

ARE INTEGRINS INVOLVED IN THE AGGREGATORY AND PHAGOCYTIC BEHAVIOUR OF FISH HAEMOSTATIC CELLS?

DARRYL J. HILL AND ANDREW F. ROWLEY*

School of Biological Sciences, University of Wales, Swansea, Singleton Park, Swansea SA2 8PP, Wales, UK

*Author for correspondence (e-mail: a.f.rowley@swansea.ac.uk)

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Summary

The involvement of a putative integrin-like fibrinogen receptor in the aggregatory and phagocytic behaviour of thrombocytes (platelet equivalents of fish) from the rainbow trout *Oncorhynchus mykiss* was studied. Aggregation of trout thrombocytes was induced by the thromboxane mimetic U-46619 in the presence of trout fibrinogen. Thrombocyte aggregation was inhibited by the tetrapeptide RGDS, but not by RGES or fibrinogen binding inhibitor peptide (HHLGGAKQAGDV). A range of monoclonal antibodies against the human platelet integrin $\alpha_{IIb}\beta_3$ (anti-CD41a, anti- β_3 and LK7r) showed no reactivity with trout thrombocytes. Subsequently, a panel of monoclonal antibodies was raised against thrombocyte membrane preparations in an attempt to obtain an antibody against the putative integrin fibrinogen receptor. Of these monoclonal antibodies, four were found to inhibit thrombocyte aggregation, namely 12G2, 30D8, 32F8 and 32H10. The antibody 32H10 was shown significantly to inhibit the attachment of thrombocytes to immobilised

trout fibrinogen, suggesting that it and the other antibodies recognise the putative fibrinogen receptor on trout thrombocytes. FITC-labelled *Bacillus cereus* were employed as test particles to prove that thrombocytes internalise bacteria *via* an active process and not simply by passive sequestration into the open canalicular system. Preincubation of bacteria with trout fibrinogen resulted in a significant increase in the number of thrombocytes exhibiting phagocytosis. This enhancement of phagocytosis by preincubation of *B. cereus* with trout fibrinogen could be inhibited by the tetrapeptide RGDS, but not by RGES, hence implicating the putative fibrinogen receptor in the internalisation of microorganisms. The relevance of these findings to the possible existence of an integrin-like receptor on trout thrombocytes is discussed.

Key words: thrombocyte, haemostasis, aggregation, phagocytosis, integrin, monoclonal antibody, rainbow trout, *Oncorhynchus mykiss*.

Introduction

Receptor recognition of extracellular molecules is a central mechanism by which cells assess and respond to their environment. Most receptors involved in cellular adhesion belong to one of a number of adhesion receptor classes including the integrins, cadherins, selectins and immunoglobulins. The integrins are a widely expressed adhesion receptor family mediating a number of important cellular adhesion events such as platelet aggregation, inflammation and tumour metastasis. All integrins identified are transmembrane dimeric molecules made up of a single α and β subunit joined in a Ca^{2+} -dependent complex. To date, 14 α and eight β subunits have been identified, yet only 20 integrin molecules have been found, indicating that the subunit interactions may be subject to some specificity (Hynes, 1992; Humphries *et al.* 1993). The platelet receptor for fibrinogen belongs to the integrin family of adhesion receptors. In integrin nomenclature, this receptor is known as $\alpha_{IIb}\beta_3$ (glycoprotein IIb/IIIa) and represents approximately 18% of all the membrane-associated protein in human platelets (Calvete,

1994). Of all the integrins, $\alpha_{IIb}\beta_3$ is the most widely studied, and it was the first integrin to be purified (Jennings and Phillips, 1982). Subsequently, cloning and sequencing have led to the identification of the fibrinogen-binding recognition sites (Calvete, 1994). Synthetic peptides have been identified that inhibit platelet aggregation by interfering with the binding of fibrinogen to platelets (Pierschbacher and Ruoslahti, 1984; Ginsberg *et al.* 1988). The first peptide shown to inhibit platelet aggregation was the tetrapeptide RGD-X, where X represents a number of amino acids (including S and V). The domain of fibrinogen that binds to purified $\alpha_{IIb}\beta_3$ was identified as the D domain using plasmin digests of purified human fibrinogen (Nachman *et al.* 1984). Because the binding region was located in the D domain, fibrinogen was able to cross-link to the $\alpha_{IIb}\beta_3$ receptors of two adjacent platelets and cause aggregation. Three sites within fibrinogen have been shown to interact with $\alpha_{IIb}\beta_3$, one at the amino terminus of the A α chain (E domain; GRPV) and two sites in the D domain (Thorsen *et al.* 1986). The carboxyl terminus of the A α chain contains a tetrapeptide

sequence RGDS (α 572–575) and the carboxyl terminus of the fibrinogen γ chain contains a dodecapeptide sequence HHLGGAKQAGDV (γ 400–411; Hawiger *et al.* 1989; Siess, 1989). The critical amino acid within the RGDS sequence appears to be D, as substitution to RGEs results in a greatly reduced effect on fibrinogen binding to $\alpha_{IIb}\beta_3$ (Parise *et al.* 1987). Many synthetic peptides containing these sequences have been shown to inhibit both human platelet aggregation and the binding of ^{125}I -labelled fibrinogen to platelets (Pierschbacher and Ruoslahti, 1984; Gartner and Bennett, 1985; Timmons *et al.* 1989).

The interaction of platelets with other cells plays an important role, not only in haemostasis but also in regulating defence against infection. Integrins have also been shown to participate in the binding and internalization of both microorganisms and senescent cells (Savill *et al.* 1990, 1992; Isberg and Tran Van Nhieu, 1994). For example, the vitronectin receptor integrin $\alpha_v\beta_3$ has been shown to mediate the recognition and clearance of apoptotic cells by macrophages (Savill *et al.* 1990, 1992). A range of integrins, including the mammalian fibrinogen receptor $\alpha_{IIb}\beta_3$, have been shown to bind to microorganisms (Isberg and Tran Van Nhieu, 1994). This suggests that mammalian platelets may possess defensive as well as haemostatic functions, although no 'true' phagocytic activity is thought to be possessed by human platelets. For instance, the apparent uptake of latex beads by these cells has been attributed to their passage into the canalicular system by a mechanism not involving pseudopod formation (White, 1972; Clawson and White, 1980). Human platelets have been shown to endocytose liposomes (Male *et al.* 1992), and there is evidence that the integrin $\alpha_{IIb}\beta_3$ is involved in the uptake of fibrinogen-coated particles (Ylanne *et al.* 1995). As can be seen from these and other reports, there is conflicting evidence regarding the contribution of platelets to bacterial clearance and the nature of the mechanisms involved.

The blood cells of various invertebrates, including the ascidian *Halocynthia roretzi*, have been shown to have dual haemostatic and phagocytic functions, both of which can be inhibited by monoclonal antibodies to a common glycoprotein on the surfaces of blood cells (Takahashi *et al.* 1995). A glycoprotein cell adhesion factor with an RGD motif, which promotes the ensheathment of microorganisms by crayfish blood cells, has also been purified (Kobayashi *et al.* 1990). The implications of these studies are that the glycoproteins responsible for aggregation and phagocytosis by invertebrate blood cells are integrins or integrin-like molecules displaying a dual function. Recently, a β subunit showing 38–42% homology to integrins from other invertebrates (*Caenorhabditis elegans* and *Drosophila melanogaster*) as well as human β_1 and β_3 integrins has been successfully isolated and sequenced from haemocytes of the freshwater crayfish *Pasifastacus leniusculus* (Holmblad *et al.* 1997).

Despite the extensive work in mammals and some initial studies with invertebrates, nothing is known of adhesion receptors in 'lower' vertebrates such as fish. Fish blood does

not have anucleate platelets similar to those of mammals. Instead, they possess a nucleated cell type termed the thrombocyte thought to be the evolutionary forerunner of platelets (Rowley *et al.* 1988, 1997). We have recently reported the aggregation of thrombocytes in the rainbow trout (*Oncorhynchus mykiss*) and the requirement of fibrinogen for this reaction, suggesting the presence of an integrin-like receptor on these cells (Hill and Rowley, 1996). The present study was therefore undertaken to investigate the role of this putative fibrinogen receptor in thrombocyte aggregatory and phagocytic behaviour.

Materials and methods

Adult rainbow trout [*Oncorhynchus mykiss* (Walbaum)] were obtained from Lliw Mill Trout Farm (Pont Lliw, South Wales). Fish were maintained in external tanks at temperatures ranging from 10 to 14 °C and fed *ad libitum* on a commercial pelleted feed (Mainstream Expanded Diet, BP Nutrition Ltd, Cheshire, UK). Fluorescein isothiocyanate (FITC), RPMI 1640 medium, the tetrapeptide sequences RGDS and RGEs, and fibrinogen binding inhibitor peptide (FBIP) were obtained from Sigma Chemical Co. (Poole, UK), while anti-CD41a monoclonal antibody was obtained from TCS Biologicals (Botolph Claydon, Bucks, UK). The thromboxane mimetic U-46619 (9,11-dideoxy-9 α ,11 α -methanoepoxy, PGF $_{2\alpha}$) was purchased from Cascade Biochem (Reading, UK) and aminoethyl benzosulphonyl fluoride (AEBSF) was obtained from Melford Laboratories Ltd (Suffolk, UK). Anti- β_3 integrin antibody was purchased from Affiniti Research Products Ltd (Plymouth Meeting, USA). The monoclonal antibody LK7r, which reacts with platelet membrane β_3 integrin (Liu *et al.* 1995), was kindly supplied by Professor S. Karparkin, New York University Medical School, New York, USA. All other chemicals were of the highest grade available.

Thrombocyte isolation

Prior to blood collection, trout were given terminal anaesthesia by immersion in MS-222 (final concentration 0.1 g l $^{-1}$). A two-step density gradient centrifugation procedure was employed to isolate thrombocytes from the peripheral blood of rainbow trout, as described previously (Lloyd-Evans *et al.* 1994; Hill and Rowley, 1996).

Thrombocyte aggregation studies

The aggregatory response of trout thrombocyte suspensions purified on Percoll gradients was measured turbidimetrically by a method similar to that of Born (1962) using a Payton Minigator II aggregometer (Payton Associates, Scarborough, Ontario, Canada) as detailed previously (Hill and Rowley, 1996). Thrombocyte suspensions (500 μl samples; 1×10^7 cells ml $^{-1}$ in Ca $^{2+}$ /Mg $^{2+}$ -containing Hank's balanced salt solution, HBSS) were incubated with the thromboxane mimetic U-46619 (0.3 $\mu\text{mol l}^{-1}$) in the presence of trout fibrinogen (200 $\mu\text{g ml}^{-1}$). The percentage aggregation was determined against a cell-free blank. To assess the involvement

of a putative integrin receptor in thrombocyte aggregation, the effects of tetrapeptides, RGDS ($0\text{--}200\mu\text{mol l}^{-1}$) and RGES ($0\text{--}100\mu\text{mol l}^{-1}$), and fibrinogen binding inhibitor peptide (FBIP; $0\text{--}200\mu\text{mol l}^{-1}$) on thrombocyte aggregation were tested. Each peptide was incubated with thrombocyte suspensions for 15 min at room temperature ($18\text{--}21^\circ\text{C}$) prior to the induction of aggregation. The effect of thrombocyte-specific monoclonal antibodies (produced as described below) on aggregation was studied by preincubating thrombocyte suspensions for 15 min at room temperature in hybridoma supernatant or an RPMI control, prior to inducing aggregation with U-46619.

Production of monoclonal antibodies

Monoclonal antibodies were raised against trout thrombocyte membrane preparations using standard techniques employed in this laboratory (e.g. Mullet *et al.* 1993; Bowden *et al.* 1997). Thrombocytes from Percoll gradients were pelleted by centrifugation ($2800g$, for 10 min at 4°C) and resuspended in 10 vols of $\text{Ca}^{2+}/\text{Mg}^{2+}$ -containing HBSS + 1 mmol l^{-1} AEBSF + 1 % (v/v) Triton X-100. The resulting cell lysate was centrifuged ($30\,000g$, for 15 min at 4°C) to remove cytoskeletal elements, frozen in liquid N_2 and stored at -80°C .

Balb/c mice (4–6 weeks old) were injected intraperitoneally with thrombocyte membrane preparation containing $200\mu\text{g}$ of protein mixed 1:1 with Freund's complete adjuvant. Two weeks later, the injections were repeated without adjuvant. After a further 2 weeks, blood was taken from the tail of each mouse, and the serum was tested for antibody to trout thrombocyte preparations by immunocytochemistry. Mice received booster injections after a further 4 weeks, and 5 days later the spleen from one mouse (chosen on the tail bleed immunocytochemistry results) was removed for the fusion. Splenocytes were fused with SP2 myeloma cells in the presence of polyethylene glycol 1500 in RPMI supplemented with 20 % foetal calf serum (FCS), 2 mmol l^{-1} L-glutamine, 1 mmol l^{-1} sodium pyruvate, $2\times 10^{-5}\text{ mol l}^{-1}$ 2-mercaptoethanol, 100 i.u. ml^{-1} penicillin, $0.1\mu\text{g ml}^{-1}$ streptomycin, 0.1 mmol l^{-1} hypoxanthine and $1\mu\text{g ml}^{-1}$ azaserine. Hybridomas were screened with an enzyme-linked immunosorbent assay (ELISA) using 96-well plates containing adherent formalin-fixed thrombocyte preparations and immunocytochemistry for antibody reactivity to thrombocyte preparations. Hybridomas of interest were subcloned three times by a limiting dilution technique to ensure monoclonality. The resulting hybridomas were grown to confluence in RPMI supplemented with 10 % FCS, 2 mmol l^{-1} L-glutamine, 1 mmol l^{-1} sodium pyruvate, $2\times 10^{-5}\text{ mol l}^{-1}$ 2-mercaptoethanol, 100 i.u. ml^{-1} penicillin and 0.1 mg ml^{-1} streptomycin in 250 ml flasks and stored at -20°C .

Immunocytochemistry

Thrombocytes were allowed to adhere to glass slides for 10 min, washed three times with $\text{Ca}^{2+}/\text{Mg}^{2+}$ -containing HBSS to remove any unbound cells and fixed with 1 % formaldehyde (30 min at room temperature). Thrombocyte monolayers were

washed three times with phosphate-buffered saline (PBS), and endogenous peroxidase was blocked with 3 % H_2O_2 and 40 % methanol in PBS (30 min at room temperature). Following washing (three times with PBS), non-specific binding sites on the monolayers were blocked with blocking solution (10 % goat serum, 3 % bovine serum albumin (BSA), 0.1 mol l^{-1} ammonium chloride, 0.1 mol l^{-1} glycine, 0.05 % Tween 20 in PBS) for 1 h at room temperature. After this time, the blocking solution was removed, and the monolayers were overlaid with primary antibody diluted 50:50 in blocking solution for 1 h at room temperature. Monolayers were washed (three times with PBS) and overlaid with goat anti-mouse immunoglobulin G peroxidase conjugate diluted 1:100 with blocking solution, for 1 h at room temperature. After washing three times with PBS, monolayers were incubated with chromogenic substrate (0.1 % diaminobenzidine, 0.02 % H_2O_2 in PBS) for 15 min at room temperature. Following this time, slides were washed three times with PBS, counterstained with Cole's haematoxylin and mounted with Kaiser's glycerol gelatine.

Immunoblot analysis

Dot blots were performed to ascertain the reactivity of antibodies (anti-CD41a, anti- β_3 integrin, LK7r), and a panel of monoclonal antibodies raised against trout thrombocyte membranes, to soluble trout thrombocyte or human platelet antigens. Thrombocyte membrane preparations, platelet membrane preparations or human fibroblast lysate containing approximately $10\mu\text{g}$ of protein were spotted onto nitrocellulose membrane (Amersham International) and allowed to dry. Non-specific binding sites on the nitrocellulose were blocked by incubation with 1 % milk protein (Marvel, Cadbury Ltd), 1 % BSA, 1 % goat serum in PBS for 1 h at room temperature. After this time, the membrane was transferred to an antibody solution of appropriate dilution (50:50 for hybridoma supernatant) for 1 h at room temperature. After five washes in PBS, each for 5 min, the secondary antibody was added (in all cases this was goat anti-mouse peroxidase conjugate, diluted 1 in 100 in blocking solution) and incubated for 1 h at room temperature. The nitrocellulose membrane was washed five times for 5 min in PBS and treated with 4-chloronaphthol solution in the dark for 30 min at room temperature, after which time the reaction was stopped by washing in water.

Thrombocyte adherence to fibrinogen

Trout fibrinogen, prepared as described previously (Hill and Rowley, 1996), was immobilised onto glass slides for 1 h at room temperature. The immobilised fibrinogen was washed three times with $\text{Ca}^{2+}/\text{Mg}^{2+}$ -containing HBSS to remove any unbound protein. Immobilised fibrinogen was overlaid with blocking solution for 1 h at room temperature, to block any non-specific binding sites exposed on the glass slide, and washed three times with $\text{Ca}^{2+}/\text{Mg}^{2+}$ -containing HBSS. Thrombocyte preparations were fixed with 1 % formaldehyde (30 min at room temperature) and incubated with hybridoma supernatant or RPMI as a control for 15 min at room

temperature. Following this time, the thrombocyte suspensions (4×10^6 cells ml^{-1}) were overlaid onto the immobilized fibrinogen slides for 1 h at room temperature. Subsequently, the fibrinogen-immobilized slides were washed five times with $\text{Ca}^{2+}/\text{Mg}^{2+}$ -containing HBSS and stained with Cole's haematoxylin to allow attached cells to be visualised. The number of thrombocytes attached to immobilised fibrinogen was counted in 16 fields of view of a $\times 100$ objective under brightfield microscopy.

Phagocytic studies with trout thrombocytes

Bacillus cereus (NCTC 2599) were used as target bacteria in a phagocytosis assay. They were grown overnight in nutrient broth (Difco Ltd, Surrey, UK) at 37°C , heat-killed (1 h at 100°C) and washed three times by centrifugation (2000g for 10 min) in $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free HBSS. After the final wash, the pellet was resuspended in 0.2 mol l^{-1} sodium carbonate, 0.2 mol l^{-1} sodium bicarbonate, pH 9.4, and the bacterial concentration was adjusted to give 1×10^9 bacteria ml^{-1} . Fluorescein isothiocyanate (FITC; 0.1 mg ml^{-1}) was added to the bacterial suspension and incubated for 30 min in the dark with constant mixing on a rotary test tube rack. The bacteria were washed four times in $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free HBSS (2000g for 10 min) before a final resuspension in $\text{Ca}^{2+}/\text{Mg}^{2+}$ -containing HBSS at a concentration of 1×10^8 bacteria ml^{-1} . This bacterial suspension was stored at -20°C . Prior to incubation with a thrombocyte suspension, some FITC-labelled *B. cereus* were incubated with trout fibrinogen ($200 \mu\text{g ml}^{-1}$) for 1 h at room temperature with constant mixing on a rotary test tube rack. Bacterial suspensions were washed four times with $\text{Ca}^{2+}/\text{Mg}^{2+}$ -containing HBSS to remove any unbound fibrinogen.

Thrombocyte suspensions (2×10^6 cells ml^{-1}) were incubated with either fibrinogen-incubated or untreated FITC-labelled *B. cereus* at a ratio of 1:50 respectively. Incubations were carried out at 18°C for either 15 or 30 min in the dark. In later experiments, incubations were performed for 30 min with the addition of either RGDS ($100 \mu\text{mol l}^{-1}$), which has been shown to inhibit the binding of human fibrinogen to its integrin receptor, or the control tetrapeptide RGEs ($100 \mu\text{mol l}^{-1}$). Following incubation, thrombocytes were allowed to adhere to glass slides for 10 min and subsequently washed three times with $\text{Ca}^{2+}/\text{Mg}^{2+}$ -containing HBSS to remove any unbound cells or bacteria. External FITC fluorescence was quenched by overlaying cell monolayers with 0.2% Trypan Blue in $\text{Ca}^{2+}/\text{Mg}^{2+}$ -containing HBSS for 5 min (Hed, 1986). Monolayers were washed five times with $\text{Ca}^{2+}/\text{Mg}^{2+}$ -containing HBSS and fixed with 1% formaldehyde (30 min at room temperature). Monolayers were finally washed three times with $\text{Ca}^{2+}/\text{Mg}^{2+}$ -containing HBSS and mounted in Kaiser's glycerol gelatine for examination. Approximately 200 cells per slide were examined with a Zeiss Photomicroscope II equipped with a barrier and excitation filter set no. 487709 for FITC epifluorescence.

Following fixation, some thrombocyte monolayers were used for immunocytochemistry, being reacted with the

monoclonal antibodies 21G6 (previously shown to react with all trout leucocytes but not thrombocytes; Bowden *et al.* 1997) and 32H10 (a thrombocyte-specific marker).

Ultrastructural examination of the phagocytic behaviour of thrombocytes

Following incubation with fibrinogen-incubated *B. cereus* for 45 min at room temperature, thrombocytes were fixed for 30 min in ice-cold 2.5% glutaraldehyde in 0.2 mol l^{-1} sodium cacodylate buffer, pH 7.2. Subsequently, cells were washed by centrifugation (1000g, 10 min) and resuspended in 0.2 mol l^{-1} sodium cacodylate buffer containing 0.5% cetyl pyridinium chloride (1 h at room temperature). Following this, cells were washed twice by centrifugation (1000g for 10 min) and post-fixed in ice-cold 1% osmium tetroxide in distilled water for a further 60 min at room temperature. Cells were pelleted by centrifugation, washed in distilled water, dehydrated in a graded series of acetone:water at room temperature and embedded in Araldite resin. Sections were cut on Huxley Mk II ultramicrotomes, attached to 300 mesh copper grids and stained with lead citrate (Reynolds, 1963) and alcoholic uranyl acetate. They were examined with a Jeol 1200-EX electron microscope operated at 80 kV.

Statistical analyses

Statistical analyses were performed with Instat2 (Graphpad Software, California, USA) using Student's *t*-tests or the Student-Newman-Keuls multiple-comparison test as appropriate.

Results

Fibrinogen interaction with thrombocytes during aggregation

The previously reported capacity of human and trout fibrinogen to support trout thrombocyte aggregation (Hill and Rowley, 1996) suggests that binding of fibrinogen to thrombocytes occurs *via* an integrin-like receptor. To investigate the involvement of this putative integrin receptor in the binding of fibrinogen to thrombocytes during aggregation, thrombocyte suspensions were incubated with the tetrapeptides RGDS or RGEs prior to inducing aggregation by the addition of U-46619 ($0.3 \mu\text{mol l}^{-1}$) in the presence of trout fibrinogen ($200 \mu\text{g ml}^{-1}$). Preincubation of thrombocyte preparations with the tetrapeptide RGDS ($5\text{--}100 \mu\text{mol l}^{-1}$) resulted in a dose-dependent decrease in U-46619-induced aggregation in the presence of trout fibrinogen (Fig. 1). The maximum inhibition of thrombocyte aggregation seen in the presence of RGDS ($200 \mu\text{mol l}^{-1}$) was approximately 52%. In contrast, RGEs ($5\text{--}100 \mu\text{mol l}^{-1}$) preincubated with thrombocytes exhibited no significant effect on U-46619-induced aggregation in the presence of trout fibrinogen (Fig. 1). The fibrinogen binding inhibitor peptide (FBIP), representing the dodecapeptide sequence in human fibrinogen γ -chain (Hawiger *et al.* 1989), was also added to thrombocyte preparations prior to the addition of U-46619 in the presence of trout fibrinogen. This, however, had no significant effect on

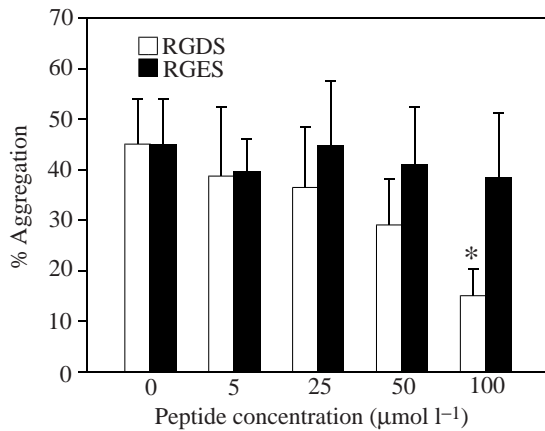


Fig. 1. Effect of the tetrapeptides RGDS and RGES (5–100 $\mu\text{mol l}^{-1}$) on thrombocyte aggregation induced by U-46619 (0.3 $\mu\text{mol l}^{-1}$) in the presence of trout fibrinogen (200 $\mu\text{g ml}^{-1}$). *Value significantly different from the control value, $P < 0.05$. Mean values + S.E.M., $N=3$.

U-46619-induced aggregation at the concentrations employed (10–200 $\mu\text{mol l}^{-1}$; data not shown).

Following the aggregation inhibition studies with RGDS, attempts were made to identify a putative $\alpha_{\text{IIb}}\beta_3$ -like receptor on trout thrombocytes using commercially available monoclonal antibodies (anti-CD41a, LK7r and anti- β_3) raised against various regions of the human platelet $\alpha_{\text{IIb}}\beta_3$ integrin. All antibodies reacted with the positive controls used (human platelets and fibroblasts), but not with trout thrombocytes (data not shown). This was tested by both immunocytochemistry and immunoblotting.

Because of the lack of reactivity of trout thrombocytes with these commercially available anti-integrin monoclonal antibodies, antibodies were raised against trout thrombocyte membranes. Previous studies with human platelets have demonstrated that this is a suitable approach for obtaining antibodies to the integrin as it represents a large percentage (approximately 18%) of the membrane-associated proteins (Calvete, 1994). From the fusion, 24 hybridoma cell lines were generated that secreted antibody against the cells present in thrombocyte preparations as identified by ELISA and immunocytochemistry. Of these 24 hybridomas, 13 clones reacted with all cells present (thrombocytes, lymphocytes, granulocytes and monocytes) except erythrocytes. Three clones were specific for lymphocytes (designated 20B11, 9H3 and 2H3) and eight clones secreted antibody specific for thrombocytes (designated 12G2, 8A9, 33B10, 18G3, 30D8, 32H10, 32F8 and 20H12) as assessed by immunocytochemistry (Fig. 2A,B). The 24 hybridomas were tested for their effects on thrombocyte aggregation. Four hybridoma supernatants were found significantly to inhibit U-46619-induced thrombocyte aggregation in the presence of trout fibrinogen (Figs 3, 4). These four hybridoma supernatants, namely 12G2, 30D8, 32F8 and 32H10, and one further monoclonal antibody (8A9), which had no effect on thrombocyte aggregation but which was thrombocyte-specific, showed reactivity to thrombocyte membrane preparations

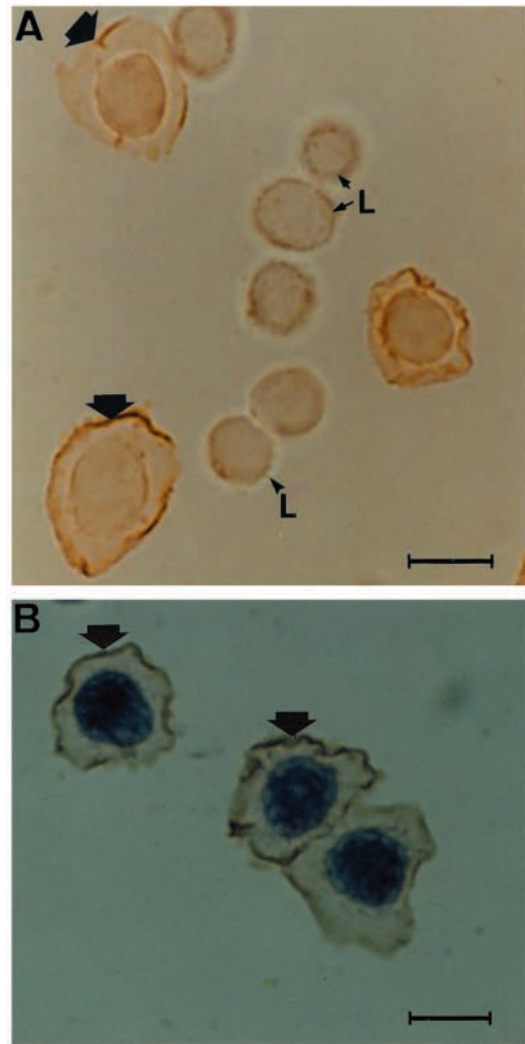


Fig. 2. Immunocytochemical reactivity of monolayers of trout leucocytes showing the specificity of monoclonal antibodies 12G2 (A) and 30D8 (B) for thrombocytes. Note the characteristic immunoreactivity (brown staining in A) with cell surfaces of thrombocytes (unlabelled arrows) and the lack of reactivity associated with contaminating lymphocytes (L). Cells in B were counterstained with Cole's haematoxylin. Scale bars, 10 μm .

under native conditions only, as assessed by immunoblot analysis. No reactivity was observed under reducing or denaturing conditions.

To assess whether the inhibitory effect of the antibodies on thrombocyte aggregation was due to interference in the thrombocyte–fibrinogen interaction, the effect of monoclonal antibodies 32H10 and 8A9 on the attachment of fixed thrombocytes to immobilised fibrinogen was studied. It was found that 32H10 significantly reduced the number of thrombocytes attached to immobilised fibrinogen compared with the RPMI control, while the 8A9 monoclonal antibody was without significant effect (Fig. 5).

Phagocytic activity of trout thrombocytes

Initial experiments using FITC-labelled *B. cereus* and

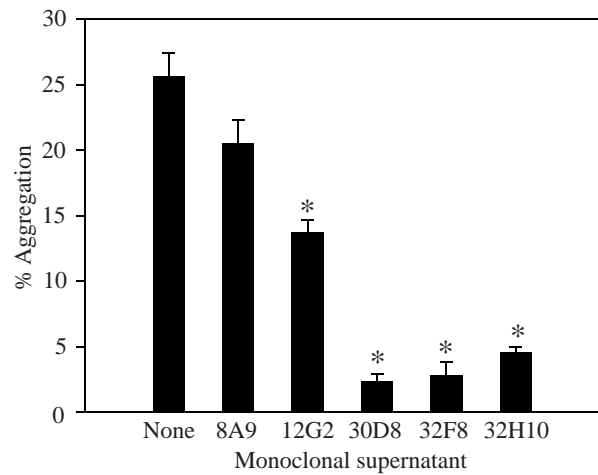


Fig. 3. Effect of monoclonal hybridoma supernatants on thrombocyte aggregation induced by U-46619 ($0.3\mu\text{mol l}^{-1}$) in the presence of trout fibrinogen ($200\mu\text{g ml}^{-1}$). *Value significantly different from the control value, $P<0.05$. Mean values + S.E.M., $N=3$.

Trypan Blue quenching showed that bacteria were internalised by thrombocytes (Fig. 6). Using this method, intracellular bacteria retained FITC fluorescence, while extracellular forms showed a reduction in fluorescence due to quenching. The use of the monoclonal antibodies 21G6 and 30D8 allowed clear identification of thrombocytes and internalised bacteria from other contaminating phagocytic cell types present such as granulocytes (Fig. 7). Ultrastructurally, examples of all stages of phagocytosis were observed, in particular the formation of pseudopodial extensions around bacteria and subsequent internalisation into phagosomes (Fig. 8).

To assess any role of the putative fibrinogen receptor in the internalisation of foreign material by thrombocytes, bacteria were either preincubated with trout fibrinogen and subsequently washed to remove unbound fibrinogen or added directly to thrombocyte preparations. The percentage of phagocytic thrombocytes was significantly higher ($P<0.001$) with fibrinogen-incubated rather than control *B. cereus* (Fig. 9). The tetrapeptides RGDS or RGES were also co-incubated with thrombocytes in the presence of fibrinogen-incubated and control bacteria (Fig. 9). Incubation of fibrinogen-treated bacteria in the presence of RGDS resulted in a significant ($P<0.001$) reduction in uptake of bacteria compared with the control (i.e. incubated with fibrinogen but not RGDS). Also, RGDS had no significant effect on the uptake of bacteria not previously incubated with fibrinogen. Finally, RGES did not cause any significant reduction in bacterial uptake using test particles incubated with or without fibrinogen compared with the appropriate controls.

Discussion

Fibrinogen interaction with thrombocytes

To date, only a limited number of plasma-borne components of the haemostatic process in fish have been purified. For

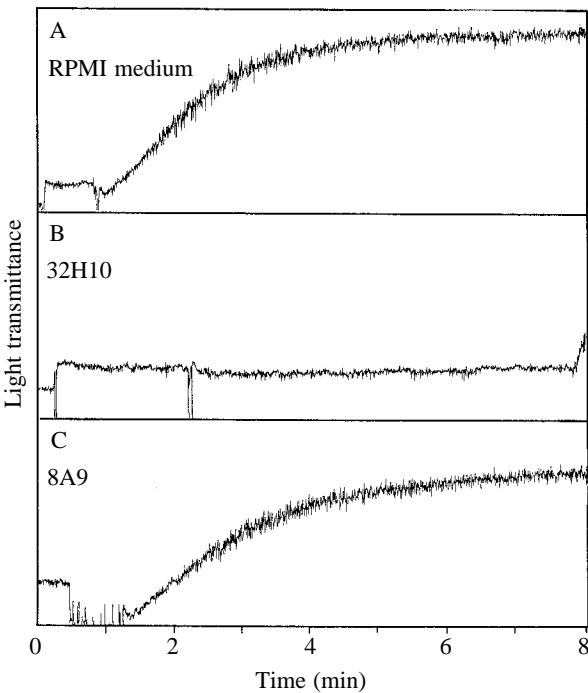


Fig. 4. Aggregatory response (measured as light transmittance) of trout thrombocytes induced by U-46619 ($0.3\mu\text{mol l}^{-1}$) and trout fibrinogen ($200\mu\text{g ml}^{-1}$) in the presence of (A) RPMI medium, (B) hybridoma supernatant 32H10 or (C) hybridoma supernatant 8A9.

example, fibrinogen and thrombin have been isolated from lamprey plasma (Doolittle, 1965). These proteins have been shown to react with thrombin and fibrinogen, respectively, from sheep, dog and bovine sources (Doolittle, 1965). The release of fibrinopeptides from the various fibrinogen–thrombin interactions invariably occurs faster for fibrinogen–thrombin pairs from the same species. The differences in the clotting times can be accounted for by amino

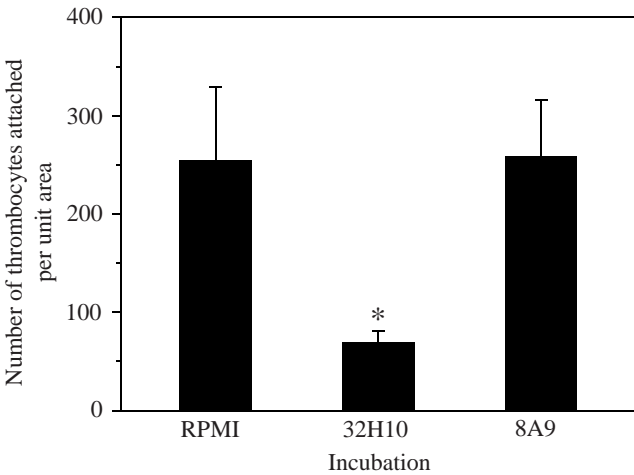


Fig. 5. Effect of monoclonal hybridoma supernatants on the attachment of formalin-fixed thrombocytes to immobilised trout fibrinogen. *Value significantly different from the control value, $P<0.05$. Mean values + S.E.M., $N=4$.

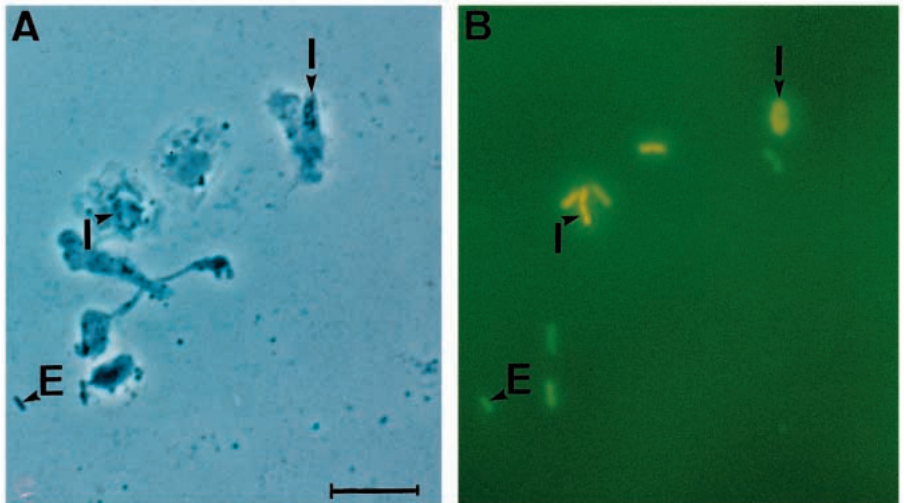


Fig. 6. Phagocytic thrombocytes previously incubated with FITC-labelled *Bacillus cereus* viewed under (A) phase contrast microscopy and (B) epifluorescence. Note the variation in the intensity of fluorescence from intracellular (I) and extracellular (E) bacteria caused by Trypan Blue quenching. Scale bar, 10 µm.

acid composition differences of the thrombin and fibrinogen molecules between phylogenetically distinct organisms. Despite there being some differences in amino acid composition between the mammalian and fish fibrinogen, there are clearly significant areas of homology between the different species. For example, the sequence on the fibrin side of the junction split by the various thrombins in lamprey fibrinogen is conserved and is virtually identical to that in mammalian fibrinogen (Doolittle *et al.* 1962, 1976; Doolittle, 1965; Cottrell and Doolittle, 1976).

The ability of human fibrinogen to support the aggregation of trout thrombocytes, observed in previous studies (Lloyd Evans *et al.* 1994; Hill and Rowley, 1996), indicates the possibility of a significant degree of sequence homology between fibrinogen and its receptor in trout and humans. The

inhibition of thrombocyte aggregation by the tetrapeptide RGDS also shows that this key sequence involved in binding the integrin complex is conserved during the evolution of fibrinogen. The apparent lack of inhibition by the dodecapeptide sequence (FBIP) is, however, indicative of a lack of sequence homology between trout and human fibrinogen within the α chain. From the range of monoclonal antibodies generated against thrombocyte membrane preparations, four were identified that inhibited trout thrombocyte aggregation induced by U-46619 in the presence of trout fibrinogen. No inhibition of aggregation was observed in the presence of unrelated monoclonal antibodies or culture medium alone. This suggests that the monoclonal antibodies may interfere with the thrombocyte–fibrinogen interaction. The specific inhibition of thrombocyte attachment to immobilised trout fibrinogen by

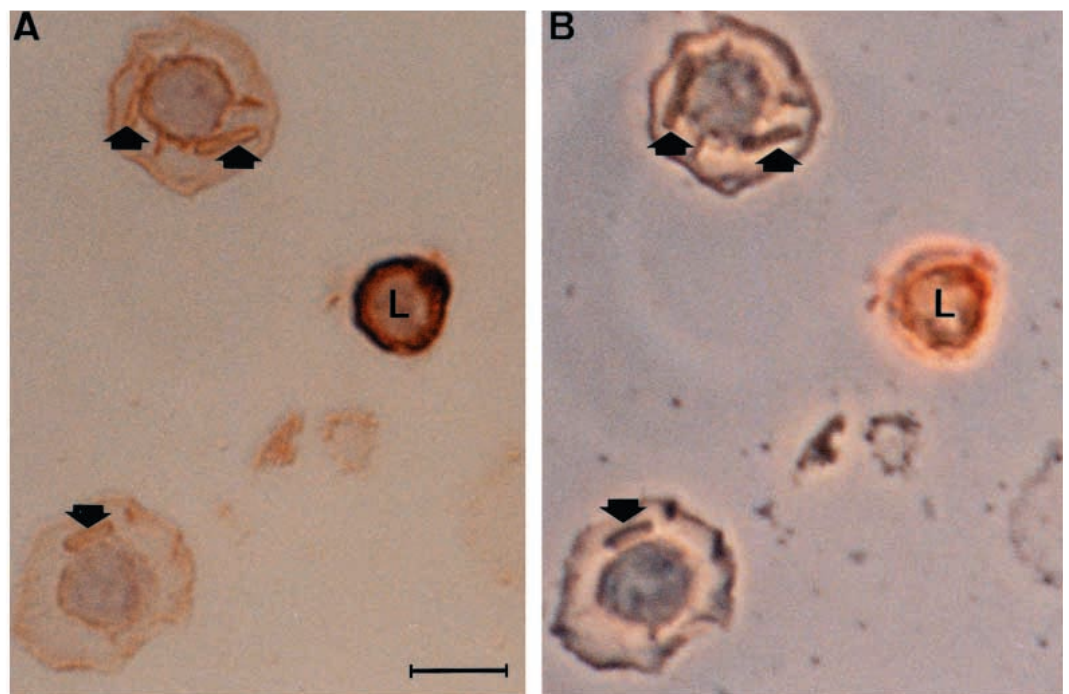


Fig. 7. Brightfield (A) and phase contrast (B) micrographs of a monolayer of trout thrombocytes incubated with *Bacillus cereus* and reacted with monoclonal antibody 21G6. Note the intracellular bacteria (arrows) in the thrombocytes and the immunopositivity of a contaminating lymphocyte (L). Scale bar, 5 µm.

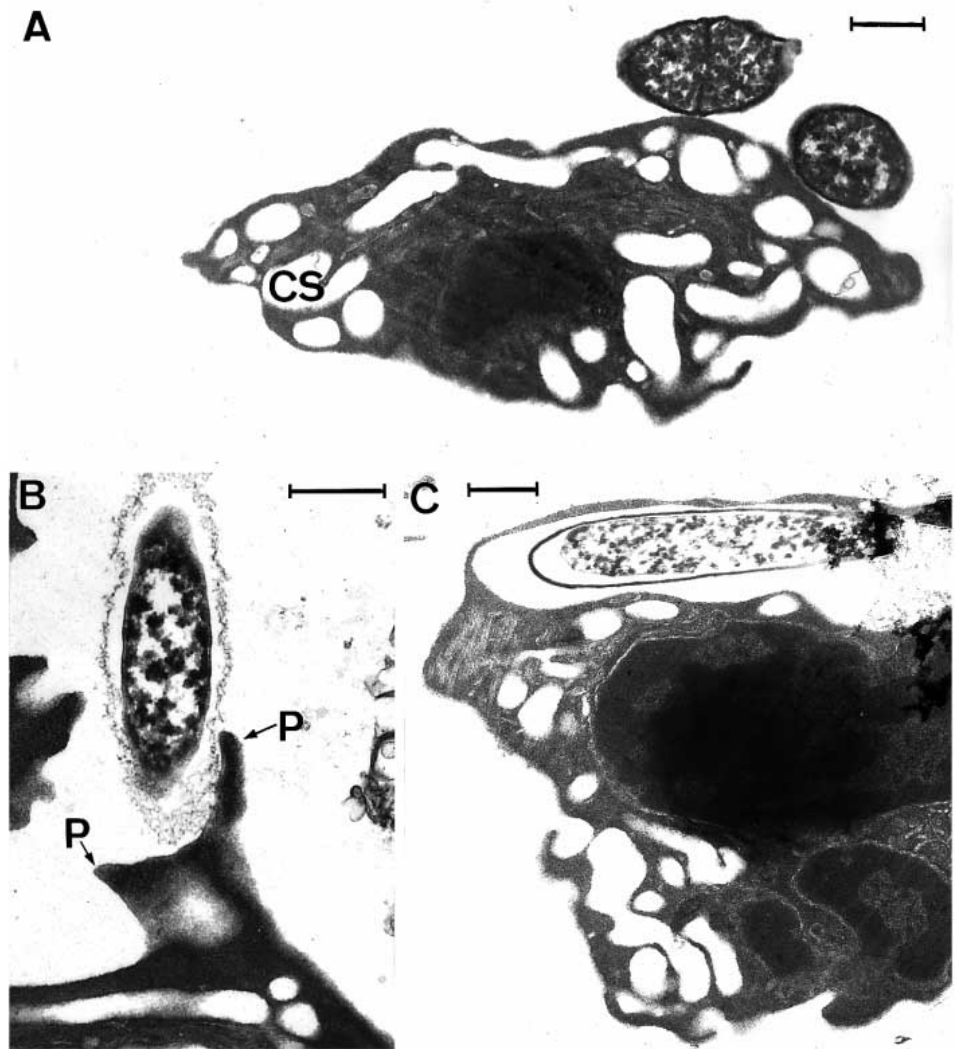


Fig. 8. Electron micrographs showing the stages in the uptake of *Bacillus cereus* by trout thrombocytes. (A) Attachment of bacteria to thrombocyte. Note the characteristic prominent canalicular system (CS) in this cell. (B) Early stage of the ingestion of the bacterium by the production of pseudopodial extensions (P). (C) A bacterium in the phagosome of a thrombocyte. Scale bars, 0.5 μm .

monoclonal antibody 32H10 provides further evidence that the antibodies recognise the putative fibrinogen receptor. The non-reactivity of these monoclonal antibodies with thrombocyte membrane preparations in the presence of SDS or under reducing conditions implies that all the antibodies tested recognise a conformational binding site on the thrombocytes. Assuming that the putative fibrinogen receptor is present on the surface of trout thrombocytes in the same proportions as $\alpha_{\text{IIb}}\beta_3$ on human platelets, it would account for approximately 20% of the total membrane-associated protein (Calvete, 1994), making the chances of obtaining antibodies to the putative fibrinogen receptor favourable.

Phagocytic activity of trout thrombocytes

Of some significance in the present study was the observation of phagocytic activity by trout thrombocytes. Previous studies with dogfish, *Scyliorhinus canicula* (a cartilaginous fish), have shown that thrombocytes are apparently avidly phagocytic *in vivo* (Hunt and Rowley, 1986), but their initial *in vitro* experiments using bacteria as test particles failed to detect any significant uptake by these cells

compared with granulocytes and monocytes, which were highly phagocytic. The thrombocytes of other vertebrate species are thought to be phagocytic (e.g. in birds; Chang and Hamilton, 1979) but in the case of mammalian platelets, White (1972) considered that they have no 'true' phagocytic capacity because the uptake of latex beads observed in thin sections of such material was due to simple passage into the canalicular system by a passive process. More recently, however, Simonet *et al.* (1992) demonstrated that *Yersinia pseudotuberculosis* bacilli were apparently ingested by human platelets and that the bacteria came to lie in phagosomes not in continuity with the extracellular environment. The observation of pseudopodial extensions from trout thrombocytes around bacteria in the present and previous studies (Hill and Rowley, 1996) is evidence that the incorporation of foreign material by these cells is *via* an active phagocytic mechanism and not simply by incorporation within the canalicular system. Additional evidence for such internalisation comes from the results using FITC-labelled bacteria following Trypan Blue quenching, where internalised bacteria possess intense fluorescence compared with extracellular bacteria.

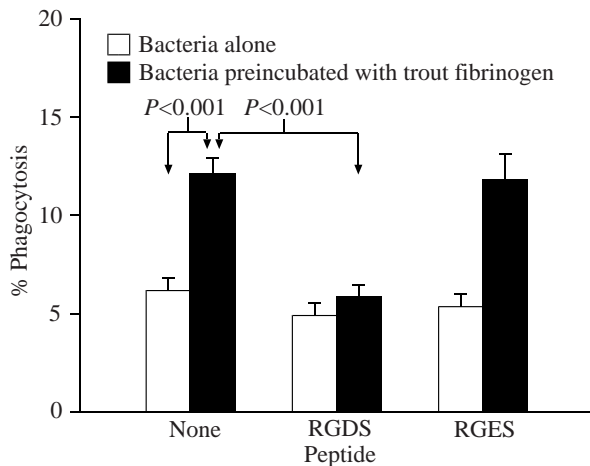


Fig. 9. Effect of tetrapeptides RGDS or RGES on the percentage of phagocytic thrombocytes present following a 15 min incubation with fibrinogen-incubated or control *Bacillus cereus*. Mean values + S.E.M., $N=7$.

In some instances, the binding and internalisation of microorganisms by mammalian cells have been shown to involve integrins (Isberg and Tran Van Nhieu, 1994). Haemocytes from the urochordate *Halocynthia roretzi* possess an integral membrane glycoprotein, which has been shown to regulate both phagocytic and aggregatory activity of these cells (Takahashi *et al.* 1995). A glycoprotein with an RGD motif has been implicated in the encapsulation of microorganisms by crayfish haemocytes (Kobayashi *et al.* 1990). Furthermore, the encapsulation of foreign particles by the wax moth *Galleria mellonella* has also been shown to occur *via* an RGD-dependent mechanism (Pech and Strand, 1995). The results of the present study, in which the number of phagocytic thrombocytes increases following the incubation of bacteria with trout fibrinogen, suggest that the putative fibrinogen receptor on the surface of thrombocytes may be involved in the uptake process. The observation that the tetrapeptide RGDS inhibits the increase in the number of phagocytic thrombocytes further implies that the uptake of fibrinogen-coated *B. cereus* by trout thrombocytes occurs *via* an RGD-dependent mechanism.

In conclusion, the inhibition of fibrinogen-dependent processes (aggregation and phagocytosis) by the tetrapeptide RGDS and the fact that only activated thrombocytes aggregate in the presence of fibrinogen provide evidence for the existence of a putative integrin-like fibrinogen receptor on thrombocytes from *O. mykiss*. It may be that fish thrombocytes, like the ancestral haemostatic cells in advanced deuterostome invertebrates, such as tunicates and echinoderms, have both immunological (phagocytic) and haemostatic (aggregatory) functions helping to maintain fish pathogen-free following vascular damage. Open wounds through which pathogens could freely pass into fish would be quickly sealed by the aggregatory response of thrombocytes. Hence, the first cells that any invading pathogen is likely to come into contact with are thrombocytes, until granulocytes, monocytes and

lymphocytes are attracted to the periphery of aggregates in the later stages of haemostasis (Hill and Rowley, 1996). Some aspects of this dual functionality observed in thrombocytes may have been lost with the evolution of the anucleate mammalian platelet. It is probable that aggregation and phagocytosis are mediated *via* a common integrin receptor on fish thrombocytes. The monoclonal antibodies raised in this present work should enable the purification and characterisation of the putative fibrinogen receptor from *O. mykiss* thrombocytes by immunoaffinity chromatography.

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