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Interaction between non-specific electrostatic forces and humoral factors in haemocyte attachment and encapsulation in the edible cockle, *Cerastoderma edule*

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Summary

In invertebrates, encapsulation is the common immune defence reaction towards foreign bodies, including multicellular parasites, which enter the haemocoel and are too large to be phagocytosed. This immune response has been most extensively studied in insects, in which it is highly complex, involving a diversity of cellular and molecular processes, but little is known of this process in bivalve molluscs. Non-specific physicochemical properties are known to influence parasite–haemocyte interactions in many invertebrates, and these may provide the common basis of encapsulation on which highly specific biochemical interactions are imposed. The present study uses synthetic beads and thread to mimic inactive metacercarial cysts of trematodes, and thus investigates factors involved in the basic, non-specific mechanisms of cell attachment and encapsulation in the edible cockle, *Cerastoderma edule*. Results showed that positively charged targets stimulated the most vigorous response, and further detailed experiments revealed that non-specific electrostatic forces and humoral plasma factors have a synergistic role in haemocyte attachment and the encapsulation response of *C. edule*.

Key words: invertebrate immunity, encapsulation, cell attachment, surface charge, electrostatic force, humoral factor, haemocyte, bivalve, *Cerastoderma edule*.

Introduction

In invertebrates, encapsulation is the common immune defence reaction towards foreign bodies, including multicellular parasites, which enter the haemocoel and are too large to be phagocytosed (Ratcliffe and Rowley, 1987). In general, a capsule of haemocytes encloses the foreign body and cytotoxic products, such as degradative enzymes and free radicals, are released by the haemocytes in an attempt to destroy the invader. This immune defence reaction has been most extensively studied in insects and research has shown this process to be highly complex (Ratcliffe, 1993), involving a diversity of cellular and molecular processes (e.g. Cheng and Garrabrant, 1977; Ouaissi et al., 1990; Nappi et al., 1995; Loret and Strand, 1998; Choi et al., 2002; Whitten et al., 2004). Physicochemical properties such as surface charge and hydrophobicity also influence parasite–haemocyte interactions (e.g. Walter and Williams, 1967; Lackie, 1983; Lackie, 1986; Lavine and Strand, 2001); however, these factors have received less attention than the highly specific biochemical and molecular mechanisms.

Since the 1920s, scientists have shown considerable interest in the biological relevance of surface charge within vertebrate systems (Mehrishi and Bauer, 2002), and there is much evidence to support a role for surface charge in immunological-based reactions. For example, sialic acids, which are present on leucocytes to help prevent non-specific interactions between cells, are removed upon cellular activation during early 'non-self' recognition, resulting in a decreased cell surface negative charge and unmasking of further cell receptors and ligands. This increases cellular interactions and leads to effective immune defence (Rieu et al., 1992; Kelm and Schauer, 1997; Crocker and Varki, 2001). Such a reaction highlights the synergistic action of non-specific electrostatic forces and highly specific receptor–ligand interactions.

Electrostatic forces are also implicated in antibody-antigen interactions involving complement and major histocompatibility complex (MHC) (Morikis and Lambris, 2004a; Morikis and Lambris, 2004b), and cationic antimicrobial peptides exploit their positive surface charge in order to interact with anionic lipids of microorganism membranes (Zasloff, 2002). In addition, parasites and pathogens use their negative surface charge to help avoid host immune responses (Crocker and Varki, 2001). Surface charge also has a pivotal role in the development of gene/drug/antigen delivery systems to target macrophages, which may aid treatment of globally important diseases such as

schistosomiasis, cancer, HIV and tuberculosis (Ashan et al., 2002).

In invertebrates, cell surface charge has generally been implicated in host–parasite interactions (e.g. Saraiva et al., 1989; Monteiro et al., 1998; Akaki et al., 2001; Souto-Padrón, 2002), and in multicellular parasites, surface charge has been largely investigated with respect to the encapsulation response. In insects, chromatography beads and thread are used to mimic parasites (e.g. Vinson, 1974; Dunphy and Nolan, 1980; Ratner and Vinson, 1983; Paskewitz and Riehle, 1994; Lavine and Strand, 2001), probably because they lack the common pathogen-associated molecular patterns (PAMPs; e.g. lipopolysaccharide, peptidoglycan and β-1,3-glucan). The absence of all parasite-associated molecules from synthetic targets allows the basic mechanism of encapsulation, involving non-specific physicochemical properties and electrostatic forces, to be studied. This mechanism is potentially common to all host–parasite interactions, and provides the basis on which highly specific biochemical interactions are imposed. Studies using beads have generally shown that positively charged beads are encapsulated most vigorously (e.g. Walter and Williams, 1967; Vinson, 1974; Dunphy and Nolan, 1980; Lackie, 1983; Ratner and Vinson, 1983), but few detailed investigations justify on the role of electrostatic forces and their interaction with opsonic humoral factors in invertebrate immunity.

One extensively studied multicellular parasite system in invertebrates is the trematode blood fluke, *Schistosoma mansoni* and its intermediate host, the gastropod mollusc, *Biomphalaria glabrata* (see reviews by Bayne et al., 2001; Loker and Bayne, 2001; Yoshino et al., 2001). Marine bivalve molluscs are also common hosts to multicellular parasites, including trematodes (de Montaudouin et al., 2000); however, information on host resistance mechanisms to such parasites is very limited, despite the growing aquaculture industry and the high commercial value of molluscan shellfish (FAO, 2005). Cheng and Rifkin (Cheng and Rifkin, 1970), who recognised the high prevalence of metazoan parasites in marine bivalves, examined the host response to these parasites histologically, and proposed five different types of encapsulation. Research, however, has not continued into understanding the dynamics of capsule formation in bivalve molluscs.

The present study investigates the non-specific basic mechanisms involved in cell attachment and encapsulation in the edible cockle, *Cerastoderma edule*. Synthetic beads and thread were used to mimic the inert and inactive metacercarial cysts of the trematode, *Himasthla* sp., commonly found encapsulated in the foot of *C. edule*. The investigation describes how non-specific electrostatic forces and humoral plasma factors interact to mediate haemocytic immune responses.

Materials and methods

Experimental animals

Cerastoderma edule L. (>2.0 cm diameter) were collected at low tide from Mumbles Bay, Swansea, South Wales, UK, and animals were maintained in a free-flowing aquarium for 48 h prior to use. A minimum of 10 individuals were investigated for all experimental conditions. Chemicals were purchased from Sigma-Aldrich Company Ltd, Dorset, UK, unless otherwise stated.

Observations of trematode capsules

Squash preparations and histology were used to confirm that *C. edule* uses haemocytic encapsulation as an immune defence reaction. The cockle foot was examined for metacercarial cysts of *Himasthla* spp., parasitic trematodes of *C. edule* (e.g. Wegeberg et al., 1999; de Montaudouin et al., 2000) commonly found in this tissue. For squash preparations, the cockle foot was dissected longitudinally, and each section squashed between two glass slides until *Himasthla* sp. cysts were clearly visible under a dissecting microscope. Standard paraffin wax sections were also used to examine the structure of the haemocytic capsule surrounding *Himasthla* sp. cysts. The cockle foot was stored in an excess volume of Bouin's seawater fixative, double wax embedded (Humarson, 1979), sectioned and then stained with Cole's Haematoxylin and Eosin.

In vivo and in vitro cell attachment and encapsulation experiments

Factors involved in mediating haemocyte attachment and encapsulation were studied, both *in vivo* and *in vitro*, using chromatography beads of various matrices, charges and functional groups, and Nylon monofilament (thread) as encapsulation targets (Table 1). Trematode metacercarial cysts were not used in encapsulation studies as they could not be removed intact from host tissues. Prior to use, beads were washed 10× in 0.05 mol l^{-1} Tris-buffered saline (TBS; 0.05 mol l^{-1} Tris/HCl containing 2% NaCl, pH 7.4) and resuspended to produce a 15% v/v stock solution of beads/TBS. The beads ranged in size (Table 1), which prevented the use of precise haemocyte:bead ratios in the experiments. Bead preparations were stored at 4°C and then vortexed immediately before use. Nylon thread was cleaned with detergent, rinsed 5× with distilled water followed by 70% ethanol, before final washing with ultrapure water. The thread was air dried and stored in sterile tubes at room temperature.

Characterization of encapsulation

The encapsulation response varies greatly from one invertebrate species to another and may or may not involve the formation of multi-layers of flattened haemocytes (see Discussion for details). The encapsulation response of *C. edule* to synthetic beads, both *in vivo* and *in vitro*, involves the attachment of haemocytes to the beads to mediate their aggregation, without the formation of classical multi-layered capsules. For clarity and quantification, this reaction is designated encapsulation and justification for this is provided in the Discussion.

For the present study, the *C. edule* encapsulation process is divided into two different stages. The first stage is identified as

Table 1. *Physicochemical properties of synthetic encapsulation targets**

Target	Size (diameter; μm)	Matrix	Functional group	Charge
DEAE Sepharose CL 6B	45–165	Agarose	Diethylaminoethyl	Positive
QAE Sephadex	40–125	Dextran	Quaternary aminoethyl	Positive
CM Sepharose CL 6B	45–165	Agarose	Carboxymethyl	Negative
Nylon monofilament	350	Polyamide	Amide	Negative
Sepharose CL 6B	45–165	Agarose	None	Neutral
Toyopearl HW 55S	20–40	Methacrylate polymer	Ether and hydroxyl	Neutral

*As stated by Sigma-Aldrich Company Ltd, Dorset, UK, except for Nylon monofilament, which was purchased from Shakespeare Fishing Tackle, Columbia, SC, USA.

the onset of encapsulation, during which haemocytes first recognise and attach to beads, both *in vivo* and *in vitro* (Fig. 1A). The second stage is the end-point (or completion) of encapsulation, characterized by the formation of large haemocyte/bead aggregates and by the lack of further incorporation of haemocytes or beads into these structures (Fig. 1B). In addition, *in vivo*, the end-point could be identified by the formation of aggregates containing >100 beads, which prevented any outflow of beads from the dissected foot. The time difference between these two stages was recorded and reflected the rate of the encapsulation response.

At these two stages, other parameters also recorded *in vitro* were: the approximate number of beads in the aggregate, the compactness of the bead/haemocyte aggregate, the approximate percentage of the haemocytes in the well attached to the beads, and whether the haemocytes were rounded or spread on the bead surface.

With nylon thread, the first stage (or onset) of encapsulation was identified as the attachment of individual haemocytes and the second stage (or end-point) was characterized as the attachment of large haemocyte clumps or the formation of a haemocytic sheath around the thread.

The times investigated to determine the rate of encapsulation of targets, *in vivo*, were 10 min, 30 min, at hourly intervals up to 6 h, at 12, 18, 24, 48 h, or until no further response was observed. *In vitro*, times were as for *in vivo* but only up to 24 h. For each target, the time taken for the two stages of encapsulation to be reached was recorded.

In vivo experiments

Bead suspension (100 μl of 15% v/v beads/TBS) was injected into the proximal region of the cockle foot, using a 25-gauge needle. The foot was then removed and placed in 0.05 mol l^{-1} TBS. The tissue was dissected and the recovered beads added to 500 μl Baker's formol calcium (4% formaldehyde, 1% calcium chloride, 2% NaCl) in sterile 24-well tissue culture plates (Fisher Scientific UK, Leicestershire, UK) for subsequent examination. Nylon thread was treated similarly, except that it was threaded longitudinally though the foot of the cockle inside a 21-gauge needle, which was removed to leave the thread *in situ*. Both beads and nylon thread were examined using an inverted microscope and the haemocytic response recorded.

In vitro *experiments*

The encapsulation response using whole haemolymph (i.e. haemocytes with plasma) was compared with that of haemocytes suspended in 0.05 mol l^{-1} TBS (i.e. haemocytes without plasma), thus also assessing the role of humoral factors and opsonisation in cell attachment and encapsulation.

For whole haemolymph samples, haemolymph (500 μl per individual) was withdrawn from the posterior adductor muscle using a 26-gauge needle, into an equal volume of ice-cold 0.05 mol l^{-1} TBS. For haemocyte preparations suspended in 0.05 mol l^{-1} TBS (i.e. without plasma), whole haemolymph was withdrawn, as above, into an equal volume of ice-cold anticoagulant buffer (0.05 mol l^{-1} TBS containing 0.5% EDTA and 2% glucose, pH 6.2) (Pipe et al., 1995). This was centrifuged at 55 ***g*** for 12 min at 4°C, the cell pellet washed once with anticoagulant buffer and then resuspended in 1 ml 0.05 mol l^{-1} TBS. Haemocyte viability tests were performed on all samples using both a dye exclusion assay (0.2% Eosin in

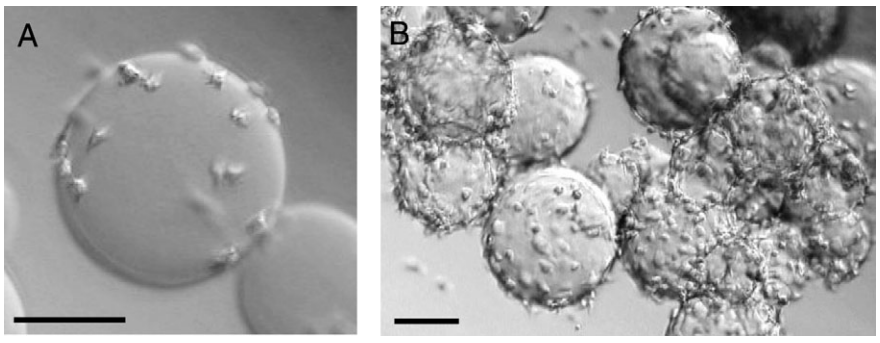

Fig. 1. Encapsulation of positively charged, DEAE Sepharose beads by haemocytes in the presence of plasma. (A) Onset of encapsulation (10 min incubation), characterized by attachment of individual haemocytes to beads. (B) End point of encapsulation (2 h incubation), characterized by formation of bead/haemocyte aggregates with large numbers of haemocytes attaching to the beads. Phase-contrast microscopy. Scale bars, 80 mm.

0.05 mol l^{-1} TBS) and a cell viability kit (L-7013, Molecular Probes Inc, Eugene, OR, USA). Results from both tests were consistent with each other, so only the dye exclusion assay was used subsequently. Samples with a viability of <90% were always rejected.

Sterile tissue culture plates (24-well, Fisher Scientific UK) were coated with 1% ECM gel (Sigma-Aldrich Company Ltd, Dorset, UK) in 0.05 mol l^{-1} TBS for 1 h at room temperature to prevent haemocytes from attaching to the bottom of the wells. The wells were washed 5× with 0.05 mol l^{-1} TBS, haemocyte samples (with or without plasma) from each individual were added to duplicate wells (400 μl $well^{-1}$; $1.21\pm0.81\times10^6$ cells $well^{-1}$) and 10 μl of 15% v/v beads/TBS solution (ca. 1500 beads) or five pieces of Nylon thread (5 mm in length) added to each well. The plate was incubated at 9°C on a rocking platform and the wells examined for encapsulation at the designated time intervals. Cell viability was monitored throughout the experiment.

Role of surface charge and plasma in cell attachment and encapsulation, in vitro

Comparisons were made between living and dead haemocytes, both in the presence and absence of plasma, towards beads of original, and then reversed, surface charge, in order to establish the role of electrostatic forces and humoral opsonic factors in cell attachment and encapsulation.

The surface charge of beads was reversed in order to investigate the role of electrostatic forces. The positive surface charge of DEAE Sepharose beads was reversed to negative by binding heparin, poly-L-glutamate or poly-L-aspartate to the bead surface. Stock bead solution was incubated with 100 units ml^{-1} heparin or 1 mg ml^{-1} poly-L-glutamate/poly-L-aspartate for 16 h at 9°C with agitation. Alternatively, negatively charged, CM Sepharose beads were incubated with 1 mg ml^{-1} poly-L-lysine in order to reverse their surface charge to positive. After incubation, all beads were washed 3× with 0.05 mol l^{-1} TBS and confirmation of binding, and thus reversal of surface charge, was determined using FITC-poly-L-lysine labelling (see below).

Dead haemocyte preparations were incorporated into this experiment in order to eliminate cell attachment due to active cell signalling and 'non-self' recognition processes of living cells. This allowed attachment to be investigated solely based on the surface properties of haemocytes and beads. Dead haemocyte preparations (both with and without plasma) were produced by incubating haemolymph samples (see above for preparation) at 15°C until haemocyte viability was zero (between 48–72 h).

The experimental set-up followed that of the *in vitro* experiments described above, with duplicate wells for each bead type and haemocyte preparation. Plates were incubated for 15 h at 9°C on a rocking platform and the encapsulation response towards each bead type recorded as either +++, ++, or + (see Table 2 for descriptions). Cell viability was also measured after 15 h incubation.

The role of humoral opsonic factors was examined in two control experiments. First, foetal calf serum (FCS) was incorporated into experiments to act as a *C. edule* plasma control. Haemolymph from five animals (500 μl per individual) was withdrawn into ice-cold anticoagulant buffer and pooled on ice. Cell pellets were produced, as above, from three 1.5 ml portions and resuspended either in 1.5 ml cell-free haemolymph, FCS (200 μg FCS protein ml^{-1} TBS, i.e. equivalent cell-free haemolymph protein concentration) or TBS. The encapsulation of DEAE Sepharose beads (positively charged) or CM Sepharose beads (negatively charged) was then studied *in vitro*, as above, using the three haemocyte preparations. Second, DEAE and CM Sepharose beads were pre-incubated with either cell-free haemolymph, FCS or TBS overnight at 4°C with agitation. Beads were then washed 3 times with TBS. Encapsulation of these bead preparations was then studied *in vitro* using haemocytes without plasma (i.e. cells resuspended in TBS). For both control experiments, presence/absence of encapsulation was recorded for each well after a 15 h incubation.

Table 2. *Role of surface charge and plasma in haemocyte attachment and encapsulation after 15 h in vitro incubation*

		Target (surface charge)			
Sample	Haemocyte viability	DEAE (+)	DEAE+heparin (–)	CM (–)	CM+poly-L-lysine (+)
With plasma	Viable	+++	++[a]	++[a]	+++
Without plasma	Viable	++[b]	0	0	++[b]
With plasma	Non-viable	+	0	0	+
Without plasma	Non-viable	++[b]	0	0	++[b]

+++, very strong encapsulation response, characterized by >90% haemocytes in well attached to beads, formation of large, tightly-bound bead/haemocyte aggregates (containing >150 beads) with haemocytes flattened and spread on the bead surface.

++, less strong encapsulation response, characterized by ca. 75% haemocytes in well attached to beads, formation of smaller bead/haemocyte aggregates (containing <10 beads). [a]Tightly bound bead/haemocyte aggregates, haemocytes flattened and spread, [b]loosely bound bead/haemocyte aggregates, haemocytes remain rounded after attachment.

+, weak encapsulation response, characterized by <30% haemocytes in well attached to beads, formation of very large, loosely bound bead/haemocyte aggregates (containing >250 beads), held together with few rounded haemocytes.

0, no encapsulation.

N=10 for each preparation.

Measurement of surface electronegativity

A modified method of Pendland and Boucias (Pendland and Boucias, 1991) was used to label negatively charged surfaces. The method uses binding of FITC-labelled poly-L-lysine, a highly positively charged compound, to surfaces to establish surface electronegativity. The effectiveness and reliability of FITC-poly-L-lysine binding was initially confirmed using encapsulation targets of known surface charge (Table 1). Following this, the surface electronegativity of all haemocyte preparations, modified beads and *Himasthla* sp. cysts was measured. Samples were incubated with FITC-poly-L-lysine (0.1 mg ml^{-1} in 0.05 mol l^{-1} TBS) for 30 min at 9°C with agitation, washed 1× in 0.05 mol l^{-1} TBS and examined using a Zeiss photomicroscope II (450–490 nm filter). Fluorescing samples represented a negative surface charge.

Statistical analyses

For statistical analyses, data were first checked for normality using the Kolmogorov–Smirnov test. Gaussian populations were analysed using a one-way analysis of variance (ANOVA) with a Tukey's multiple comparison post test, whilst non-Gaussian populations were either transformed to normality (i.e. Gaussian) and analysed as above, or analysed using the non-parametric equivalent (Prism, Graphpad Software Inc., San Diego, CA, USA).

Results

Observations of trematode capsules

Of the *C. edule* population studied, individuals of >3 years old showed >90% infection rate. Squash preparations of the cockle foot revealed both encapsulated and un-encapsulated cysts, often within the same individual (Fig. 2A), with 29.3±1.5% (mean ± s.e.m.) of the cysts encapsulated. Sections showed thick haemocytic capsules surrounding encapsulated cysts (Fig. 2B); however, no distinct haemocyte layers within the cyst were evident.

In vivo and in vitro cell attachment and encapsulation experiments

Encapsulation of beads in *C. edule*, as described in the Materials and methods, was divided into two stages, onset and completion, for comparisons of the rate of the response towards each target. Onset of encapsulation was characterized by the attachment of individual cells to the target, and completion was identified by the formation of bead/haemocyte aggregates. The size and compactness of these aggregates depended on the surface charge of the bead and the presence/absence of plasma.

In vivo experiments

Times recorded for the onset and completion of encapsulation are presented in Fig. 3A. Statistical analyses revealed significant differences in the rate of encapsulation, i.e. in both the onset and completion, between targets of different surface charges, but not within targets of the same surface charge. Positively charged targets (DEAE Sepharose and QAE Sephadex) were encapsulated significantly more rapidly than both negatively charged (Nylon and CM Sepharose) and neutral targets (Sepharose and Toyopearl) (P<0.001). In addition, negatively charged targets were encapsulated significantly faster than neutral ones (P<0.001).

In vitro experiments

In agreement with the *in vivo* study, *in vitro* studies showed that there were significant differences in the rate of cell attachment and encapsulation between targets of different surface charge (P<0.001), but not within targets of the same surface charge (Fig. 3B).

In the presence of plasma, positively charged targets were encapsulated most rapidly (P<0.001), followed by negatively charged ones, and then neutral targets, which were encapsulated least rapidly and to a lesser extent. In addition, *in vitro* encapsulation of neutral targets was noticeably lower when compared with the *in vivo* response (Fig. 3A). Only after 13.20±0.80 h (mean ± s.e.m.) and 13.80±0.92 h (mean ± s.e.m.), for Sepharose and Toyopearl beads, respectively, did individual haemocytes attach to beads, and no further progression of encapsulation was observed.

In the absence of plasma, haemocytes only attached to positively charged targets (DEAE Sepharose and QAE Sephadex). The response, however, was significantly slower than in the presence of plasma (P<0.001; Fig. 3B). In addition, unlike in the presence of plasma where haemocytes became flattened and spread upon attachment (Fig. 4A,C), haemocytes

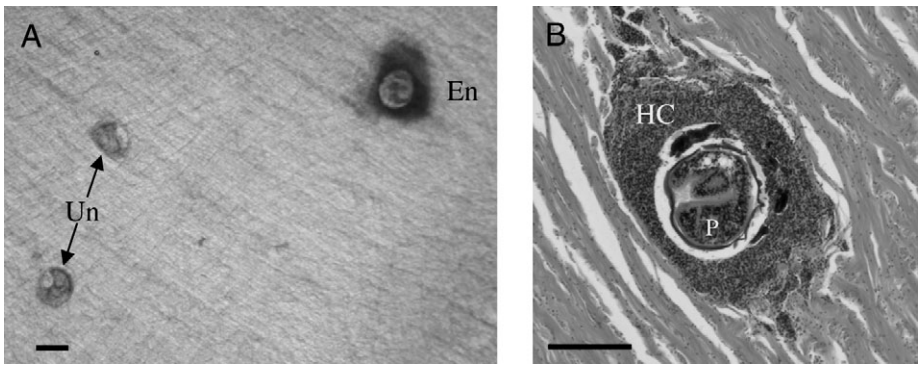

Fig. 2. Metacercarial cysts of *Himasthla* sp. in the foot of *C. edule*. (A) Squash preparation showing both un-encapsulated (Un) and encapsulated (En) cysts within the same individual. (B) Histological section stained with Cole's Haematoxylin and Eosin showing a thick haemocytic capsule (HC) surrounding the parasite cyst (P). Scale bars, 200 μm.

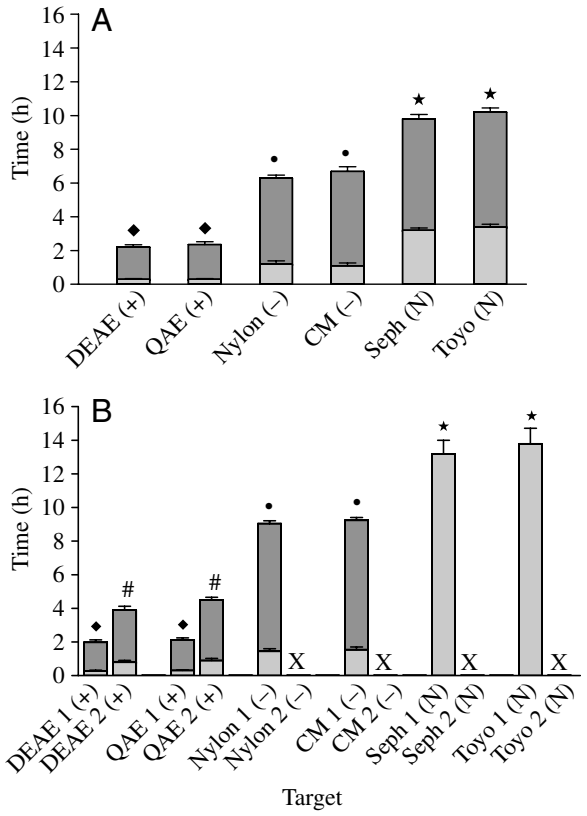

Fig. 3. *In vivo* and *in vitro* cell attachment and encapsulation experiments. Times were recorded for the time taken for onset (light grey) and completion (dark grey) of cell attachment/encapsulation. The rate of encapsulation (both onset and completion) was significantly different between (but not within) targets of different surface charges (P<0.001). (A) *In vivo* response. (B) *In vitro* response, in which times were not recorded for completion of cell attachment/encapsulation of neutral targets (N) as no further progression of encapsulation was observed after initial attachment of individual haemocytes. DEAE, DEAE Sepharose; QAE, QAE Sephadex; Nylon, Nylon thread; CM, CM Sepharose; Seph, Sepharose; Toyo, Toyopearl; +, positive; –, negative; N, neutral; 1, haemocytes in the presence of plasma; 2, haemocytes in the absence of plasma; ×, no encapsulation. N=10 for each target. Samples with different symbols are significantly different.

in the absence of plasma retained a rounded morphology even after attachment (Fig. 4B,D).

Assessment of cell viability showed a decrease of 1.1±0.21% and 1.4±0.27% (mean ± s.e.m.) for haemocytes with and without plasma, respectively, after a 1 h incubation. This decreased further to 3.8±0.24% and 7.6±0.40%, after 5 h incubation, 8.7±0.48% and 16.8±1.0% after 10 h (by which time most encapsulation reactions were complete), 10.9±0.82% and 17.6±0.98% after 15 h, and finally 27.5±0.81% and 45.3±1.2% after 24 h.

Role of surface charge and plasma in cell attachment and encapsulation, in vitro

Results from this investigation are summarized in Table 2. The extent of encapsulation towards each target depended upon the surface charge of the bead, whether or not plasma was present, and whether the haemocytes were alive or dead.

With respect to surface charge, haemocytes from all preparations (i.e. in the presence and absence of plasma, living and dead) attached to all positively charged targets. The haemocytic response did not vary with different surface molecular patterns (Table 2). In contrast, haemocytes from only one preparation (living haemocytes in the presence of plasma) attached to negatively charged targets. Also, changing the bead surface charge from positive to negative [i.e. DEAE (+) to DEAE plus heparin (–)], resulted in decreased haemocyte attachment, whereas changing the bead surface charge from negative to positive [i.e. CM (–) to CM plus poly-L-lysine (+)] increased the attachment of cells. Reversal of DEAE Sepharose surface charge to negative, through binding of poly-L-glutamate and poly-L-aspartate, produced very similar encapsulation responses to that of heparin binding (data not shown).

The presence of plasma also strongly influenced haemocyte attachment to all bead types, including those with reversed surface charge. With live haemocytes, plasma resulted in a higher degree of cell attachment when compared with its absence. This was illustrated by haemocytes spreading on the bead surface, larger numbers of haemocytes attaching to beads, and by production of larger, more tightly bound, bead/haemocyte aggregates (see Table 2). In contrast, with dead haemocyte preparations, the presence of plasma lowered the degree of cell association with positively charged beads.

The role of humoral opsonic factors in the enhanced encapsulation response of living haemocytes in the presence of plasma was confirmed, since haemocytes resuspended in FCS did not encapsulate negatively charged CM Sepharose beads. Neither did haemocytes resuspended in TBS. In contrast, haemocytes resuspended in cell-free haemolymph encapsulated both DEAE Sepharose and CM Sepharose beads. The specificity of the response was further confirmed by haemocytes resuspended in TBS encapsulating CM Sepharose beads pre-incubated in cell-free haemolymph after 15 h incubation, but not beads pre-incubated in FCS or TBS. In contrast, DEAE Sepharose beads pre-incubated in either cell-free haemolymph, FCS or TBS were all encapsulated after 15 h.

Cell viability had decreased by 10.2±0.47% and 18.7±1.1%, for haemocytes in the presence and absence of plasma respectively, after the 15 h incubation.

Measurement of surface electronegativity

Fluorescence of negatively charged beads (Fig. 5A), but not positively charged or neutral ones, confirmed that FITC-poly-L-lysine only bound to negatively charged surfaces. Both living and dead haemocytes in the absence of plasma also fluoresced, confirming a negative surface charge (82.41±1.46% and 85.39±1.68% (mean ± s.e.m.) respectively, Fig. 5B). Unfortunately, the presence of plasma precipitated FITC-poly-L-lysine, therefore a negative surface charge could not be

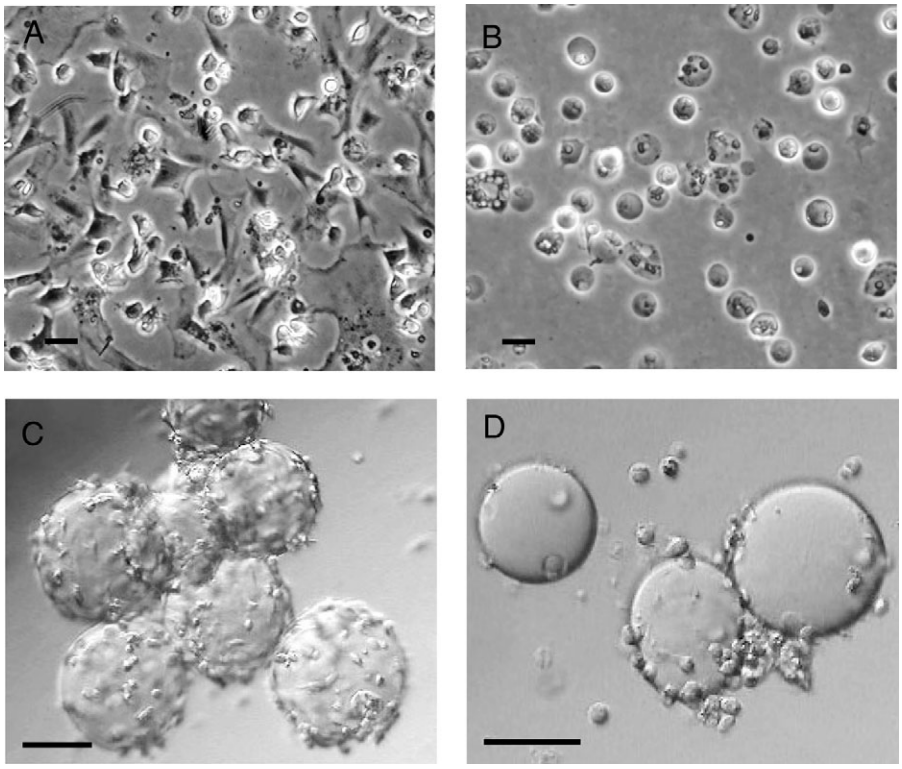

Fig. 4. Differences in haemocyte morphology observed *in vitro,* in the presence and absence of plasma. (A) Haemocytes with plasma, spread on uncoated 24-well culture plate (1 h incubation); scale bar, 10 μm. (B) Rounded haemocytes without plasma, attached to uncoated 24-well culture plate (1 h incubation); scale bar, 10 μm. (C) Haemocytes with plasma, spread on surface of DEAE Sepharose beads (1 h incubation); scale bar, 80 μm. (D) Rounded haemocytes without plasma, attached to surface of DEAE Sepharose beads (3 h incubation); scale bar, 80 μm.

confirmed for haemocytes in these preparations. *Himasthla* sp. cysts were also negatively charged (Fig. 5C,D). In addition, FITC-poly-L-lysine binding confirmed reversal of bead surface charge.

Discussion

Little is known about the encapsulation response in bivalve molluscs, although five distinct types of encapsulation have been described in these animals in response to metazoan parasites (Cheng and Rifkin, 1970). In the present study, details of the encapsulation response of the edible cockle, *Cerastoderma edule*, are described and detailed information is revealed of a synergistic interaction between surface charge and humoral plasma factors. This synergistic relationship is probably relevant to encapsulation responses of other invertebrates groups.

Encapsulation occurred in response to metacercarial cysts of the trematode, *Himasthla* sp. in the foot of *C. edule*; however, not all cysts were encapsulated. Seawater containing dye failed to penetrate *Himasthla* sp. cyst walls (E. C. Wootton, unpublished), suggesting that *Himasthla* sp. secretory products were not responsible for the lack of encapsulation towards some cysts. Why only one third of cysts induce a host response is currently unknown.

The nature of the invertebrate encapsulation response is highly species-specific, but the basic mechanisms, involving non-specific physicochemical

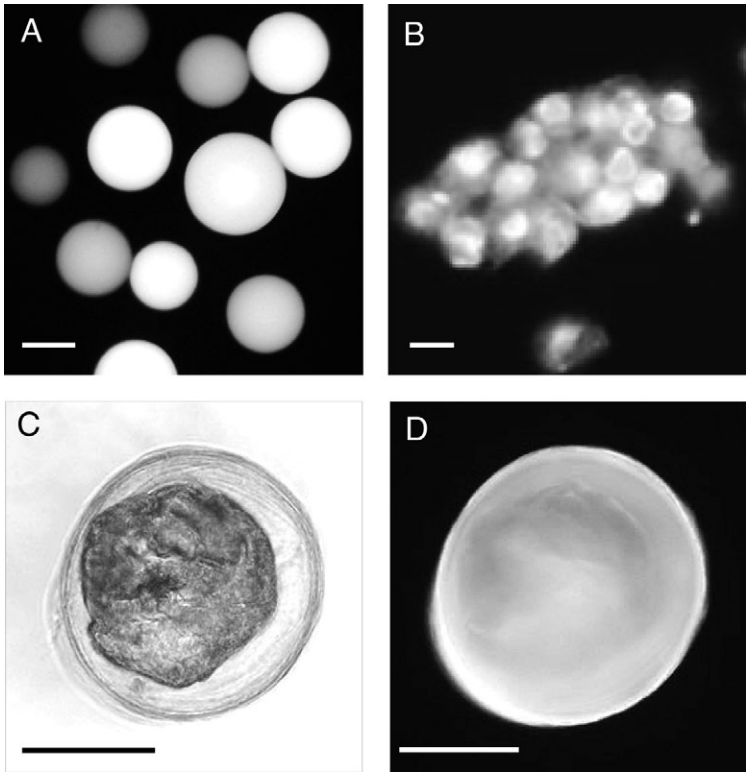

Fig. 5. Identification of negatively charged surfaces using FITC-poly-L-lysine. (A) Negatively charged CM Sepharose beads; scale bar, 80 μm. (B) Negatively charged viable haemocytes (without plasma); scale bar, 10 μm. (C) *Himasthla* sp. cyst under phase contrast; scale bar, 100 μm. (D) Identical cyst showing negatively charged cyst wall; scale bar, 100 μm.

factors such as electrostatic forces, are potentially common to all encapsulation reactions. It is the modification of these basic mechanisms, by highly specific biochemical interactions, which produce unique responses to individual host–parasite associations. These biochemical interactions are determined by many factors including parasite species, viability and stage of development as well as the susceptibility of the host and the particular host tissue invaded (e.g. Cheng et al., 1966; Lie et al., 1987; Ataev et al., 1999; Laruelle et al., 2002). The gastropod mollusc, *Biomphalaria glabrata*, for example, exhibits a variety of encapsulation responses towards invading trematodes, including haemocytic infiltration of rounded haemocytes, type I capsules involving flattened cells, type II capsules involving polygonal cells, and amoebofibrous capsules (Lie et al., 1987). In addition, five distinct encapsulation types have been described in bivalve molluscs in response to such parasites (Cheng and Rifkin, 1970), including aggregation, as observed in the present work. It is justified, therefore, to designate 'encapsulation' to describe the haemocytic responses exhibited by *C. edule* during the present study.

All our experiments provided strong evidence for a role for surface charge and non-specific electrostatic forces in the encapsulation response of *C. edule*. Investigations, both *in vivo* and *in vitro*, into rate of encapsulation revealed that all positively charged targets elicited the strongest and most rapid response, regardless of their functional group, whilst negatively charged and neutral targets mediated a less vigorous response. This illustrates that haemocytes are responding to surface charge and not surface molecular patterns and this may be partly due to the beads not possessing common PAMPs, such as LPS, peptidoglycan or β-1,3 glycan.

Further experiments, *in vitro*, revealed that live and dead cells (both with and without plasma) associated with, or encapsulated, positively charged targets. This contrasts with negatively charged targets, which were only encapsulated by living haemocytes in the presence of plasma. These observations, combined with the enhancement of cell attachment through changing the bead surface charge from negative to positive, and the converse change in bead surface charge lowering the haemocytic response, strongly imply that non-specific electrostatic forces are influencing bead–haemocyte interactions. As *C. edule* haemocytes were shown to be negatively charged, electrostatic attractions between haemocytes and positively charged beads are entirely feasible.

In our experiments, heparin, despite its anticoagulant properties, was used to reverse the surface charge of positive DEAE Sepharose beads. It was considered an appropriate compound as its binding forces are largely electrostatic (Lindahl, 1997). In addition, the prophenoloxidase (PpO) cascade in *C. edule* is minimal as cockles do not exhibit melanization reactions (E. C. Wootton, unpublished), nor do the haemocytes contain phenoloxidase (Wootton et al., 2003). Thus, the interaction of heparin with immunological serine proteases to activate a prophenoloxidase system is unlikely. To confirm that heparin was not interfering with such proteins, two additional compounds, poly-L-glutamate and poly-L-asparate, were also used to reverse the surface charge of DEAE Sepharose beads, and these beads produced very similar encapsulation responses to those bound with heparin.

In other invertebrates, particularly insects, positively charged targets commonly elicit strong encapsulation responses (Walter and Williams, 1967; Vinson, 1974; Dunphy and Nolan, 1980; Lackie, 1983; Ratner and Vinson, 1983), and non-specific electrostatic forces have been considered an influential factor (e.g. Walters and Williams, 1967; Vinson, 1974; Lackie, 1983; Wiesner, 1992). It has been suggested that humoral recognition molecules bind more readily to positively charged targets (Vinson, 1974; Wiesner, 1992; Strand and Pech, 1995), mediating 'non-self' recognition and immune defence reactions. It has also been proposed that negatively charged targets adsorb humoral components, become disguised as 'self', and thus reduce host immune responses (Walter and Williams, 1967; Paskewitz and Riehle, 1994). In addition, the stability of recognition molecules in the vertebrate complement pathway increases on contact with surfaces of a particular charge (Toufik et al., 1995), and this has been suggested to occur in insect immunity too (Lackie, 1988). As of yet, these hypotheses explaining enhanced encapsulation of positively charged targets by invertebrate haemocytes have not been fully tested

Thus, non-specific electrostatic forces are probably only one of many factors involved in cell attachment during immune defence reactions. Opsonins and pattern recognition receptors, for example, will undoubtedly play an important role. In this respect, our study, like many others (e.g. Davies et al., 1988; Paskewitz and Riehle, 1998; Lavine and Strand, 2001), revealed a strong involvement of humoral components. This is highlighted by the encapsulation of negatively charged targets exclusively by living haemocytes in the presence of plasma. The specificity of this reaction was confirmed by the lack of encapsulation of negatively charged beads in the presence of the plasma substitute, FCS, and by the opsonization of beads only by pre-incubation in *C. edule* plasma. In the present study, humoral opsonisation appeared to reduce the electrostatic repulsion between the negatively charged haemocytes and negatively charged targets, possibly through electrostatic interactions, thus allowing cells and beads to interact. Humoral factors, therefore, mediate 'non-self' recognition processes in *C. edule* and allow for encapsulation of targets with different surface charges. This is important for effective immune defence, as many parasites and pathogens, including *Himasthla* sp. cysts, carry a negative surface charge, which is thought to be an evasive strategy to avoid host immune responses (Crocker and Varki, 2001). Dead haemocytes in the presence of plasma, however, did not attach to negatively charged targets, and this highlights that humoral opsonization, and subsequent encapsulation, involves active cell-to-cell communication.

Humoral opsonisation, however, does not completely override the non-specific electrostatic forces. Our study shows that the two factors act simultaneously in influencing cell

attachment. For example, positively charged targets are more vigorously encapsulated than negatively charged or uncharged targets in the presence of plasma. The electrostatic attraction between positively charged targets and negatively charged haemocytes is an additional influence to humoral opsonisation, and thus enhances the response. In addition, although dead cells attached to positively charged beads in the presence of plasma, the response was weaker than that of dead cells in the absence of plasma. This reveals that humoral factors were interfering with, but not completely masking, the electrostatic attraction between cells and beads. As the haemocytes were dead, cell attachment must be due to non-specific electrostatic forces, because, as suggested previously, encapsulation requires active cell-to-cell communication.

Although previous studies in insects have often postulated that surface charge is an important influencing factor of haemocytic encapsulation in invertebrates (e.g. Walters and Williams, 1967; Vinson, 1974; Lackie, 1983; Wiesner, 1992; Paskewitz and Riehle, 1994), detailed investigations into proving this hypothesis are very limited. Lackie (Lackie, 1981) proposed that immune recognition in insects is based on a 'two-tiered' system, in which electrostatic forces have an additive or synergistic effect on the highly specific biochemical 'non-self' recognition processes. Our results support the 'two-tiered' hypothesis by highlighting the importance of electrostatic interactions in cellular communication, and by providing substantial evidence for an interactive and influential role for both non-specific electrostatic forces and humoral factors in haemocyte attachment and encapsulation.

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References

Ahsan, F., Rivas, I. P., Khan, M. A. and Torres Suárez, A. I. (2002). Targeting to macrophages: role of physicochemical properties of particulate carriers – liposomes and microspheres – on the phagocytosis by macrophages. *J. Control. Release* **79**, 29-40.

Akaki, M., Nakuno, Y., Nagayasu, E., Nagakura, K., Kawai, S. and Aikawa, M. (2001). Invasive forms of *Toxoplasma gondii*, *Leishmania amazonensis* and *Trypanosoma cruzi* have a positive charge at their contact site with host cells. *Parasitol. Res.* **87**, 193-197.

Ataev, G. L. and Coustau, C. (1999). Cellular responses to *Echinostoma caproni* infection in *Biomphalaria glabrata* strains selected for susceptibility/resistance. *Dev. Comp. Immunol*. **23**, 187-198.

Bayne, C. J., Hahn, U. K. and Bender, R. C. (2001). Mechanisms of molluscan host resistance and of parasite strategies for survival. *Parasitology* **123**, S159-S167.

Cheng, T. C. and Rifkin, E. (1970). Cellular reactions in marine molluscs in response to helminth parasitism. In *A Symposium on Diseases of Fishes and Shellfishes*, Special Publication 5 (ed. S. F. Snieszko), pp. 443-496. American Fisheries Society.

Cheng, T. C. and Garrabrant, T. A. (1977). Acid phosphatase in granulocytic capsules formed in strains of *Biomphalaria glabrata* totally and partially resistant to *Schistosoma mansoni*. *Int. J. Parasitol.* **7**, 467-472.

Cheng, T. C., Shuster, C. N. and Anderson, A. H. (1966). A comparative study of the susceptibility and response of eight species of marine pelecypods to the trematode *Himasthla quissetensis*. *Trans. Am. Microsc. Soc*. **85**, 284-295.

Choi, J. Y., Whitten, M. M. A., Cho, M. Y., Lee, K. Y., Kim, M. S., Ratcliffe, N. A. and Lee, B. L. (2002). Calreticulin enriched as an early-stage encapsulation protein in wax moth *Galleria mellonella* larvae. *Dev. Comp. Immunol.* **26**, 335-343.

Crocker, P. R. and Varki, A. (2001). Siglecs, sialic acids and innate immunity. *Trends Immunol.* **22**, 337-342.

Davies, D. W., Hayes, T. K. and Vinson, S. B. (1988). Preliminary characterization and purification of *in vitro* encapsulation promoting factor: a peptide that mediates insect haemocyte adhesion. *Dev. Comp. Immunol.* **12**, 241-253.

de Montaudouin, X., Kisielewski, I., Bachelet, G. and Desclaux, C. (2000). A census of macroparasites in an intertidal bivalve community, Arcachon Bay, France. *Oceanol. Acta* **23**, 453-468.

Dunphy, G. B. and Nolan, R. A. (1980). Response of eastern hemlock looper hemocytes to selected stages of *Entomophthora egressa* and other foreign particles. *J. Invertebr. Pathol.* **36**, 71-84.

FAO (2005). *The State of World Fisheries and Aquaculture (SOFIA)*. FAO Fisheries Department, 153 pp.

Humarson, G. L. (1979). *Animal Tissue Techniques* (4th edn.). San Francisco: W. H. Freeman and Company.

Kelm, S. and Schauer, R. (1997). Sialic acids in molecular and cellular interactions. *Int. Rev. Cytol.* **175**, 137-240.

Lackie, A. M. (1981). Immune mechanisms in insects. *Dev. Comp. Immunol.* **5**, 191-204.

Lackie, A. M. (1983). Effect of substratum wettability and charge on adhesion *in vitro* and encapsulation *in vivo* by insect hemocytes. *J. Cell Sci.* **63**, 181-190.

Lackie, A. M. (1986). The role of substratum surface-charge in adhesion and encapsulation by locust hemocytes *in vivo*. *J. Invertebr. Pathol.* **37**, 377-378.

Lackie, A. M. (1988). Immune mechanisms in insects. *Parasitol. Today* **4**, 98-105.

Laruelle, F., Molloy, D. P. and Roitman, V. A. (2002). Histological analysis of trematodes in *Dreissena polymorpha*: their location, pathogenicity, and distinguishing morphological characteristics. *J. Parasitol.* **88**, 857-863.

Lavine, M. D. and Strand, M. R. (2001). Surface characteristics of foreign targets that elicit an encapsulation response by the moth, *Pseudoplusia includens*. *J. Insect Physiol.* **47**, 965-974.

Lie, K. J., Jeong, K. H. and Heyneman, D. (1987). Molluscan host reactions to helminth infection. In *Immune Responses in Parasitic Infections: Immunology, Immunopathology and Immunoprophylaxis* (ed. E. J. L. Soulsby), pp. 212-270. Boca Raton: CRC Press.

Lindahl, U. (1997). Heparin sulphate – a polyanion with multiple messages. *Pure Appl. Chem.* **69**, 1897-1902.

Loker, E. S. and Bayne, C. J. (2001). Molecular studies of the molluscan response to digenean infection. In *Phylogenetic Perspectives on the Vertebrate Immune System* (ed. G. Beck, M. Sugumaran and E. Cooper), pp. 209-222. USA: Kluwer Academic/Plenum Publishers.

Loret, S. M. and Strand, M. R. (1998). Follow-up of protein release from *Pseudoplusia includens* hemocytes: a first step toward identification of factors mediating encapsulation in insects. *Eur. J. Cell Biol.* **76**, 146-155.

Mehrishi, J. N. and Bauer, J. (2002). Electrophoresis of cells and the biological relevance of surface charge. *Electrophoresis* **23**, 1984-1994.

Monteiro, V. G., Soares, C. P. and De Souza, W. (1998). Host cell surface sialic acid residues are involved in the process of penetration of *Toxoplasma gondii* into mammalian cells. *FEMS Microbiol. Lett.* **16**, 323-327.

Morikis, D. and Lambris, J. D. (2004a). The electrostatic nature of C3d-complement receptor 2 association. *J. Immunol.* **172**, 7537-7547.

Morikis, D. and Lambris, J. D. (2004b). Physical methods for structure, dynamics and binding in immunological research. *Trends Immunol*. **25**, 700-707.

Nappi, A. J., Vass, E., Frey, F. and Carton, Y. (1995). Superoxide anion generation in *Drosophila* during melanotic encapsulation of parasites. *Eur. J. Cell Biol*. **68**, 450-456.

Ouaissi, M. A., Taibi, A., Cornette, J., Velge, P., Marty, B., Loyens, M., Esteva, M., Rizvi, F. S. and Capron, A. (1990). Characterization of major surface and excretory secretory immunogens of *Trypanosoma cruzi* trypomastigotes and identification of potential protective antigens. *Parasitology* **100**, 115-124.

Paskewitz, S. M. and Riehle, M. A. (1994). Response of *Plasmodium* refractory and susceptible strains of *Anopheles gambiae* to inoculated Sephadex beads. *Dev. Comp. Immunol.* **18**, 369-375.

Paskewitz, S. M. and Riehle, M. (1998). A factor preventing melanization of

Sephadex CM C-25 beads in *Plasmodium*-susceptible and refractory *Anopheles gambiae*. *Exp. Parasitol.* **90**, 34-41.

Pendland, J. C. and Boucias, D. G. (1991). Physicochemical properties of cell surfaces from the different developmental stages of the entomopathogenic hyphomycete, *Nomuraea rileyi*. *Mycologia* **83**, 264-272.

Pipe, R. K., Coles, J. A. and Farley, S. R. (1995). Assays for measuring immune response in the mussel *Mytilus edulis*. In *Techniques in Fish Immunology – 4. Immunology and Pathology of Aquatic Invertebrates* (ed. J. S. Stolen, T. C. Fletcher, S. A. Smith, J. T. Zelikoff, S. L. Kaattari, R. S. Anderson, K. Söderhall and B. A. Weeks-Perkins), pp. 93-100. Fair Haven: SOS Publications.

Ratcliffe, N. A. (1993). Cellular defence responses of insects: unresolved problems. In *Parasites and Pathogens of Insects*, vol. I (ed. N. E. Beckage, S. M. Thompson and B. A. Federici), pp. 267-304. San Diego: Academic Press.

Ratcliffe, N. A. and Rowley, A. F. (1987). Insect responses to parasites and other pathogens. In *Immune Responses in Parasitic Infections: Immunology, Immunopathology, Immunoprophylaxis* (ed. E. J. L. Soulsby), pp. 271-332. Boca Raton: CRC Press.

Ratner, S. and Vinson, S. B. (1983). Encapsulation reactions *in vitro* by haemocytes of *Heloithis virescens*. *J. Insect Physiol.* **29**, 855-863.

Rieu, P., Porteu, F., Bessou, G., Lesavre, P. and Halbwachsmecarelli, L. (1992). Human neutrophils release their major membrane sialoprotein, leukosialin (CD43), during cell activation. *Eur. J. Immunol.* **22**, 3021-3026.

Saraiva, E. M. B., Vannier-Santos, M. A., Silve Filho, F. C. and De Souza, W. (1989). Anionic site behaviour in *Leishmania* and its role in the parasite-macrophage interaction. *J. Cell Sci.* **93**, 481-489.

Souto-Padrón, T. (2002). The surface charge of trypanosomatids. *An. Acad. Bras. Cienc.* **74**, 649-675.

Strand, M. R. and Pech, L. L. (1995). Immunological basis for compatibility in parasitoid-host relationships. *Annu. Rev. Entomol.* **40**, 31-56.

Toufik, J., Carreno, M. P., Jozefowicz, M. and Labarre, D. (1995). Activation of the complement system by polysaccharidic surfaces bearing carboxymethyl, carboxymethylbenzylamide and carboxymethylbenzylamide sulfonate groups. *Biomaterials* **16**, 993-1002.

Vinson, S. B. (1974). The role of foreign surface and female parasitoid secretions on the immune response of an insect. *Parasitol*. **68**, 27-33.

Walter, D. R. and Williams, C. M. (1967). Reaggregation of insect cells as studied by a new method of tissue and organ culture. *Science* **154**, 516-517.

Wegeberg, A. M., de Montaudouin, X. and Jensen, K. T. (1999). Effect of host size (*Cerastoderma edule*) on infectivity of three *Himasthla* species (Echinostomatidae, Trematoda). *J. Exp. Mar. Biol. Ecol.* **238**, 259-269.

Whitten, M. M. A., Tew, I. F. and Ratcliffe, N. A. (2004). A novel role for an insect apolipoprotein (apolipophorin III) in β-1,3-glucan pattern recognition and cellular encapsulation reactions. *J. Immunol.* **172**, 2177-2185.

Wiesner, A. (1992). Characteristics of inert beads provoking humoral immune responses in *Galleria Mellonella* larvae. *J. Insect Physiol.* **38**, 533-541.

Wootton, E. C., Dyrynda, E. A. and Ratcliffe, N. A. (2003). Bivalve immunity: comparisons between the marine mussel (*Mytilus edulis*), the edible cockle (*Cerastoderma edule*) and the razor-shell (*Ensis siliqua*). *Fish Shellfish Immunol.* **15**, 195-210.

Yoshino, T. P., Boyle, J. P. and Humphries, J. E. (2001). Receptor-ligand interactions and cellular signalling at the host-parasite interface. *Parasitology* **123**, S143-S157.

Zasloff, M. (2002). Antimicrobial peptides of multicellular organisms. *Nature* **415**, 389-395.