Isolation and Purification of a Cell Adhesion Factor from Crayfish Blood Cells

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Abstract. Isolated granular haemocytes (blood cells) from the crayfish *Pacifastacus leniusculus* attached and spread in vitro on coverslips coated with a lysate of crayfish haemocytes. No cell adhesion activity was detected in crayfish plasma. The cell adhesion activity was only present in haemocyte lysates in which the prophenoloxidase (proPO) activating system (Söderhäll and Smith, 1986*a*, *b*) had been activated; either by lipopolysaccharide (LPS), the beta-1,3-glucan laminarin, or by preparing the lysate in 5 mM Ca^{2+}. Both lysates of granular or of semigranular haemocytes could mediate adhesion.

After A23187-induced exocytosis of the granular cells, cell adhesion activity could be generated in the secreted material if it was incubated with laminarin.

The factor responsible for cell adhesion was isolated from an active haemocyte lysate and purified by ammonium sulfate precipitation, cation exchange chromatography and Con A–Sepharose; it had a molecular mass of ~76 kD on an SDS–polyacrylamide gel. An antibody to this 76-kD band inhibited cell adhesion.

Ca^{2+} was necessary in the medium for the cells to adhere to the adhesion factor. With cyanide or azide, the cells attached but failed to spread.

It is suggested that in vivo the cell adhesion factor is stored in the secretory granules of the semigranular and the granular cells in a putative inactive pro-form, which can be released during exocytosis and, in the presence of beta-1,3-glucans or LPS, be activated outside the cells to mediate cell attachment and spreading, processes of essential importance in arthropod host defense.

IT is a general feature of invertebrate host defense that the circulating haemocytes (blood cells) immobilize invasive microorganisms and injected foreign particles (9, 30). Smaller entities are phagocytosed or, at higher concentrations, entrapped in haemocyte aggregates (nodules), whereas larger particles are encapsulated by several layers of flattening haemocytes (9, 30). Ratcliffe and Gagen (28) observed that the encapsulation response in vivo in the insect *Galleria mellonella* appeared to involve some "sticky" material, which seemed to be secreted from the haemocytes and which was believed to mediate adhesion of haemocytes to the foreign implant. In spite of the importance of these reactions in invertebrate defense, virtually nothing is known at the molecular level about the ways by which their blood cells adhere to a substratum.

In inflammation and haemostasis of vertebrates, factors of complement and of the coagulation system appear to mediate adhesion of granulocytes and platelets (7, 8, 31). Cell-substratum adhesion in general, however, has been best characterized for fibronectin (11, 16, 27, 47), which together with vitronectin (14) seem to constitute the most important adhesive molecules of vertebrate sera (12, 15). Using antisera against vertebrate fibronectin, fibronectin-like molecules have been detected in a few invertebrates: in the freshwater sponge, *Ephydatia mulleri* (23), and in embryos of several sea urchin species (5, 21, 43, 44, 46). A collagen-binding

fibronectin-like molecule, which however did not cross-react with an antiserum against human fibronectin, was isolated from the sea urchin *Pseudocentrotus depressus* (17). This molecule could mediate spreading in vitro of baby hamster kidney cells (18), but no information on the biological role of this factor was given, since activity was not tested on any sea urchin cell type. On the other hand, vertebrate fibronectin has recently been found to induce migration in vitro of primary mesenchyme cells from this sea urchin species (20).

Recently, several observations have implicated a role for the so-called prophenoloxidase (proPO)[1] activating system of arthropods (for reviews, see 39, 40) in haemocyte adhesion (35, 41) and locomotion (45). The proPO system has been suggested to be a complement-like cascade; it consists of one or several serine proteases, which can be activated by minute amounts of the microbial polysaccharides beta-1,3-glucans or lipopolysaccharides (LPS) (3, 25, 29, 39, 40), and which can, in turn, activate proPO. Contrary to vertebrate complement, the proPO system resides within the blood cells in crustaceans (39, 40) and in some insects (25, 29).

The haemocytes (amoebocytes) of the horseshoe crabs

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1. *Abbreviations used in this paper*: CFS, crayfish saline; HLS, haemocyte lysate supernatant; LPS, lipopolysaccharide; proPO, prophenoloxidase; SITS, 4-acetamido-4'-isothiocyanatostilbene-2,2'-disulfonic acid disodium salt; STI soybean trypsin inhibitor.

(e.g., *Limulus polyphemus*), which are ancient arthropods, have, although they lack the enzyme phenoloxidase, a similar enzyme cascade, the well-studied *Limulus* clotting system (for reviews, see 2, 26). In vitro, clotting of a *Limulus* amoebocyte lysate can be triggered by extremely low concentrations of LPS through a series of proteolytic events that ultimately leads to the cleavage of the clottable protein coagulogen to coagulin, which then polymerizes to a gel. After extravasation or trauma, *Limulus* amoebocytes aggregate, degranulate and release the clotting system, which forms an extracellular gel.

In crustaceans, the serine protease and prophenoloxidase (proPO) are confined to the semigranular and the granular cells, and absent from the third haemocyte type: the hyaline cell (34, 38). The enzymes appear to be contained in the secretory granules of these cells and are secreted during degranulation (exocytosis) in their inactive proenzyme forms, which can be activated by adding LPS or beta-1,3-glucans to the released material (19).

Upon activation of a crude proPO system in a crustacean haemocyte lysate, several "sticky" proteins and nonenzymic factors, which have as yet not been isolated, appear to be generated (39, 40), the biological activities of which include: lysis of semigranular cells and degranulation of granular cells in vitro (33, 34, 42), stimulation of phagocytosis carried out by the crab hyaline cells in vitro (33, 42), and enhancement of the in vivo encapsulation of injected fungal spores in crayfish (41). In addition, injection of beta-1,3-glucans caused haemocyte aggregation and a drop in the number of free circulating haemocytes in crayfish and crabs (33, 35).

Taken together, all these observations indicate that the proPO system is involved in blood cell adhesion processes in arthropods. The objective of this study was, therefore, to demonstrate that the proPO system upon release from the cells and activation indeed generates a factor capable of mediating cell adhesion of isolated crayfish haemocytes in vitro, and to purify this factor. To our knowledge, this is the first report on an invertebrate blood molecule promoting cell-substratum adhesion in vitro of a defined invertebrate cell population.

Materials and Methods

Animals

Freshwater crayfish, *Pacifastacus leniusculus*, were collected from Lake Halmsjön, Uppland, Sweden, and kept in aquaria in aerated tap water at 10°C. Only intermoult animals were used in the experiments.

Separation of Haemocytes

The different crayfish haemocyte populations were separated by density gradient centrifugation with the method of Söderhäll and Smith (38), modified for freshwater crustaceans, using gradients of 70% Percoll (Pharmacia AB, Uppsala, Sweden) in 0.15 M NaCl (34).

Preparation of Cell Lysate Supernatants and Plasma

Haemolymph (blood) from 12 crayfish specimens was collected with 1.2-mm needles in 10 ml ice-cold buffer of 10 mM Na cacodylate, 0.25 M sucrose and 100 mM $CaCl_2$, pH 7.0. The haemocytes were spun down at 800 g for 10 min (4°C), and the supernatant was removed and used as a plasma preparation. The cell pellet was washed with the same buffer and homogenized with a glass piston homogenizer in 1–3 ml of 10 mM Na caco-

dylate, pH 7.0, containing either 5 mM $CaCl_2$, a Ca^{2+} concentration that yields "spontaneously" activated serine protease and phenoloxidase in a haemocyte lysate (36, 37), or containing 100 mM $CaCl_2$, to keep the enzymes of the proPO system in their inactive pro-forms (36, 37). These homogenates were then centrifuged at 70,000 g for 20 min at 4°C and the resulting haemocyte lysate supernatants (HLS) were used in the experiments.

In one set of experiments, the inactive HLS (prepared in 100 mM Ca^{2+}) was activated by incubation with an equal volume of 0.1 mg/ml laminarin (Calbiochem, La Jolla, CA) or 1 µg/ml lipopolysaccharide (LPS) (*E. coli* 011-B4) (Sigma Chemical Co., St. Louis, MO) for 1 h at 37°C. Control inactive HLS was incubated with H_2O.

Lysates of isolated semigranular or granular cells were prepared as described (42), except that the cells were homogenized in 10 mM Na cacodylate, 5 mM $CaCl_2$, pH 7.0.

Enzyme Assays and Protein Measurement

Phenoloxidase and protease activities were assayed towards the substrates L-dihydroxyphenylalanine and the chromogenic peptide S-2337 ([bz-ile-glu-(gamma-O-piperidyl)-gly-arg-paranitroanilide], Kabi Vitrum, Mölndal, Sweden), respectively, as described (37). Phenoloxidase activity is expressed as $\Delta A_{490}(min \times mg\ protein)^{-1}$. Protein concentration was determined with the Bradford method (4) using BSA as standard.

Exocytosis of Granular Cells

Exocytosis (degranulation) of isolated suspended granular cells was induced as in (19) with some modifications. Bands of granular cells from six Percoll gradients were pooled, diluted 1:2 with 0.15 M NaCl and centrifuged at 800 g for 10 min at 4°C in tubes containing 90% Percoll, 0.15 M NaCl at the bottom. The concentrated cell bands were withdrawn with a Pasteur pipette and split into two aliquots. One aliquot of the cell suspension was treated with $CaCl_2$ (10 mM) and the Ca^{2+} ionophore A23187 (Sigma Chemical Co.) (10 µM, diluted from a stock solution of 5 mM in dimethyl sulfoxide); the other, the control, contained $CaCl_2$ without ionophore, but with the same final concentration of dimethyl sulfoxide. The volume of both samples was 1 ml. After incubation for 15 min at 20°C, the samples were diluted and buffered to 1.5 ml with 10 mM Na cacodylate, 100 mM $CaCl_2$, 0.15 M NaCl, pH 7.0 (final concentrations), and the cells were spun down at 800 g at 4°C for 10 min. To remove the ionophore, the supernatants were then dialyzed for 2 × 1 h at 4°C against 1 l of 10 mM Na cacodylate, 100 mM $CaCl_2$, pH 7.0. From each of the two supernatants, 180 µl was incubated for 1 h at room temperature (20°C) with 20 µl 1 mg/ml laminarin and another 180 µl was incubated with H_2O. Finally, all four samples were assayed for cell adhesion activity as described below.

Cell Adhesion Assay

All glassware was rendered pyrogen-free by incubation at 180°C for 4 h. Glass coverslips (No. 1.5, 22 × 22 mm; Chance Proper Ltd., Warley, England) were placed in Linbro multiwell plates (Flow Laboratories, Irvine, Scotland) and coated with 100 µl of the sample to be tested at 20°C for 5 min. After coating, the coverslips were washed with distilled water three times, dried at 40°C, quenched with 100 µl 1% BSA for 5 min, and finally washed and dried again as before. Control coverslips received only BSA.

Isolated granular cells, obtained from a Percoll gradient, were diluted 1:2 with 0.15 M NaCl. The cell density of this cell suspension was determined in a haemocytometer and was generally found to be 3–8 × 10^4 cells/ml. The cell suspension was added to the coated coverslips together with $CaCl_2$ (final concentration 10 mM). Each coverslip was overlaid with a total volume of 200 µl, covering an approximate area of 20 × 20 mm².

After incubation for 1 h at room temperature, the coverslips were washed with 0.15 M NaCl and fixed in 10% formalin in 0.15 M NaCl. The percentage of attached cells was assessed by counting the cells at 200× magnification in at least 20 observation fields in the phase contrast microscope (corresponding to 2.8% of the area initially covered by the cell suspension), or at least 200 cells in the positive experiments. In certain experiments, the proportion of spread cells was determined.

The general procedure hitherto outlined was somewhat modified in some experiments, see below and under Results.

Purification of the Cell Adhesion Factor

A "spontaneously" activated HLS was prepared from 60 crayfish in 5 ml cacodylate buffer with 5 mM $CaCl_2$ as described above, and precipitated with

ammonium sulfate (50% saturation) for 2 h at 0°C. All subsequent steps were performed at 4°C. The precipitate was collected by centrifugation at 10,000 g for 15 min. The resulting pellet was dissolved in 600 µl cacodylate buffer (10 mM Na cacodylate, pH 7.0) and dialyzed against 1 l of the same buffer overnight.

A slight precipitation which appeared in the dialyzed fraction was spun down at 5,000 g for 15 min, and then the supernatant was applied to a column of carboxymethyl-cellulose (0.8 × 3.0 cm) (Sigma Chemical Co.), previously equilibrated with cacodylate buffer. This column was eluted with 0.25 M NaCl in cacodylate buffer.

Fractions containing cell adhesion activity from three runs on a carboxymethyl-cellulose column were pooled (an activity from 180 crayfish), and $CaCl_2$ and $MnCl_2$ were added to final concentrations of 1 mM and NaCl to 0.5 M. This fraction was then applied to a column of Con A-Sepharose 4B (Pharmacia) (0.8 × 5.0 cm) equilibrated with 10 mM Na cacodylate, 0.5 M NaCl, 1 mM $CaCl_2$, 1 mM $MnCl_2$, pH 7.0. First the column was incubated for 2 h with 0.25 M alpha-methylamannoside in 10 mM Na cacodylate, 0.5 M NaCl, pH 7.0, and eluted with this buffer. Then, the active cell adhesion factor was obtained by incubation of the Con A-Sepharose column overnight in 0.75 M alpha-methylmannoside in 10 mM Na cacodylate, 0.5 M NaCl, pH 7.0, and elution.

It was established that dilution of a crude active HLS or of the purified cell adhesion factor gave a concentration-dependent cell attachment, so that the percentage of attached cells decreased roughly linearly when plotted against the logarithm of protein concentration. For the purpose of purification, one unit of cell adhesion activity was arbitrarily defined as the amount of a fraction needed to mediate attachment of 50% of the cells. Thus, several dilutions of each fraction after a purification step were always assayed for cell adhesion and the dilution giving 50% cell attachment was calculated.

SDS-PAGE

After running the whole purification scheme as above, the active cell adhesion fraction was dialyzed against 4 l of 0.1 M ammonium acetate for 2 × 2 h at 4°C, lyophilized and finally dissolved in 10 mM Na cacodylate, pH 7.0. The molecular weight of the purified factor was then determined by electrophoresis in a 7.5% polyacrylamide gel in the presence of SDS (SDS-PAGE) (24), both under nonreducing and reducing conditions. Reduction was achieved by treating the sample with dithiothreitol (DTT) (5 mg/ml final concentration) and heating at 100°C for 3 min. 6 µg of cell adhesion factor was applied together with a low molecular mass electrophoresis calibration kit (Pharmacia) (4 µg of each protein). Gels were stained with Coomassie Brilliant Blue.

Amino Acid Analysis

Crayfish cell adhesion factor; purified as above, dialyzed against H_2O and freeze-dried; was analyzed for amino acid composition. The samples (8 µg) were hydrolyzed for 24 h at 110°C with 1 ml 6 M HCl containing 2 mg/ml phenol in thoroughly evacuated and sealed pyren ignition tubes. Half-cysteine and methionine were determined as cysteic acid and methionine sulfone following oxidation with performic acid. The hydrolyzates were analyzed with a LKB model 4151 alpha-plus amino acid analyzer equipped with a Shimadzu CR2AX integrator.

Antiserum

Initially, a rabbit antiserum was made against a partially purified cell adhesion factor. A fraction after $(NH_4)_2SO_4$ precipitation and Con A-Sepharose chromatography (see above under "Purification") was, after dialysis, lyophilization and redissolution in 3 ml H_2O, mixed with an equal volume of Freund's adjuvant and injected intradermally in three rabbits, 20 × 100 µl (120 µg protein) in each. 100 d after immunization, each rabbit was boostered with 100 µg protein, and 2 w later the rabbits were bled.

Purification of Antibodies

From this antiserum and from normal rabbit serum, IgG was isolated using Protein A-Sepharose (Pharmacia) according to the manufacturer's instructions. The anti-IgG was then purified against the cell adhesion factor 76-kD band after transfer to nitrocellulose, essentially as described (32). Cell adhesion factor was purified, dialyzed, freeze-dried, redissolved, and subjected to SDS-PAGE under reducing conditions as above (6 µg per lane). After electrophoresis, the gel was soaked in transfer buffer (25 mM tris-glycine, pH 9.0) for 1 h and the proteins were electrotransferred overnight

at 0.1 A to nitrocellulose filter in the same buffer in a Bio-Rad transblot cell according to the manufacturer. After transfer, the nitrocellulose paper was first incubated in 0.1% KOH for 5 min, then one lane was stained for protein with India ink (13) and three lanes (i.e., with totally 18 µg protein applied) were used for antibody purification. Thus, a strip of nitrocellulose corresponding to the protein band at ∼76 kD was cut out from these lanes, quenched in washing buffer (50 mM Tris, 150 mM NaCl, 5 mM EDTA, 0.05% Triton X-100 and 0.25% gelatin, pH 8.0) at 37°C for 4 h and overlaid with 1 ml of the anti-IgG, diluted 1:10 in washing buffer (final IgG concentration 0.18 mg/ml) overnight at 20°C. (All following steps were also performed at this temperature). After washing the nitrocellulose paper for 4 × 15 min with washing buffer, bound antibodies were eluted as follows: The filter was vortexed for 30 s with 100 µl of first elution buffer (5 mM glycine, 0.5 M NaCl, 0.5% Tween-20, 0.01% BSA, pH 2.3), the buffer was removed and neutralized with 10 µl 0.1 M Tris. This was repeated twice, then the nitrocellulose was similarly vortexed with 3 × 100 µl washing buffer and finally with 3 × 100 µl of second elution buffer (3 M KSCN, 150 mM KCl, 10 mM Na phosphate, 0.01% BSA, pH 6.0). The total eluate, i.e., 930 µl, was dialyzed against 1 l of crayfish saline (CFS)(33) overnight at 4°C. The amount of purified antibody was estimated by applying this solution to a small column of Protein A-Sepharose (0.8 × 0.5 cm), equilibrated in 0.1 M Na phosphate buffer, pH 7.0; after washing thoroughly with this buffer bound material was eluted with 0.1 M glycine-HCl, pH 3.0, and the amount of eluted protein was measured. By this method the concentration of antibody in the eluate from the 76-kD band on nitrocellulose was calculated to be 38 µg/ml.

To investigate which proteins in a haemocyte lysate these antibodies recognized and whether there was any difference between inactive and active lysates, inactive HLS was prepared in 100 mM $CaCl_2$ and activated with laminarin as described above. These preparations were run on reduced SDS-PAGE (96 µg protein per lane), transferred to nitrocellulose and stained for protein with India ink as above. In addition, on one lane of each sample antibody binding was visualized. Thus, the paper was, after soaking in 1% KOH for 5 min, blocked with washing buffer at 37°C for 4 h, incubated with the antibody eluted from the 76-kD band (diluted 1:10 in washing buffer, i.e., 3.8 µg/ml antibody) overnight at 4°C, washed for 4 × 15 min, overlaid with sheep anti-rabbit IgG peroxidase conjugated, IgG fraction (United States Biochemical Corporation, Cleveland, OH) (diluted 1:1,000 in washing buffer) for 4 h at 4°C, washed again as before plus three times with H_2O and finally assayed with a mixture of 20 ml 3 mg/ml 4-chloronaphthol in methanol and 100 ml 0.06% H_2O_2 in 10 mM Tris, 0.15 M NaCl, pH 8.1.

The Effect of Anti-76-kD Band Antibodies on Cell Adhesion

Cell adhesion was assayed as before, except that after the glass coverslips had been coated with purified cell adhesion factor, they were then coated for 2 h with the antibodies eluted from the 76-kD band (38 µg/ml). Several control experiments were performed, in which cell adhesion factor–coated coverslips were instead overlaid with dialyzed elution buffer, with only CFS, or with IgG from normal rabbit serum in CFS (also 38 µg/ml).

Proteolytic Digestion of the Cell Adhesion Factor

Purified cell adhesion factor, 54 µl of 20 µg/ml, was digested with 6 µl of 1 mg/ml trypsin (i.e., final trypsin concentration 0.1 mg/ml) for 1 h at 37°C. The reaction was stopped by treatment with a 100-fold excess of soybean trypsin inhibitor (STI), 60 µl of 11.11 mg/ml, for 15 min at room temperature. Glass coverslips were then coated with 100 µl of this mixture and its cell adhesion activity was assayed as described above. In the control, glass coverslips were coated with cell adhesion factor, which had first been incubated with only H_2O and then with STI. Also, another control experiment was performed, in which a mixture of 1 vol of 0.1 mg/ml trypsin and 1 vol of 11.11 mg/ml STI was used to coat the coverslips (It was established that with this ratio between inhibitor and enzyme, no trypsin activity could be detected).

Chemical Modifications of the Substratum

In one set of experiments, glass coverslips, coated with the purified cell adhesion factor as above, were, after washing and drying, incubated with 100 µl formaldehyde (1.5%), to block free amino groups, or with 100 µl N-ethylmaleimide (10 mM), to block free thiol groups (10). The coverslips

were then washed and dried again and blocked with BSA as above before assaying cell adhesion.

The Effect of Various Dissolved Molecules on Cell Adhesion

The effect of different dissolved molecules on the adhesion process was examined in a number of experiments by adding the chemical to be tested to the cell suspension (in 0.15 M NaCl and 10 mM $CaCl_2$) immediately before incubation of the cells on the substratum. Glass coverslips, coated with the purified cell adhesion factor, were used as substrata in these experiments. The chemicals tested included: the exocytotic inhibitor SITS (1 mM) (4-acetamido-4'-isothiocyanatostilbene-2,2'-disulfonic acid disodium salt, Fluka AG, Buchs, Switzerland); the metabolic inhibitors KCN (1 mM) and NaN_3 (5 mM); and, finally, a spectrum of different sugars at a final concentration of 50 mM: glucose, mannose, galactose, fucose, N-acetylglucosamine, N-acetylneuraminic acid, alpha-methylglucoside and alpha-methylmannoside.

Scanning Electron Microscopy

Small round coverslips (12-mm wide) were coated with 25 μl of purified cell adhesion factor. To each coverslip, 50 μl suspension of isolated granular cells were added either with or without KCN (1 mM final concentration). After 1 h incubation, the coverslips were washed in CFS and the attached cells were fixed in 2.5% glutaraldehyde in CFS for 4 h. The coverslips were then washed three times in "rinse buffer" (0.1 M Na phosphate, 0.002% $CaCl_2$, 2% sucrose, pH 7.2), incubated for 3 × 4 h in this buffer and postfixed for 1 h in 2% osmium tetroxide in "rinse buffer". After washing with water for 2 × 15 min, the cell monolayers were dehydrated in a graded series of ethanol, transferred to freon TF, critical point-dried in CO_2 and finally coated with gold–palladium. The cells were examined and photographed with a JEOL JSM 35 scanning electron microscope, operated at 12 kV.

Results

Activated Haemocyte Lysates or Released Granule Contents Mediate Adhesion of Crayfish Granular Cells

Separated granular haemocytes from the crayfish *Pacifastacus leniusculus* adhered in vitro to glass coverslips coated with a crayfish HLS, in which the (proPO) system (39, 40)

*Table I. Attachment of Crayfish Granular Cells to Various Substrata*

Substratum*	Attached cells (mean ±SD)
	%
HLS in 5 mM Ca^{2+}	88 ± 15
HLS in protease inhibitors + 100 mM Ca^{2+}‡	7 ± 3
HLS in 100 mM Ca^{2+}, activated with LPS	78 ± 8
HLS in 100 mM Ca^{2+}, activated with laminarin	77 ± 8
HLS in 100 mM Ca^{2+}, control with H_2O	25 ± 8
Plasma	4 ± 2
Control (only BSA)	14 ± 10

* Substrata consisted of glass coverslips, coated with 100 μl preparation (haemocyte lysate supernatant [HLS] or plasma) and quenched with 100 μl 1% BSA as in Materials and Methods. The protein concentrations of all HLS preparations were adjusted to 0.75 mg/ml; the protein concentration of the plasma preparation was 20 mg/ml. Phenoloxidase activity was measured as described (37) in the preparations before coating and was found to be 20–24 in the HLS in 5 mM $CaCl_2$, 0.42–0.64 in the HLS in 100 mM $CaCl_2$ and protease inhibitors, 0.57–1.5 in the HLS in 100 mM $CaCl_2$ (control) and 4–10 in the HLS in 100 mM $CaCl_2$ after activation with LPS or laminarin. All activities are expressed as ΔA_{490} (min × mg protein)$^{-1}$.
‡ Haemocytes were homogenized in cacodylate buffer containing 100 mM $CaCl_2$, 5 mM benzamidine, and 2.5 mM leupeptin. Other preparations and the cell adhesion assay were as in Materials and Methods. Each experiment was performed three times.

had been activated, regardless whether the system had been triggered "spontaneously" by homogenizing the haemocytes in 5 mM Ca^{2+} (36, 37) or by incubating an inactive HLS (in 100 mM Ca^{2+}) (36, 37) with LPS or the beta-1,3,-glucan laminarin (Table I) (Fig. 1 *a*). LPS or laminarin alone could not mediate attachment of cells.

An HLS in which the proPO system was kept inactive did not mediate cell adhesion (Table I) (Fig. 1 *b*), this was particularly evident if a mixture of protease inhibitors, known to completely inhibit proPO activation, was included in the homogenizing buffer (Table I). The protease inhibitors per
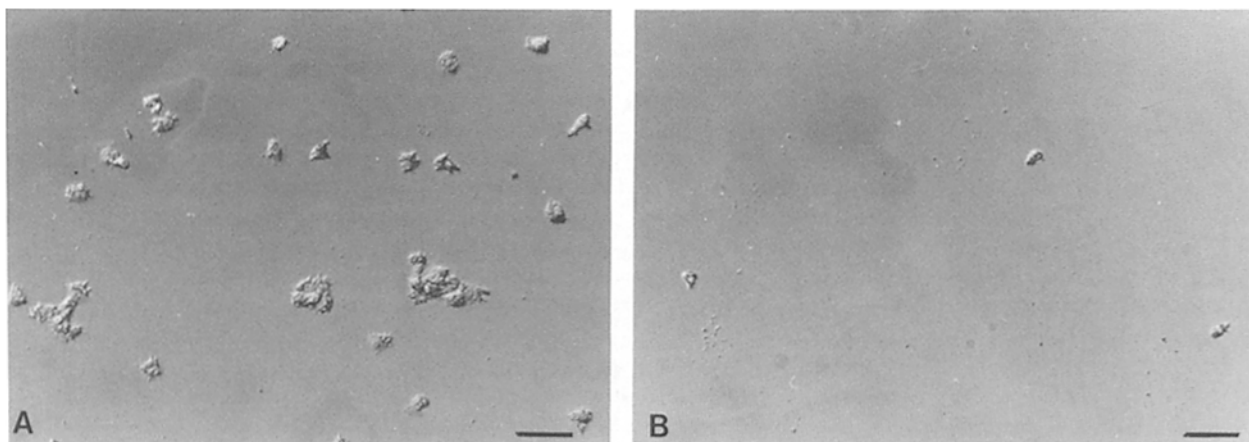
Figure 1. Isolated crayfish granular blood cells (haemocytes) added to glass coverslips coated with different crayfish haemocyte lysates. (*A*) Coverslips were coated with 100 μl of a haemocyte lysate (0.75 mg protein/ml) in which the prophenoloxidase system was activated (by preparing the lysate in 5 mM $CaCl_2$). The granular cells attached and spread to this substratum. (*B*) Coverslips were coated with 100 μl of a haemocyte lysate (0.75 mg protein/ml) in which the prophenoloxidase system was kept inactive (by preparing the lysate in 100 mM $CaCl_2$). The granular cells did not attach to this substratum. For details, see Materials and Methods. Nomarski differential interference contrast microscopy. Bars, 100 μm.

Table II. Purification of Crayfish Cell Adhesion Factor from a "Spontaneously" Activated Haemocyte Lysate*

Purification step	Volume	Total protein	Total activity	Specific activity‡	Apparent yield	Apparent purification
	ml	*mg*	*U*	*U/mg*	*%*	*n-fold*
Haemocyte lysate supernatant	15	15.0	150	10	100	1
Ammonium sulfate precipitation	1.8	10.0	290	29	190	2.9
Carboxymethyl-cellulose	5.1	0.47	210	450	140	45
Con A–Sepharose	3.9	0.062	90	1450	60	145

* For details, see Materials and Methods.
‡ For definition, see Materials and Methods.

se did not inhibit the cell adhesion activity when added to an already activated haemocyte lysate.

The adhesion activity of an HLS in which the proPO system had been activated was quite stable: it was not affected by freezing for several months and heating a preparation to 58°C for 10 min did not significantly decrease the adhesion activity; however, it was totally destroyed at 70°C. Coated coverslips, i.e., immobilized cell adhesion factor, on the other hand, could be dried at 105°C for 5 min (but not at 180°C) without loss of activity.

Lysates of separated cells were also tested: both lysates of semigranular and of granular cells could mediate cell adhesion of the granular cells (not shown). Unfortunately, it is not possible to obtain a sufficient number of hyaline cells for preparation of a lysate from this crayfish species. No activity was detected in plasma preparations (Table I).

Finally, cell adhesion activity was released from granular cells when they were triggered to degranulate (undergo exocytosis) by the calcium ionophore A23187. As with whole cell lysates, the secreted material had to be incubated with laminarin before it could mediate cell adhesion. A substratum of such a laminarin-activated supernatant from iono-phore-treated cells gave 57% cell attachment, whereas the control, the supernatant from ionophore-treated cells incubated with water, mediated attachment of 7% of the cells. To supernatants from control non-degranulated cells less than 5% of the cells attached.

Purification of the Cell Adhesion Factor

A factor responsible for this cell adhesion activity could be isolated from an active haemocyte lysate (theoretically, the factor could have been isolated from an activated supernatant of degranulated cells; to collect sufficient protein this way would however have been more time-consuming) and purified to apparent homogeneity using ammonium sulfate precipitation, cation exchange chromatography and affinity chromatography on Con A–Sepharose (Table II). A chromatofocusing experiment showed that the cell adhesion activity was present in a fraction with a pI between 7 and 9. The purified cell adhesion activity fraction contained no detectable protease or phenoloxidase activity. The cell adhesion activity of purified fractions varied somewhat, generally an amount of 0.5–3 μg protein per coverslip was needed to give 50% cell attachment (i.e., a specific activity ranging from 300–2000 units /mg protein [Table II]). The amino acid composition of this fraction is shown in Table III.

*Table III. Amino Acid Composition of Crayfish Cell Adhesion Factor*

Amino acid*	mol
	%
asx	7.59
thr	4.04
ser	16.96
glx	14.46
pro	3.31
gly	20.07
ala	6.09
cys	1.04
val	3.44
met	0.68
ile	2.44
leu	4.10
tyr	2.12
phe	1.89
his	1.99
lys	7.94
arg	1.84

* The amino acid composition of purified crayfish cell adhesion factor was determined as described in Materials and Methods. Tryptophan was not determined.

With SDS–PAGE, a molecular mass of ∼76 kD of the purified cell adhesion factor fraction was found, under both reducing or non-reducing conditions (Fig. 2, *a* and *b*). Moreover, when used for coating coverslips already coated with the cell adhesion factor, antibodies eluted from this 76-kD band on nitrocellulose could inhibit cell attachment (Table IV), indicating that the cell adhesion factor is indeed the 76-kD polypeptide. These antibodies recognized a corresponding 76-kD band in haemocyte lysates (either inactive, in 100 mM CaCl₂ (Fig. 3); or activated, with laminarin (Fig. 3), or by homogenizing in 5 mM CaCl₂ [not shown]) on nitrocellulose after reduced SDS–PAGE and protein blotting. No protein in crayfish plasma was detected to bind these antibodies.

Nature of Adhesion

First, it was confirmed that the active factor was indeed a protein: To a substratum which consisted of purified cell adhesion factor that had been treated with trypsin (followed by the addition of STI) only 38% of the cells attached;
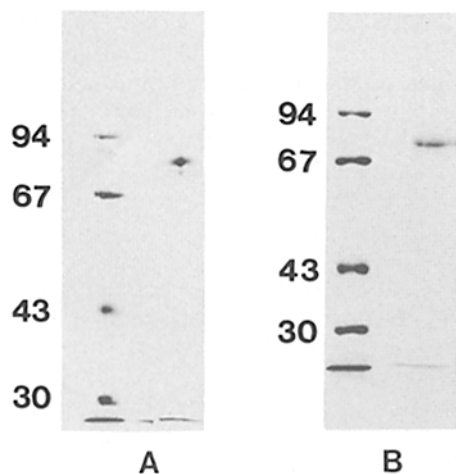
Figure 2. SDS–PAGE of purified crayfish cell adhesion factor under reducing (A) and nonreducing (B) conditions. Gels were stained with Coomassie Blue. (Left lanes) Molecular mass standards (kD). (Right lanes) Cell adhesion factor (6 μg on each lane). The molecular mass of the crayfish cell adhesion factor was ∼76 kD both under reducing and nonreducing conditions. For details, see Materials and Methods.

Figure 3. Nitrocellulose blots of inactive and laminarin-activated crayfish blood cell lysate after SDS-PAGE under reducing conditions. (A) Laminarin-activated cell lysate, protein staining with India ink. (B) Laminarin-activated cell lysate, visualization of binding of anti–76–kD band antibodies with secondary antibodies coupled to horseradish peroxidase. (C) Inactive cell lysate, visualization of binding of anti–76 kD band antibodies with secondary antibodies coupled to horseradish peroxidase. (D) Inactive cell lysate, protein staining with India ink. To the right: the positions of molecular mass standards (kD). For the preparation of antibodies and other details, see Materials and Methods.

whereas the control, nondigested cell adhesion factor with STI added, gave 79% cell attachment (STI alone did not affect the activity of the cell adhesion factor). A mixture of trypsin and STI, without the cell adhesion factor, did not have any effect on the cells.
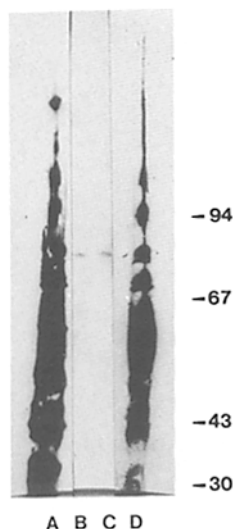
Furthermore, it was established that Ca²⁺ was always necessary in the cell medium for the cells to adhere to the purified cell adhesion factor (Table V). To try to further characterize the process, coated coverslips were treated with formaldehyde, to block free amino groups, or with N-ethylmaleimide, to block free thiol groups (10). None of these modifications affected the adhesion activity of the cell adhesion factor (not shown). The possible participation of carbohydrates was examined by adding different sugars (see Materials and Methods) together with the cells. No inhibition of adhesion was seen with any of the monosaccharides at the

concentration tested. Higher concentrations of some of the sugars appeared to be toxic for the cells, so any involvement of carbohydrates in the binding can still not be completely excluded.

Typically, the initial cell attachment was followed by cell spreading. Cyanide or azide retarded spreading, but did not change the number of attached cells (Table VI) (Fig. 4, a and b).

Cell attachment did not change significantly in the presence of 1 mM SITS (not shown), a drug that inhibits degranulation (exocytosis) of granular cells more than 80% (42), indicating that the initial attachment or binding of a cell to the adhesion factor can occur without degranulation.

Adhesion of Semigranular Cells

Due to the stability in vitro of the granular cells, they were used throughout this study. It was confirmed, however, that isolated semigranular cells also adhered in a Ca²⁺-dependent manner to the purified cell adhesion factor (not shown).

Discussion

It is evident from this study that crayfish semigranular and granular haemocytes (blood cells), the two cell types that

Table IV. The Effect of Anti–76–kD Band Antibodies on the Attachment of Crayfish Granular Cells to a Substratum of Cell Adhesion Factor*

Substratum	Degree of inhibition of cell attachment
	%
Cell adhesion factor + anti–76–kD band antibodies	61
Controls:	
Cell adhesion factor + dialyzed elution buffer	0
Cell adhesion factor + normal IgG	5
Cell adhesion factor + crayfish saline	0

* Cell adhesion factor was isolated and purified as in Materials and Methods. The protein concentration of this fraction was 20 μg/ml. Substrata consisted of coverslips, first coated with 100 μl of this fraction and then coated with 100 μl of antibodies, which had been eluted from the 76-kD band of the cell adhesion factor and dialyzed. The concentrations of anti–76–kD antibodies and of normal IgG were 38 μg/ml.
For the cell adhesion assay, the preparation of antibodies and IgG from normal rabbit serum, and other details, see Materials and Methods.

Table V. The Calcium Dependence of the Attachment of Crayfish Granular Cells to a Substratum of Cell Adhesion Factor*

Cell medium‡	Attached cells (mean ±SD)
	%
CaCl₂ (10 mM)	62 ± 15
Control (only 0.15 M NaCl)	24 ± 12
EDTA (10 mM)	2 ± 1

* Cell adhesion factor was isolated and purified as in Materials and Methods. The protein concentration of this fraction was 29 μg/ml. Substrata consisted of glass coverslips, coated with 100 μl of this fraction and quenched with 100 μl 1% BSA as in Materials and Methods.
‡ The chemicals were added to the cell suspension (in 0.15 M NaCl) immediately before incubation of the cells on the substratum. Otherwise, cell adhesion assay was as in Materials and Methods. Each experiment was performed three times.

Table VI. The Effect of Metabolic Inhibitors on Attachment and Spreading of Crayfish Granular Cells to a Substratum of Cell Adhesion Factor*

Cell medium‡	Attached cells (mean ±SD)	Spread cells (% of attached cells) (mean ±SD)
	%	%
KCN (1 mM)	52 ± 15	17 ± 6
NaN₃ (5 mM)	62 ± 20	22 ± 6
Control (only 0.15 M NaCl + 10 mM CaCl₂)	62 ± 15	52 ± 15

* Cell adhesion factor was isolated and purified as in Materials and Methods. The protein concentration of this fraction was 29 μg/ml. Substrata consisted of coverslips, coated with 100 μl of this fraction and quenched with 1% BSA as in Materials and Methods.

‡ The inhibitors were added to the cell suspension (in 0.15 M NaCl and 10 mM CaCl₂) immediately before incubation of the cells on the substratum. Otherwise, cell adhesion assay was as in Materials and Methods. Each experiment was performed three times.

contain the serine protease, proPO and the "degranulating factor" of the so-called proPO system (34, 39, 40, 42) (see Introduction), also contained a factor, able to mediate attachment and spreading of isolated granular cells in vitro. No cell adhesion factor could be detected in crayfish plasma.

It was observed that the cell adhesion factor, as judged by antibody binding, was present in a nonactivated haemocyte lysate, but without exhibiting any biological activity. The cell adhesion activity, however, appeared concomitantly with the activation of the proPO system. The mechanism by which the inactive cell adhesion molecule becomes biologically active is presently unknown.

It is known that, upon activation of the proPO system, several proteins, among them the active enzyme phenoloxidase itself, become "sticky" and attach to various surfaces (36, 39, 40, 41). Although both active phenoloxidase and protease of a crude haemocyte lysate attached to glass, and might have been likely candidates for cell adhesion activity, the cell adhesion factor appeared to be distinct from these enzymes,

since the purified cell adhesion factor did not have any protease or phenoloxidase activity and since these enzymes did not bind to the columns used in the present purification procedure. The cell adhesion factor seems to be a basic glycoprotein with a molecular mass of ∼76 kD.

Most probably, all components of the proPO system are contained in their inactive pro-forms in the secretory granules of the semigranular and the granular cells, wherefrom they are secreted during degranulation (exocytosis) (19). Similarly, the present cell adhesion factor is located in these vesicles, conceivably in an inactive pro-form, since the protein could be released by degranulation, e.g., stimulated by Ca²⁺ ionophore, and the cell adhesion activity was only evident after the secreted crude granule contents were incubated with activators of the proPO system, e.g., laminarin. In addition, recent experiments with the antibodies to the cell adhesion factor and immunogold-labeled protein A have shown that this protein is strictly located in the granules of both semigranular and granular cells (Latgé, J. P., A. Beauvais, M. W. Johansson, and K. Söderhäll, unpublished results).

Therefore, in crayfish with their open circulatory system, a cell adhesion factor is contained within the blood cells. We suggest that this molecule is of vital significance in vivo in crustacean host defence; that the factor is secreted from the cells when they degranulate encountering non-self entities (19, 33, 42), that it is activated and attached to surfaces, then able to mediate haemocyte adhesion to and encapsulation of the foreign particles. Furthermore, it is likely that this factor was responsible for the haemocyte adhesion events in vivo observed in the experiments described in the introduction: e.g., beta-1,3-glucan-induced haemocyte aggregation (33, 35) and haemocytic encapsulation of fungal spores stimulated by haemocyte lysate (41).

In wound healing and haemostasis in the horseshoe crab *Limulus* (see Introduction), the sealing of wounds is achieved in part by the formation of a cellular clot as extravasated amoebocytes are activated and adhere to the surfaces of the wound and to each other, and in part by the polymerization (gelation) of coagulogen secreted from the blood cells by ex-
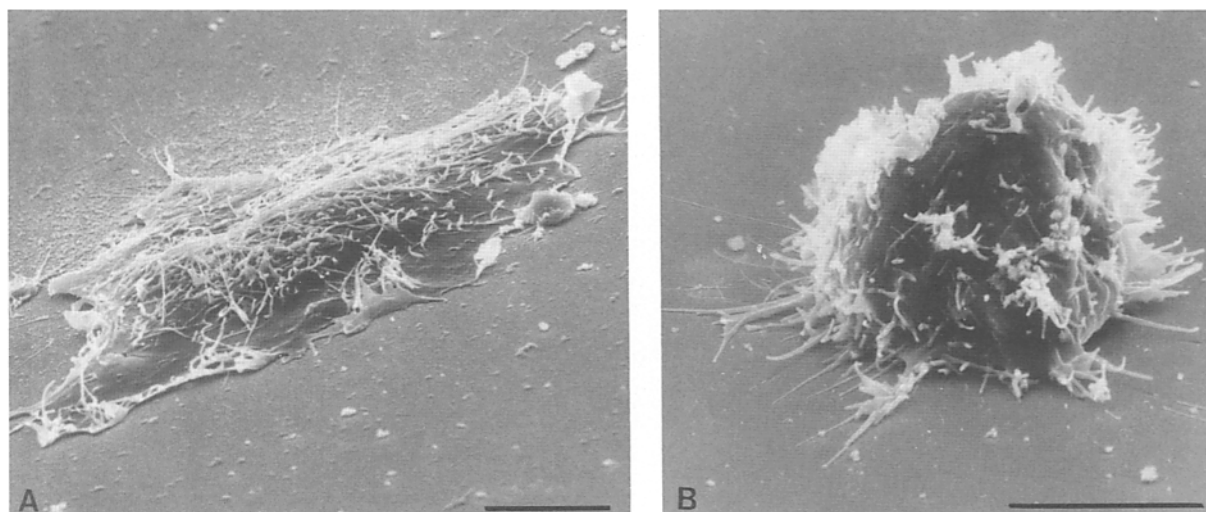
Figure 4. Scanning electron microscopy of isolated crayfish granular blood cells added to glass coverslips coated with crayfish cell adhesion factor. (*A*) Control, the cells attached and spread on the cell adhesion factor. (*B*) In the presence of KCN (1 mM), the cells attached to the cell adhesion factor but did not spread. For details, see Materials and Methods. Bars, 10 μm.

ocytosis (2). The attachment, migration and spreading of *Limulus* amoebocytes to glass, other artificial surfaces and to clotted gel in vitro has been described (1); as these processes were not effected by *Limulus* plasma, it was suggested that they were regulated by factors contained in the cells. From observations that material released from amoebocytes could restore EDTA retardation of blood cell aggregation in vitro (22), a possible role for coagulagen in mediating the aggregation of amoebocytes was proposed (2). However, as these studies have not been performed with purified coagulogen or coagulin, it may also be possible that another protein of the *Limulus* amoebocytes, either alone or in association with coagulogen, is involved in amoebocyte attachment, spreading, and aggregation.

Furthermore, it is not likely that the 76-kD cell adhesion factor in crayfish blood cells is a coagulogen-like substance, since it is not present in the plasma (as tested by biological activity as well as immunoblotting); whereas the plasma appears to be the main repository of crustacean coagulogen, which usually exists as a dimer of two 200-kD subunits (for review, see 6).

Finally, that some "sticky material", a proposed adhesion factor, is released from haemocytes during encapsulation in insects and crustaceans has been documented in several species (28, for reviews, see 9, 30, 40). Since now, in crayfish, haemocyte-substratum attachment and spreading, at least in vitro, can be attributed to a particular protein with a molecular weight of 76 kD, it seems reasonable to speculate that homologous or similar cell adhesion factors are present in other arthropod species, and can be isolated therefrom.

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