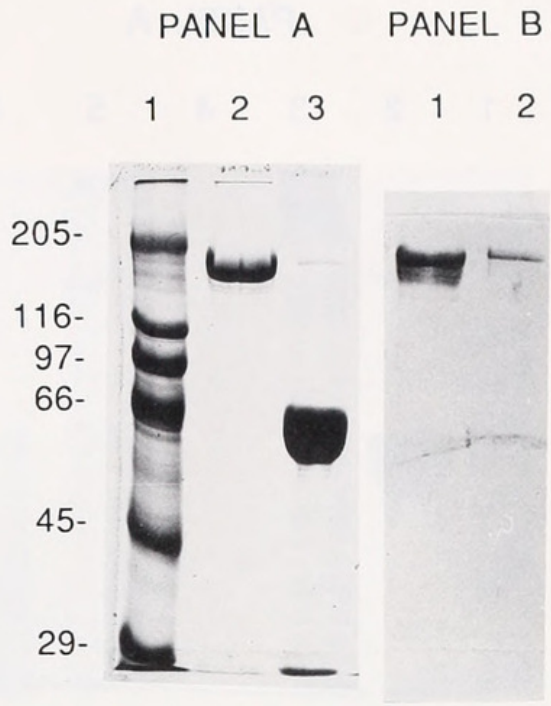

Figure 1. Characterization of the anti-*Limulus* α_2-macroglobulin antiserum by immunoblotting. The antiserum reacts specifically with a band at an apparent molecular mass of 185 kDa with purified α_2-macroglobulin (lanes A.2 and B.1) and with whole plasma (lanes A.3 and B.2). The arc-like density at about 40–60 kDa on panel B is due to a scratch on the nitrocellulose, and does not represent specific deposits of HRP reaction product. Lane A.2 contained 2.2 μg of protein; lane B.1 contained 0.55 μg of protein; and lanes A.3 and B.2 contained 0.25 μl of plasma. In Figures 1, 2, and 4, panel A is a SDS-polyacrylamide gel (7.5% polyacrylamide) run under reducing conditions and stained with Coomassie blue, and panel B is a Western blot of a parallel gel stained with the anti-*Limulus* α_2-macroglobulin antiserum.

Immunological demonstration of α_2-macroglobulin

The materials released from washed *Limulus* blood cells that had been stimulated to undergo exocytosis by exposure to A23187 were subjected to SDS-polyacrylamide gel electrophoresis under reducing conditions and then electrophoretically transferred to nitrocellulose paper. Alpha$_2$-macroglobulin was demonstrated by probing the transfers with the anti-α_2-macroglobulin antiserum. A single band at an apparent molecular weight of 185 kDa was recognized by the antibody (Fig. 2, lanes A.5 and B.6). The penultimate cell wash buffer contained no immunoreactive material (Fig. 2, lanes A.4 and B.5), indicating that the presence of α_2-macroglobulin in the material released during exocytosis of washed blood cells was not a result of contamination by plasma. Alpha$_2$-macroglobulin could likewise be demonstrated in blots of protein from whole, washed blood cells (Fig. 2, lanes A.3, B.3 and B.4). Attempts to demonstrate α_2-macroglobulin by immunoblotting of the proteins extracted from the residual pellet of cells that had undergone exocytosis were unsuccessful (Fig. 2, lanes A.6 and

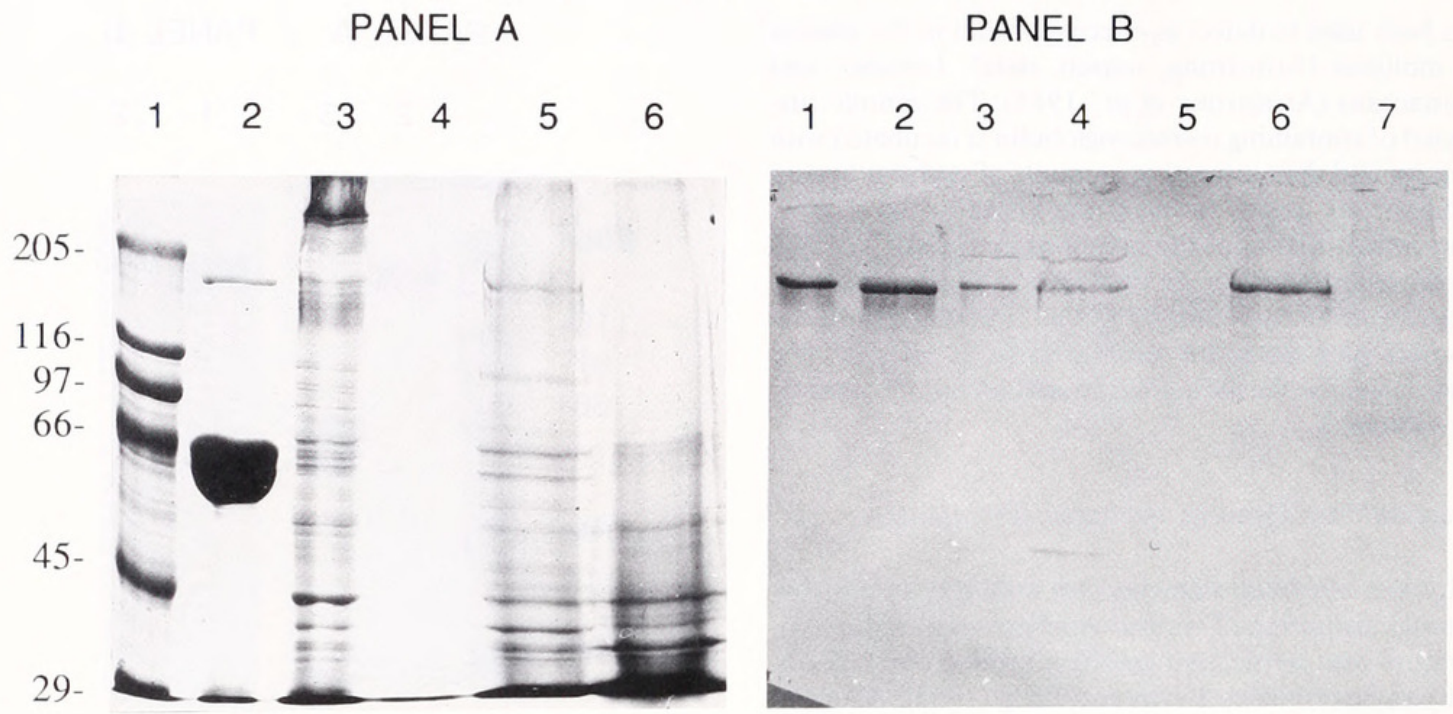

Figure 2. Immunologic demonstration of α_2-macroglobulin in *Limulus* blood cells and in the materials released during degranulation of the cells. Alpha$_2$-macroglobulin is evident in the plasma (lanes A.2 and B.2) and whole, undegranulated cells (lanes A.3, B.3 and B.4) but is not demonstrable in the penultimate wash of the cells (lanes A.4 and B.5). Alpha$_2$-macroglobulin is also demonstrable in the materials released from the cells during degranulation (lanes A.5 and B.6) but not in the degranulated cells (lanes A.6 and B.7). Lane B.1 was loaded with 1.1 μg of purified *Limulus* α_2-macroglobulin. Lanes A.2 and B.2 contained 0.25 μl of plasma; lane A.3 contained 2 μl of pelleted live, whole blood cells; lane B.3 contained 1 μl of pelleted whole, live blood cells; lanes A.4 and B.5 contained 10 μl of buffer from the penultimate cell wash; and lanes A.5 and B.6 contained 10 μl of releasate.

B.7), indicating that most or all of the cell-associated α_2-macroglobulin had been released during exocytosis. The apparent molecular weight of the subunit of cell-associated α_2-macroglobulin was identical to that of the plasma form of α_2-macroglobulin. The relative amounts of protein in different bands of Coomassie blue-stained gels were estimated spectrophotometrically with the curve integration function of a Bio Rad Model 620 scanning densitometer. Estimations of the relative amounts of α_2-macroglobulin by the optical density of the 185 kDa band on Coomassie blue-stained gels of whole cells and plasma indicate that the cells contained in a given volume of whole blood contain approximately 0.5–2% of the α_2-macroglobulin contained in the same volume of plasma.

Measurement of α_2-macroglobulin activity

The ability to protect trypsin from inactivation by soybean trypsin inhibitor was used to estimate the amounts of active α_2-macroglobulin in samples of plasma and the materials released by blood cells stimulated to undergo exocytosis. Comparable results to the immunological studies were obtained: plasma contained large quantities of α_2-macroglobulin, the cells of a given volume of blood contained about 0.5–2% of that amount of α_2-macroglobulin, and the penultimate wash buffer was negative (Fig. 3).

Alpha$_2$-macroglobulin in Limulus *amebocyte lysate (LAL)*

Limulus amebocyte lysate (LAL) is the soluble materials recovered by distilled water lysis of N-ethyl maleimide-stabilized *Limulus* amebocytes and is used for the detection of lipopolysaccharides from Gram-negative bacteria (Levin, 1979). Both immunostaining of protein blots (Fig. 4) and activity measurements (data not shown) indicated the presence of large amounts of α_2-macroglobulin in preparations of LAL. Primary extracts (Fig. 4, lanes A.2 and B.2) contained significantly more α_2-macroglobulin than secondary extracts (Fig. 4, lanes A.1 and B.1), on a per mg of total protein basis. The amounts of α_2-macroglobulin in primary extracts is significantly larger than would be expected from the amounts present in washed, live cells. Preparation of LAL involves exposing whole blood to a warm solution of n-ethyl maleimide, followed by extensive washing of

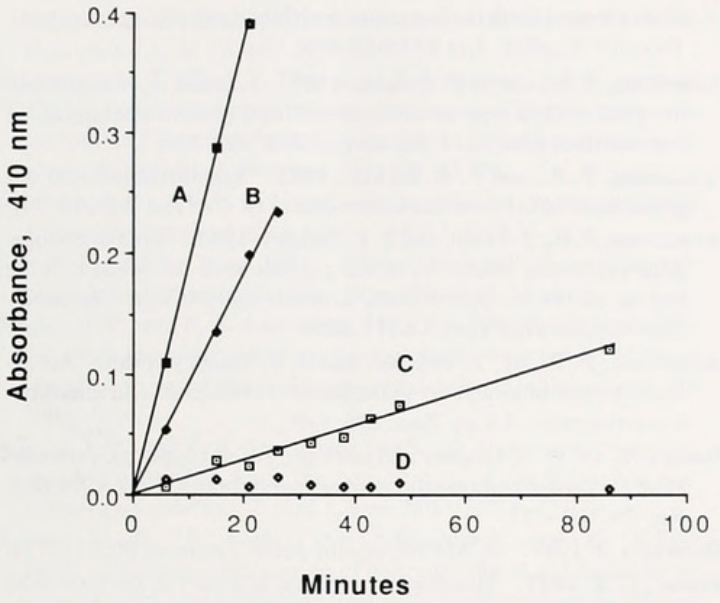

Figure 3. Demonstration of α_2-macroglobulin in materials released by saline-washed *Limulus* blood cells stimulated to degranulate by exposure to the ionophore, A23187, and in plasma from the same animal. The released materials protect trypsin from inactivation by the high molecular mass active site inhibitor, soybean trypsin inhibitor. Ten μg samples of trypsin were preincubated with 20 μl of plasma (curve B), the materials released from 12 μl of packed cells, which was the amount of cells contained in 530 μl of whole blood (curve C), or 160 μl of the penultimate saline wash (curve D) for 10 min and then 20 μg of soybean trypsin inhibitor was added. The remaining active trypsin (*e.g.*, the trypsin bound to the α_2-macroglobulin in the sample, and thereby protected from inactivation by soybean trypsin inhibitor) was assayed by its ability to hydrolyze the low molecular mass amide substrate, BAPNA. This was followed by the increase in optical absorbance at 410 nm. In the absence of added α_2-macroglobulin, the hydrolysis of BAPNA is zero (not shown). Curve A is the activity of 10 μg of trypsin in the absence of soybean trypsin inhibitor or α_2-macroglobulin. In this sample, the cells from a given volume of blood (curve C) contained 0.6% as much α_2-macroglobulin as the plasma from the same volume of blood (curve B). In other trials, the cells have contained as much as 2% of the total α_2-macroglobulin in a given volume of blood.

the cells and then their lysis in distilled water. The efficiency of washing is indicated by the minimal contamination of the LAL preparations with hemocyanin (compare the relative intensities of the hemocyanin band at 67 kDa and the α_2-macroglobulin band at 185 kDa in plasma (Fig. 2, lanes A.2 and B.2) with that in LAL (Fig. 4, lanes A.2 and B.2).

We speculate that α_2-macroglobulin in the plasma specifically becomes associated with the cells during the exposure of blood to n-ethyl maleimide and is released during the distilled water lysis step. The amounts of α_2-macroglobulin in the NEM-containing plasma phase that is the by-product of the preparation of cells for production of LAL were low or undetectable (Armstrong, unpub. data), consistent with the possibility that significant quantities of plasma α_2-macroglobulin become associated with the cells during treatment of blood with NEM.

Discussion

The blood is the principal organ involved in the defense against pathogens that have entered the body. In most animals, both plasma- and blood cell-mediated systems participate in immunity. The α_2-macroglobulin system of protease-binding proteins is a well-studied example of a humoral system of immunity, both in vertebrates (Sottrup-Jensen, 1987, 1989) and arthropods (Quigley and Armstrong, 1983, 1985; Armstrong *et al.*, 1985; Hergenhahn and Söderhäll, 1985; Spycher *et al.*, 1987; Hergenhahn *et al.*, 1988; Armstrong and Quigley, in prep.). The present report documents that the sole blood cell type of *Limulus*, the granular amebocyte, also contains α_2-macroglobulin in a form that is released during degranulation. The cell-associated form of α_2-macroglobulin is immunologically reactive with an antiserum prepared against the plasma form of *Limulus* α_2-macroglobulin and has an identical subunit molecular weight. Although the plasma of a given volume of whole blood contains much more α_2-macroglobulin than do the blood cells of that same volume of blood, the cell-associated form may play an important role in suppression of proteases in the densely cellular clot formed by aggregated blood cells at sites of wound healing (Loeb, 1920; Bursey, 1977). In this situation, exocytosis into the confined spaces between cells would be expected to yield high local concentrations of α_2-macroglobulin that might be of importance specifically because diffusion of α_2-macroglobulin from the plasma into these spaces

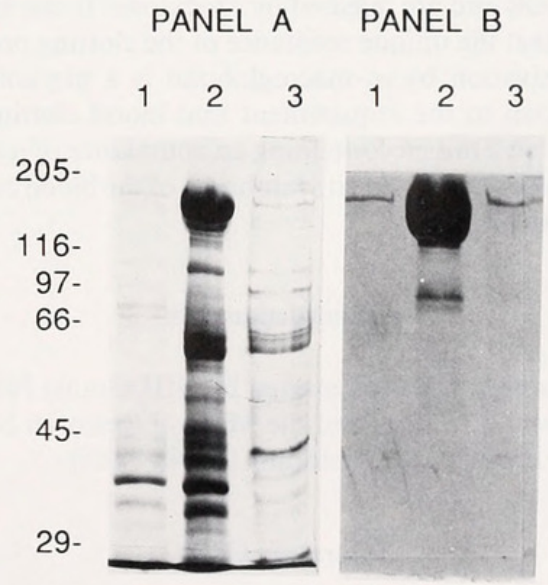

Figure 4. Demonstration of α_2-macroglobulin in preparations of *Limulus* amebocyte lysate. Lanes A.1 and B.1 show a secondary extract, lanes A.2 and B.2 show a primary extract, and lanes A.3 and B.3 show the materials released from live cells exposed to A23187. Lanes A.1 and B.1 contain 18 μg of protein, lanes A.2 and B.2 contain 125 μg of protein, and lanes A.3 and B.3 contain 10 μl of released materials (45 μg of protein).

might be expected to be slow. By forming a temporary barrier between the septic external milieu and the internal tissues of the animal, the blood clot is a critical battleground between invading pathogens and the animal. The gradual release of α_2-macroglobulin as cells of the clot degranulate may play an important role in defense during the early stages of wound healing. The mammalian blood platelet—a cell with homologous function to the *Limulus* amebocyte—also contains α_2-macroglobulin and other protease inhibitors in forms that are released by exocytosis (Nachman and Harpel, 1976; Plow and Collen, 1981). Like the *Limulus* amebocyte, the concentrations of protease inhibitors in platelets are small fractions of the total concentrations in whole blood (Nachman and Harpel, 1976; Plow and Collen, 1981).

The *Limulus* blood cells also release both acid-stable and acid-labile active site inhibitors of serine proteases during degranulation (Armstrong and Quigley, 1985; Nakamura *et al.*, 1987). The importance of α_2-macroglobulin in this situation may derive from its ability to bind such a wide selection of proteases. Although the spectrum of proteases that is susceptible to the active site inhibitors of the blood cells has not been established, most active site inhibitors are reactive only to a defined subclass of proteases, in contrast to the near universal reactivity of α_2-macroglobulin. Interestingly, the only protease that we have found to be unreactive to *Limulus* α_2-macroglobulin is the terminal protease in the blood clotting cascade (Armstrong *et al.*, 1984). In *Limulus*, both the clottable protein and the proteases involved in clotting are localized in the secretory granules of the blood cells and are released by exocytosis. It can be suggested that the unique resistance of the clotting protease to inactivation by α_2-macroglobulin is a physiological adaptation to the requirement that blood clotting can proceed in a milieu containing an abundance of α_2-macroglobulin. The active site inhibitors of the blood cells do inhibit this protease.

Acknowledgments

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