Granular cells are required for encapsulation of foreign targets by insect haemocytes

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SUMMARY

Haemocytes play an essential role in defending invertebrates against pathogens and parasites that enter their haemocoel. A primary defense response is encapsulation; a process in which haemocytes attach to the foreign organism and kill it. Whether encapsulation requires cooperation between specific subpopulations of haemocytes is unknown. Using purified subpopulations of haemocytes and an in vitro encapsulation assay, we investigated the process of capsule formation in the insect Pseudoplusia includens. Immunocytochemical staining revealed that capsule formation involves a three step process. Encapsulation began when granular cells attached to the foreign target. This was followed by attachment of multiple layers of plasmatocytes. Termination of capsule formation occurred when a subpopulation of granular cells formed a monolayer around the periphery of the capsule. Neither granular cells nor plasmatocytes were capable of forming a capsule independently. However, plasmatocytes encapsulated targets if granular cells were present or if targets were preincubated in medium conditioned by granular cells. The effect of granular cell-conditioned medium could be blocked by the addition of the cell adhesion recognition sequence, RGDS, but not by RGES. These results demonstrate experimentally that granular cells are required for encapsulation of foreign targets by plasmatocytes in vitro, and that the role of granular cells in this process involves an RGD-dependent cell adhesion mechanism.

Key words: Encapsulation, Insect immunity, Cell adhesion, Haemocyte

INTRODUCTION

Haemocytes play an essential role in defending insects and other invertebrates against invading parasites and pathogens. Metazoan parasites, such as nematodes and parasitoid wasps, are usually killed by encapsulation. During an encapsulation response, haemocytes attach to the foreign target and one another, eventually forming a smooth capsule comprised of overlapping layers of cells. The morphology of capsules is broadly similar across taxa although variation exists between species in how rapidly a target is encapsulated, the haemocyte types suggested to participate in capsule formation, and whether or not the capsule melanizes (summarized by Salt, 1970; Rowley and Ratcliffe, 1981; Lackie, 1988; Strand and Pech, 1995). Surface properties of the target also influence encapsulation by affecting the adhesive and spreading properties of haemocytes.

With the possible exception of some Diptera (Gotz, 1986), encapsulation in most insects clearly depends on a cooperative response between haemocytes. What remains unclear is whether this response is mediated by a functionally uniform population of haemocytes or through an interaction between functionally different subpopulations of cells. For example, electron microscopic studies with Galleria mellonella (Lepidoptera) and Clitumnus extradentatus (Phasmodea) suggest encapsulation is biphasic. When haemocytes called granular cells contact a foreign target, they lyse or degranulate, releasing material that promotes attachment of plasmatocytes. Multiple layers of plasmatocytes then form the capsule (Schmit and Ratcliffe, 1977, 1978). In contrast, Brehein et al. (1975) report that only a single class of cells, designated as granular haemocytes, mediates encapsulation by Locusta migratoria (Orthoptera) and Melolontha melolontha (Coleoptera).

This variation reflects in part interspecific differences in the morphology of haemocytes. However, it also reflects two major difficulties that have hindered progress in understanding how cellular defense responses in insects are coordinated. First, because haemocytes are classified primarily by morphology, it is difficult to identify the cell types in multilayered capsules or to know whether morphological classes of cells (i.e. morphotypes) are actually comprised of functionally specialized subclasses. Confusion also arises when comparing haemocytes between species, because immune functions assigned to one morphotype in one species are sometimes assigned to a different morphotype in another. Second, it is very difficult to experimentally manipulate encapsulation responses in vivo. How different targets influence encapsulation can be assessed, but it is not possible to manipulate specific haemocyte populations or putative molecules mediating haemocyte behavior. As a consequence, our understanding of how haemocytes interact in forming a capsule is based almost entirely on descriptive, microscopic studies. An obvious alternative would be to conduct complementary experiments in vitro. Unfortunately, insect haemocytes are often very unstable in culture.
with some cell types rapidly clumping or lysing upon removal from the haemocoele while others quickly spread upon contact with the surface of the culture vessel. As a result, haemocytes deplete themselves from suspension and do not form capsules around the experimental target. Methods for overcoming aggregation of cells during bleeding include collecting haemocytes in low pH/Ca²⁺ buffers (Mead et al., 1986) or collecting haemolymph from the pericardial sinus (Horohov and Dunn, 1982). Adhesion to culture vessels has also been transiently inhibited by trypsinizing haemocytes (Ratner and Vinson, 1983), culturing cells in plasma proteins such as lipophorin (Coodin and Caveney, 1992) and hemolin (Ladendorff and Kanost, 1991), or using roller/drop-type cultures (Ratcliffe and Rowley, 1975; Dunphy and Nolan, 1980). Despite these efforts, haemocytes usually remain nonadherent or viable for only short periods in vitro.

Haemocytes of the soybean looper, *P. includens* (Insecta: Lepidoptera: Noctuidae) can be divided on the basis of morphology into five classes (Strand and Noda, 1991; Pech et al., 1994). Granular cells and plasmatocytes comprise approximately 65% and 28%, respectively, of the total haemocyte population in larvae, while the remaining cells are classified as spherule cells, oenocytoids and prohaemocytes. Both granular cells and plasmatocytes adhere to foreign surfaces when cultured in vitro, but microscopic examination of capsules formed in vivo suggest they are comprised primarily of plasmatocytes. Recently, we established methods for separating the aforementioned morphotypes by gradient centrifugation and developed a panel of monoclonal antibodies (mAbs) for labelling specific cell populations (Pech et al., 1994). Two mAbs characterized previously (Strand and Johnson, 1996) were also developed an in vitro encapsulation assay that produces capsules that are morphologically indistinguishable from those formed in vivo (Pech et al., 1995). In this study, we characterized immunocytochemically the spatial and temporal distribution of selected haemocytes during capsule formation and examined whether this defense response requires cooperation between granular cells and plasmatocytes. Our results indicate that encapsulation can be divided into three discrete phases mediated by at least two antigenically distinct populations of haemocytes. Capsules are comprised primarily of plasmatocytes but granular cells mediate both the initiation of capsule formation and its termination.

**MATERIALS AND METHODS**

**Animals and haemolymph collection**

*P. includens* were reared and staged as described by Strand (1990). Insects used in experiments were 36-48 hour old fifth instar larvae. Haemocyte morphotypes were classified using previously established morphological criteria (Strand and Noda, 1991; Pech et al., 1994). Two mAbs characterized previously (Strand and Johnson, 1996) were also used as cell markers. The mAb 48F2D5 labels all haemocytes classified by morphology and ultrastructural features as granular cells, whereas the mAb 52F3D7 labels all haemocytes classified as plasmatocytes (Strand and Johnson, 1996).

For in vitro assays, haemocytes were collected from fifth instar larvae by the procedure of Pech et al. (1994). Larvae were anaesthetized with CO₂, surface sterilized with 95% ethanol, and bled from a proleg into 500 μl of anticoagulant buffer (0.098 M NaOH, 0.186 M NaCl, 0.0017 M EDTA and 0.041 M citric acid, pH 4.5). Haemocyte preparations were then used in assays as either a mixed population of unseparated haemocytes or after purification of specific morphotypes.

**Haemocyte separation procedure**

After washing in Ex-cell 400 medium (JRH Biosciences, Lenexa, KS), haemocyte morphotypes were purified on Percoll step gradients formed in sterile 12 mm × 75 mm round-bottom polystyrene tubes (Becton Dickinson, Lincoln Park, NJ) (Pech et al., 1994). Unseparated haemocytes (2.0×10⁶ cells) in 200 μl of Ex-cell 400 medium were gently layered onto the top of a gradient that consisted of 2 ml of 47.5% Percoll (Sigma, St Louis, MO) in Ex-cell 400 layered over 2 ml of 62.5% Percoll. Gradients were centrifuged for 15 minutes at 480 g in a Beckman J2-21 centrifuge with a JS-7.5 swinging bucket rotor. The band of cells at the top of the gradient, containing granular cells, was removed by aspiration, washed twice in Ex-cell 400 medium, and either placed in culture or used in encapsulation assays. The band of cells at the interface of the 47.5% and 62.5% Percoll, containing plasmatocytes, was similarly removed and used in assays. The granular cell fraction was approximately 98% pure, contaminated by plasmatocytes, whereas the plasmatocyte fraction consistently was contaminated with 4-6% granular cells. To remove these granular cells, we took advantage of the differential rate at which granular cells and plasmatocytes adhere to untreated culture plates (Pech et al., 1994). The plasmatocyte fraction from a Percoll gradient was cultured in 96-well tissue culture plates (Corning) containing Ex-cell 400 medium for 1 hour. Granular cells strongly adhere to and spread on the plate’s surface during this period while plasmatocytes do not. The plasmatocytes were then removed using a pipet, washed twice in Ex-cell 400, and used in assays.

**Immunofluorescence microscopy**

Haemocytes or capsules were fixed in 5% formalin for 10 minutes, rinsed in PBS and permeabilized for 15 minutes in PBT (PBS plus 0.1% Triton X-100) (Strand and Johnson, 1996). Cells were blocked for 1 hour in 3% bovine serum albumen (BSA) (fraction V, Boehringer Mannheim) in PBT followed by incubation with the primary antibody for 1 hour (haemocytes) or overnight (capsules). After rinsing 4× in PBT, haemocytes were incubated with fluorescein isothiocyanate (FITC) or rhodamine (Rh)-conjugated goat anti-mouse secondary antibody (IgG; Kirkegaard and Perry) diluted 1:20 in PBS plus 3% BSA. For double labelling experiments, haemocytes or beads were rinsed 4× in PBS, incubated with another primary antibody followed by a FITC or Rh-conjugated secondary antibody as described above. Samples were examined using a Nikon Diaphot fluorescence microscope with Hoffman modulation contrast optics.

**Preparation of conditioned medium**

Purified granular cells or plasmatocytes (1×10⁵ cells/well) were incubated in 70 μL of Ex-cell 400 medium in 96-well culture plates (Corning). After 24 hours, the conditioned medium was removed and filtered through 0.2 μm filters (Millipore). The resulting solution was referred to as 100% conditioned medium. Subsequent dilutions were made with Ex-Cell 400 medium. For some experiments, Dowex 1X-2 beads were preincubated for 1 hour in 33% granular cell- or plasmatocyte-conditioned medium. The beads were then washed three times with Ex-cell 400 and used in encapsulation assays.

**In vitro and in vivo encapsulation assays using unseparated haemocytes**

In vitro encapsulation assays were conducted as described by Pech et al. (1995). Tissue culture plates were coated with the murine-derived basement membrane, Matrigel (Collaborative Biomedical Products, Becton Dickinson). Matrigel was diluted 1:100 with ice-cold Ex-cell 400 medium and wells of a 96-well culture plate were coated overnight with 70 μl of the diluted solution. The resulting membrane was washed 5× with medium before addition of haemocytes and encapsulation targets. Encapsulation assays were conducted by
placing 4.0×10^5 unseparated haemocytes from *P. includens* larvae into membrane-coated wells containing 70 µl of Ex-cell 400 medium. From 150 to 200 Dowex 1X-2 beads were then added as encapsulation targets. At prescribed intervals over a 36 hour period, the beads and haemocytes present in selected wells were processed for immunofluorescence microscopy as described above. In certain assays, the viability of the haemocytes attached to capsules was also monitored by dye exclusion assay using propidium iodide. A minimum of three assay wells using haemocytes from three independent collections were monitored per time point for each treatment.

In vivo encapsulation assays were conducted by injecting fifth stadium larvae with Dowex 1X-2 beads suspended in physiological saline. A larva was first anesthetized with CO₂ followed by injection of 10-20 beads into the haemocoel using a glass needle mounted on a micromanipulator. Larvae were dissected in physiological saline at prescribed intervals over a 24 hour period. All beads found in the haemocoel of individual larvae were then processed for immunofluorescence microscopy as described previously. A minimum of three larvae were dissected per time point for each treatment. For both in vitro and in vivo assays, capsule formation was determined to have initiated when individual or small clusters of haemocytes were attached to the bead’s surface. A bead was scored as being encapsulated if it was enveloped by a multilayered sheath of cells with a minimum width of 50 µm.

**Encapsulation assays using gradient purified haemocytes**

In vitro encapsulation assays were also conducted using haemocytes purified on Percoll step gradients. Assays were conducted as described previously using either 2.4×10^5 purified granular cells, 1.6×10^5 purified plasmatocytes, or 2.4×10^5 granular cells plus 1.6×10^5 plasmatocytes into Matrigel-coated wells containing Ex-cell 400 medium. Note that the number of purified granular cells and plasmatocytes present in an assay well reflected their relative abundance in an unseparated population of 4.0×10^5 haemocytes (i.e., a mixed population of haemocytes consists of ~60% granular cells and ~40% plasmatocytes). Dowex 1X-2 beads were then added as encapsulation targets. At prescribed intervals over a 24 hour period, individual beads in each assay well were scored for encapsulation. A bead was scored as being encapsulated by the criteria described previously using unseparated haemocytes if it was enveloped by a multilayered sheath of cells with a minimum width of 50 µm. Assays were conducted in triplicate using haemocytes from three independent collections of cells from *P. includens* larvae. A previous study indicated that conjugation of the tetrapeptide Arg-Gly-Asp-Ser (RGDS) to Sepharose-4B beads promoted their encapsulation by plasmatocytes whereas conjugation of RGES to beads did not (Pech and Strand, 1995). Reciprocally, dosage studies indicated that concentrations ≥2.0 mM of RGDS, but not RGES, in culture medium inhibited spreading of plasmatocytes to culture dishes. To further assess the role of RGD-mediated adhesion by haemocytes during encapsulation, the effects of soluble RGDS and RGES peptides (Sigma) on capsule formation was examined by adding peptide concentrations of 3 mM to the culture medium used in certain assays.

**RESULTS**

**Immunofluorescence patterns during capsule formation**

A previous study indicated that mAb 48F2D5 recognized, under denaturing conditions, a 62.3 kDa protein specific to the cytoplasm of granular cells, while mAb 52F3A5 recognized proteins of 120.0 and 140.0 kDa in the cytoplasm of plasmatocytes (Strand and Johnson, 1995). Based on these characteristics, we used these antibodies here to characterize the distribution of granular cells and plasmatocytes in capsules. In vitro assays using unseparated haemocytes indicated that encapsulation occurred in three phases. Individual granular cells began to adhere to Dowex 1X-2 beads after 30 minutes (Fig. 1A,B). By 2 hours, additional granular cells had attached to targets and one another, forming a patchy distribution around the target (Fig. 1C,D). We referred to this period as the initiation phase. During this period no plasmatocytes were detected by staining with mAB 52F3A5.

After 3 hours, plasmatocytes began adhering to granular cells, the surface of the target and one another. Initially, small aggregates of overlapping plasmatocytes attached asymmetrically to one side of the target. As additional plasmatocytes attached to the target and one another, however, a confluent layer of overlapping spread cells ultimately enveloped the target (Fig. 2A,B). Few granular cells were observed in these overlapping layers of plasmatocytes. Capsule formation concluded with what we designated as the termination phase. This occurred in vitro between 16 and 24 hours. The termination phase was characterized by a monolayer of granular cells attaching to the outermost layer of plasmatocytes of the capsule (Fig. 2C,D). Once this layer of granular cells was present, no additional plasmatocytes adhered to the capsule, and capsule diameter ceased to increase. Measurement of 120 encapsulated beads at 18 hours indicated that capsule diameter ranged from 142-520 µm. Vital staining by dye exclusion assay using propidium iodide indicated that approximately 95% of the haemocytes in capsules at 18 hours were viable (data not presented). However, the granular cells that formed the outermost layer of the capsule began staining with propidium iodide at approximately 24 hours, and by 30 hours few haemocytes around the periphery of the capsule were stained by mAb 48F2D5. Instead, a diffuse outer layer, 10-20 µm in diameter, around the periphery of the capsule was stained by mAb 48F2D5 (Fig. 3A). This border subsequently narrowed to 2-7 µm and persisted for the duration of the assay (Fig. 3B).

When we injected Dowex 1X-2 beads into *P. includens* larvae, we found that they were encapsulated in a manner very similar to that we observed in vitro. The initiation phase began within 1 hour of the target being injected into the haemocoel as evidenced by the attachment of granular cells to the surface of the bead (Fig. 3C). Plasmatocytes began to attach to beads after 2 hours and formed a multilayered capsule by 8 hours. The termination phase in turn had occurred by 12 hours when we observed a monolayer of granular cells attached to the periphery of the capsule (Fig. 3D). Individual granular cells were not present around the periphery of capsules collected from larvae after 20 hours. However, a smooth outer layer stained by mAb 48F2D5, identical to what we observed in vitro (see Fig. 3A,B), was present around each capsule.

**Encapsulation of targets by gradient-purified haemocytes**

To determine whether encapsulation by *P. includens* required cooperation between granular cells and plasmatocytes, we conducted in vitro assays using haemocytes that had been purified on Percoll step gradients (Fig. 4). Log-likelihood tests for the homogeneity of replicates indicated no significant differences (P>0.05) between replicates for each treatment; thus, results for each treatment were pooled in subsequent analyses. Overall, the proportion of Dowex 1X-2 beads that was encapsulated differed between treatments (G-test; G=246.1, df=3, P<0.001).
When gradient-purified granular cells were incubated with beads, cells began attaching to targets within 2 hours of their placement in an assay chamber. However, less than 5% of the available targets were fully encapsulated after 24 hours (Fig. 4). Labeling of the beads at this time with mAbs 48F2D7 or 52F3A5 revealed that granular cells were attached to unencapsulated beads in a manner similar to the initiation phase of encapsulation described previously (see Fig 1). However, granular cells did not form overlapping layers of cells around targets as observed during the recruitment phase using unseparated haemocytes. The small number of beads that were encapsulated by the granular cell fraction were actually enveloped by overlapping layers of plasmatocytes that were a low level contaminant (see Materials and Methods). Haemocytes taken directly from the plasmatocyte fraction of gradients encapsulated 33% of available targets after 24 hours (Fig. 4). This value was significantly lower ($G= 8.7, \alpha=0.05$) than the proportion of beads encapsulated by unseparated haemocytes but was well above the proportion of beads encapsulated by granular cells. When we recombined the granular cell and plasmatocyte fractions in a 60:40 ratio that approximated their respective abundance in P. includens, the same proportion of beads was encapsulated as occurred using unseparated haemocytes ($G=2.0, \alpha=0.05$).

**Fig. 1.** Hoffman and fluorescence images of encapsulation in vitro from 30 minutes to 2 hours. Fluorescence images of haemocytes stained with the anti-granular cell antibody mAb 48F2D5 and anti-plasmatocyte antibody mAb 52F3A5. (A) Hoffman image of a bead at 30 minutes with haemocytes attached to its surface. Bar, 20 μm. (B) Immunofluorescence pattern of the same bead after double-labeling for granular cells (gr) and plasmatocytes (red). Only granular cells (gr) are attached to the bead. (C) Bead at 2 hours. Bar, 25 μm. (D) Immunofluorescence pattern after double-labeling for granular cells (green) and plasmatocytes (red). Only granular cells are attached to the bead. The orange-red color of beads in B and D is due to autofluorescence.

**Effects of haemocyte-derived spreading factors on encapsulation by plasmatocytes**

Previous study indicated that medium conditioned by both granular cells and plasmatocytes contains factors that accelerate the rate at which plasmatocytes adhere and spread to cell culture plates (Pech et al., 1994). Based on our observations here that: (1) granular cells attached to targets before plasmatocytes; and (2) purified granular cells plus plasmatocytes encapsulated more beads than plasmatocytes alone, we hypothesized that preincubation of beads in granular cell-conditioned medium would also promote encapsulation by plasmatocytes. Our results indicated that a higher proportion of beads preincubated in granular cell-conditioned medium were encapsulated at 24 hours than beads preincubated in plasmatocyte-conditioned or unconditioned medium ($G=85.3, \text{df}=2, \alpha=0.05$) (Fig. 5). Plasmatocytes began attaching to targets preincubated in granular cell conditioned medium within one hour of their preincubation in granular cell-conditioned medium. This preincubation provides a specific spreading factor that promotes the adhesion and spreading of plasmatocytes on the surface of the bead, thereby facilitating the formation of a robust and complete cellular capsule around the target.

**Fig. 2.** Hoffman and fluorescence images of encapsulation in vitro from 12-20 hours. Fluorescence images of haemocytes stained with the anti-granular cell antibody mAb 48F2D5 and anti-plasmatocyte antibody mAb 52F3A5. (A) Hoffman image of two beads at 12 hours. Bar, 60 μm. (B) Immunofluorescence pattern of the same bead after double-labeling for granular cells (red) and plasmatocytes (green). The capsule is comprised primarily of plasmatocytes. Granular cells attached directly to the surface of the bead are not clearly visible due to orange autofluorescence of the bead itself. (C) Bead at 20 hours. Bar, 25 μm. (D) Immunofluorescence pattern after double-labeling for granular cells (red) and plasmatocytes (green). Plasmatocytes (pl) comprise the core of the capsule, while granular cells (gr) form a monolayer around the periphery of the capsule. The orange color of the bead in B is due to autofluorescence.
Role of granular cells in encapsulation placement in an assay chamber. This was a much more rapid response than was observed using beads preincubated in plasmatocyte-conditioned or unconditioned medium. The proportion of beads from granular cell-conditioned medium that were encapsulated by plasmatocytes after 24 hours was similar to the proportion of beads encapsulated by unseparated haemocytes (see Fig. 4). In contrast, the proportion of beads from plasmatocyte-conditioned medium that was encapsulated was lower than the proportion of beads encapsulated by plasmatocytes alone (Fig. 4). This suggests that factors in plasmatocyte-conditioned medium that bound to beads adversely affected adhesion of plasmatocytes to the target.

Effect of contaminating granular cells on encapsulation by plasmatocytes

Since large numbers of beads were encapsulated by gradient purified plasmatocytes (Fig. 4), we considered the possibility that this occurred because the plasmatocyte fraction was contaminated with granular cells. Therefore, we further reduced the proportion of granular cells in the plasmatocyte fraction using a differential adhesion procedure (see Materials and Methods). To assess the effectiveness of this approach, we placed 2.0×10⁴ cells into culture wells (n=4 independent collections of cells, 3 wells per replicate) and scored 100 randomly selected cells per well by morphology and labeling with mAbs 48F2D5 and 52F3D7. On average 0.7% of the cells in our plasmatocyte fraction after treatment were granular cells compared to 5.4% prior to treatment. When used in our encapsulation assay, only 0.4% of available targets (n=4 replicates, 3 wells per replicate, 150-200 beads per well) were encapsulated by these highly purified plasmatocytes after 24 hours. In contrast, these cells encapsulated 24% of available targets when beads that had been preincubated in granular cell-conditioned medium were used in assays.

RGD-dependence of granular cell opsonization

Since adhesion of plasmatocytes around encapsulation targets involves an RGD-dependent mechanism (Pech and Strand, 1995), we examined whether opsonization of Dowex 1X-2 beads by granular cell conditioned medium was mediated in part by the RGD recognition sequence. Beads preincubated in granular cell-conditioned medium were bioassayed using plasmatocytes from Percoll gradients in the presence of either 3 mM RGDS or 3 mM RGES. Assays conducted in the absence of either peptide served as a positive control. No bead in any replicate was encapsulated in the presence of RGDS. In contrast, the proportion of beads encapsulated in the presence of RGES did not differ from the proportion of beads encapsulated in the absence of peptide (χ²=0.15, df=1, P>0.50) (Fig. 6).

DISCUSSION

The presence of different haemocyte morphotypes in the
assays or preincubation of targets in medium conditioned by
suspension. Both the addition of granular cells to encapsulation
presence of granular cells is an essential component of encap-
the purified granular cells do not encapsulate targets, yet the
In contrast, no circulation of haemocytes occurs in vitro.
be due to the fact that haemocytes passively circulate in the
bloodstream and therefore more readily contact foreign targets.
The similarity of capsule formation has since been articulated
in many reviews on insect cellular immunity (Ratcliffe and
Rowley, 1979; Ratcliffe, 1993). The nature of this protein is unknown but this mAb will
recognize and/or adhere to foreign targets (Schmit and
Ratcliffe, 1977, 1978), thus allowing for its use in further investigating its role during the termi-
nation phase of capsule formation.

Previous ultrastructural studies reported the presence of an
capsules formed by insects has been reported several times in
the literature (Salt, 1970; Akai and Sato, 1973; Schmit and
Ratcliffe, 1977; Wago and Ischikawa, 1979; Kurihara et al.,
1992). Electron microscopic studies of capsules formed by
Galleria mellonella (Schmit and Ratcliffe, 1977, 1978),
prompted the hypothesis that encapsulation depends on release by
granular cells of putative factors that induce plasmatocytes
to recognize and/or adhere to foreign targets (Schmit and
Ratcliffe, 1977; Ratcliffe and Rowley, 1979; Ratcliffe, 1993).
This scenario of capsule formation has since been articulated
in many reviews on insect cellular immunity (Ratcliffe and
Rowley, 1979; Ratcliffe et al., 1985; Davies and Siva-Jothy,
1992; Ratcliffe, 1993), yet to our knowledge has never been
tested experimentally.

Since plasmatocytes and granular cells are the two sub-
classes of haemocytes in P. includens that adhere to foreign
surfaces, the goal of this study was to determine whether
encapsulation requires a cooperative response between
granular cells and plasmatocytes. Immunofluorescence
microscopy of capsules formed in vivo and in vitro indicated
that encapsulation consists of three major phases: (a) an
initiation phase in which granular cells attach to the target; (b)
a recruitment phase in which concentric layers of plasmato-
cytes tightly adhere to one another to form the bulk of the
capsule; and (c) a termination phase in which granular cells
attach to the outermost layer of plasmatocytes forming a
monolayer around the periphery of the capsule. The similarity
in distribution of haemocytes between capsules formed in vivo
and in vitro indicates that our in vitro assay accurately recreates
the process of encapsulation in P. includens. However,
capsules do form more slowly in vitro than in vivo. This could
be due to the fact that haemocytes passively circulate in the
haemocoel and therefore more readily contact foreign targets.
In contrast, no circulation of haemocytes occurs in vitro.

Our in vitro manipulation experiments demonstrated that
purified granular cells do not encapsulate targets, yet the
presence of granular cells is an essential component of encap-
sulation. Both the addition of granular cells to encapsulation
assays or preincubation of targets in medium conditioned by
granular cells promoted adhesion and spreading by plasmato-
cytes to beads. In addition, when granular cell contamination
was reduced to <1% using our differential adhesion assay,
almost no targets were encapsulated by plasmatocytes. We pre-
viously reported (Pech et al., 1994) that gradient purified plas-
matocytes could encapsulate some targets in the absence of
cells from our granular cell fraction. However, results
presented here indicate that this is due to low level contami-
nation of the plasmatocyte fraction by granular cells. Adhesion
of plasmatocytes from P. includens to foreign surfaces involves an RGD-dependent cell adhesion system (Pech and
Strand, 1995). The primary cell surface receptors for RGD-
containing proteins are the integrins, with identified ligands
being primarily extracellular matrix proteins (Mecham, 1991;
Hynes, 1992). Since the capsule-promoting activity of granular
cells was inhibited by soluble RGDS, we suggest the factor(s)
produced by these cells could be an RGD-containing cell
adhesion molecule, cytokines that activate the RGD-dependent
left in the designated timepoints over a 36 hour bioassay period.

Fig. 5. Mean percentage (± s.e.m.) of beads encapsulated in vitro by
gradient purified plasmatocytes. Beads were preincubated in either
granular cell (●) conditioned medium, plasmatocyte (□) conditioned
medium, or unconditioned medium (○; Excell 400). The number of
encapsulated and unencapsulated beads in assay wells was counted at
the designated timepoints over a 36 hour bioassay period.

Fig. 6. Mean percentage (± s.e.m.) of beads encapsulated in vitro by
gradient purified plasmatocytes. Beads were preincubated in granular
cell conditioned medium. Beads were then transferred to assay wells
containing plasmatocytes in Ex-cell 400 medium plus the synthetic
peptide RGES, medium plus the synthetic peptide RGDS or medium
without peptide (opsonized). Twenty four hours after placement in
assay wells, the number of encapsulated and unencapsulated beads
was counted.
outer cellular layer in capsules formed by the moth *Ephesia kuehniella* (Grimstone et al., 1967) and annelid *Nereis diversicolor* (Porchet-Hennere, 1990), and an outer acellular sheath in capsules formed by the cockroach *Blatella germanica* (Han and Gupta, 1989). How this acellular sheath formed is unknown but once present attachment of additional haemocytes to the capsule ceased. More recently, Chain et al. (1992) described three mAbs generated against haemocytes of *Periplaneta americana* that crossreacted with the basement membrane lining the haemocoel and the periphery of capsules.

Given the prominent role ECM components play in cell adhesion generally, we find it notable that studies across several orders of insects indicate an association between haemocytes and formation of basement membranes (Wigglesworth, 1937; 1973; Salt, 1970; Ashurst, 1985; Ball et al., 1987; Chain et al., 1992; Sass et al., 1994). Fogerty et al. (1994) recently demonstrated that tiggrin, a novel ECM protein produced by *Drosophila melanogaster*, contains the RGD sequence and functions as a ligand for the PS2 integrin while Gotwals et al. (1994) demonstrated that laminin functions as a ligand for the PS1 integrin. Both tiggrin and laminin are associated with embryonic haemocytes of *D. melanogaster* but have not been implicated in adhesion events associated with cellular defense responses. Several studies suggest haemocytes produce ECM components present in the basement membrane that lines the insect haemocoel (Wigglesworth, 1973; Ball et al., 1987; Nardi and Miklus, 1989; Sass et al., 1994). Our results combined with those of Chain et al. (1992), however, suggest ECM components play a role in encapsulation. Some ECM components, working through an RGD-dependent mechanism, mediate strong adhesion of plasmatocytes during the recruitment phase, whereas other components produced during the termination phase prevent further attachment of plasmatocytes.

The only factor in insects reported to regulate haemocyte behavior during encapsulation is encapsulation promoting factor (EPF) from *Heterothis virescens* (Ratner and Vinson, 1983; Davies et al., 1988). EPF is a heat stable, low molecular mass (<3 kDa) peptide present in plasma and haemocytes that promotes capsule and nodule formation in vitro. How EPF affects capsule formation in *H. virescens* or precisely which haemocyte types EPF influences is unknown. More recently, a cDNA was sequenced for a 76.0 kDa protein from haemocytes of *Periplaneta* which crossreacted with the basement membrane components in embryonic locusts. This protein, designated peroxinectin, mediates degradation and adhesion of crayfish granular cells, and possesses peroxidase and adhesion domains. Peroxinectin also shares functional and immunological properties with a protein isolated from the insect *Blaberus craniifer* (Rantamaki et al., 1991). Whether similar molecules are involved in capsule formation by *P. includens* is unknown.

The results from this study indicate that encapsulation involves at least two antigenically distinct populations of haemocytes whose interactions result in localized adhesion of cells to a foreign intruder. Trafficking of haemocytes involved in encapsulation parallels studies with mammals where movement of immunocytes mediating both innate and acquired immune responses also involves specific adhesion mechanisms (Gumperz and Parham, 1995; Fearon and Locksley, 1996; Butcher and Picker, 1996). For example, homing of lymphocytes depends on adhesion-related molecules such as the integrins and chemokines bound to specific surfaces. Future studies of the molecules mediating encapsulation will further our understanding of how trafficking of immunocytes in invertebrates and vertebrates are related.

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