EFFECT OF SUBSTRATUM WETTABILITY AND CHARGE ON ADHESION IN VITRO AND ENCAPSULATION IN VIVO BY INSECT HAEMOCYTES

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SUMMARY

The circulating leucocytes of insects, the haemocytes, adhere to and encapsulate foreign material that enters the insect's body cavity. The thickness of the capsule depends not only on the insect species but also on the nature of the object concerned, a fact that is of great importance to invading parasites and pathogens. In this paper, some of the factors that may stimulate haemocyte adhesion and subsequent encapsulation of the object have been investigated using abiotic materials with surfaces of different charge and wettability. The negativity and wettability of surfaces of polystyrene beads and plates can be increased by pretreatment with acid, and adhesion of haemocytes to these modified surfaces has been examined in vivo and in vitro. A similar proportion of haemocytes of the locust Schistocerca gregaria adhere to the plates in vitro, irrespective of the changes in charge and wettability, but the adhesion of haemocytes of the cockroach Periplaneta americana is proportional to the increases in both parameters. These differences in cell behaviour are reflected in vivo: cockroach haemocytes form thicker capsules around more hydrophilic and more negatively charged polystyrene beads, while locust cells encapsulate both types of surface to the same, minimal, degree. Positively and negatively charged Sepharose beads are encapsulated more thickly than are neutral beads in cockroaches; negatively charged Sepharose beads are not encapsulated at all in locusts.

INTRODUCTION

One role played by insect leucocytes is that of the immunological protection of the insect from invading organisms. How the cells, the haemocytes, recognize the foreign object and interact with it and with each other presents some interesting problems, exemplified by the process of encapsulation. Small objects are phagocytosed but objects larger than about 10 μm are encapsulated within layers of tightly adhering haemocytes (Grimstone, Rotheram & Salt, 1967; Salt, 1970; Schmit & Ratcliffe, 1977); the process requires that the object's surface is recognized as 'foreign' and permits haemocyte adhesion, that this initial cell–substratum interaction is followed by recruitment of further haemocytes, which adhere to each other to build up the capsule, and that recruitment eventually ceases. Within a particular insect species capsule information is not an 'all-or-none' process, since the thickness of the capsule varies depending on the object encapsulated. This is important in biological terms, because not all parasites or potential pathogens of an insect will be encapsulated to the
same degree – some may, therefore, evade encapsulation altogether, while others may be able to escape from a thin capsule.

Capsule thickness must depend to a large extent on the strength of the stimulus for recruitment and this must be influenced in some way by the interactions between cell and foreign substratum: different substrata stimulate different degrees of encapsulation. It has been shown that the cockroach *Periplaneta americana* and the locust *Schistocerca gregaria* respond to experimentally implanted biotic materials, such as helminths and tissue transplants, in very different ways (Lackie, 1976, 1979), but the surface properties of these materials are unknown and complex; how they stimulate or prevent recognition can only be guessed. Consequently, in order to examine some of the properties of foreign surfaces that influence recognition and encapsulation a much more carefully defined system is required, such as the use of a non-toxic abiotic material whose surface characteristics can be modified in a more controlled manner. Maroudas (1975) treated the surfaces of polystyrene plates with acid for varying times and reported that the density of negative charges and the wettability of the surfaces varied correspondingly. For this reason, acid-treated polystyrene was chosen as one of the test substrata in this series of experiments, which were designed to investigate the influence of surface charge and hydrophobicity on the adhesion of insect haemocytes *in vitro* and *in vivo*.

**MATERIALS AND METHODS**

**Insects**

Adult male insects were used throughout. *P. americana* were reared and maintained at 25 ± 1 deg. C. The locusts, *S. gregaria*, were obtained as young adults from Bioserv Ltd and were maintained on a daily cycle of 35 ± 1 deg. C for 8 h light, 28 ± 1 deg. C for 16 h dark.

**Haemocyte suspensions**

Insects were anaesthetized with carbon dioxide and chilled at 4 °C; 100–200 μl of a cold calcium/magnesium-free Hanks' solution containing 5 % (w/v) disodium ethylenediamino-tetracetic acid (CMF–EDTA), pH 7·2, were injected into the abdominal cavity of each insect and the diluted blood was withdrawn into a cold Pasteur pipette from the stump of a severed hind-limb. Blood from 6–10 insects was pooled in a test-tube kept on ice, then centrifuged at 500 g for 5 min; the pellet was resuspended gently in CMF–EDTA, centrifuged once more and finally resuspended to the required density in either cold Carlson's saline, pH 7·2 (Burton, 1975), for cockroaches, or cold Hoyle's saline, pH 7·0 (Burton, 1975), for locusts.

**In vitro adhesion to polystyrene**

Bacteriological grade (BG) polystyrene dishes, 4 cm diameter (Sterilin), were treated with Analar concentrated sulphuric acid at 37 °C for times ranging from 0 to 5 min. After treatment, the dishes were washed frequently in tap water followed by distilled water, and allowed to dry.

Stainless steel cloning rings, internal diameter 6 mm, were stuck to the dry polystyrene surfaces with a small amount of silicone grease; two chambers were set up for each test surface, on separate plates. A 0·2 ml sample of the haemocyte suspension was added to each of these chambers, which were left at room temperature for 75 min. At the end of this time each cloning ring was carefully removed and the plates were washed to remove non-adherent cells by dipping 10 times in Carlson's saline before fixing in 10 % formalin (in Carlson's saline). The plaques of adherent cells were stained in Haemalum and mounted in Farrant's aqueous mounting medium. The number of adherent cells
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was counted using a 10 X 10 eyepiece grid; at least six grids were counted per chamber and the total number of adherent cells per chamber was calculated.

Beads for in vivo encapsulation assays

Sulphuric acid-treated beads. These polystyrene beads, a gift from GIBCO, were approximately 200-300 \( \mu \text{m} \) in diameter. The beads were cleaned in a 1 % solution of sodium lauryl sulphate, washed thoroughly and air-dried before being treated with concentrated sulphuric acid at 37 °C for either 10 s or 90 s. At the end of the treatment, the beads were again washed thoroughly. Because of the difficulty of handling the beads during these treatments, they were confined between two loosely packed plugs of glass wool in a Pasteur pipette, attached by polythene tubing to a 10 ml disposable syringe; fluid could thus be rapidly sucked into or expelled from the pipette.

Sepharose beads. Beads used were DEAE-Sepharose-6B-CL (weakly positive), Sepharose-6B-CL (neutral) and CM-Sepharose (weakly negative). All beads, which were stored at 4°C in a neutral buffer to which a small amount of sodium azide had been added as a bacteriostat, were washed three times in Carlson's saline immediately before use. The suspensions were filtered through clean Nitex filter (mesh size 100 \( \mu \text{m} \)) and the beads retained by the filter (100-150 \( \mu \text{m} \) diameter) were used for the injections.

Injection procedure and measurement of capsule thickness

Samples of 20-30 polystyrene beads or 30-40 Sepharose beads in approximately 30 \( \mu \text{l} \) of Carlson's saline were injected via a sharpened glass micropipette (Lackie, 1976) laterally into the abdomen of CO2-anaesthetized insects. The insects were returned to their respective constant-temperature rooms, and were killed 24 h later and dissected in Carlson's saline. Eight to ten insects were used in each experiment.

Recovered beads were fixed in 2-5 % glutaraldehyde in 0.1 M-phosphate buffer. Capsule thickness was measured under phase-contrast using an eyepiece micrometer in the shape of a graduated cross. Since the capsule was not always of uniform thickness around each bead, four measurements were made at different points on the circumference and the values averaged for each capsule. Data were analysed using the Mann–Witney \( U \)-test.

RESULTS

Sulphuric acid treatment of polystyrene

Treatment of polystyrene with concentrated sulphuric acid is known to alter the state of the surface although the exact mechanism of the alteration is still subject to much debate. Maroudas (1975) showed that acid-treated surfaces bound increased amounts of the dye crystal violet and interpreted this as indicating an increase in negative charge density on the plate due to sulphonation. Crystal violet treatment of the commercial polystyrene plates used in these experiments with insect cells also revealed increased dye binding by treated plates (Table 1).

Some preliminary measurements of zeta potentials and wettability of the polystyrene surfaces were made by D. P. Gregory and J. Mingins (Unilever Research, Port Sunlight). The zeta potential of polystyrene plates was measured using a probe particle electrophoresis technique recently described by Gregory (1981), together with an analysis according to Komagata (1933). Advancing and receding contact angles (\( \theta_A \) and \( \theta_R \)) were measured on sessile drops of water on plates, using a goniometric method (Bigelow, Pickett & Zisman, 1946), accurate to ±2° at all but low angles (<15°). Angles less than 10° could not be distinguished from zero. Contact angles on the less-accessible bead surfaces were obtained using microphotography on
Table 1. Dye-binding by acid-pretreated polystyrene

<table>
<thead>
<tr>
<th>Acid pretreatment time</th>
<th>Amount dye bound (o.d.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plates</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0.074, 0.056</td>
</tr>
<tr>
<td>1 min</td>
<td>0.245, 0.203</td>
</tr>
<tr>
<td>5 min</td>
<td>0.370, 0.450</td>
</tr>
<tr>
<td>Beads</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0.095</td>
</tr>
<tr>
<td>30 s</td>
<td>0.105</td>
</tr>
<tr>
<td>5 min</td>
<td>0.240</td>
</tr>
</tbody>
</table>

Bound dye was eluted from the plates and beads with acid/ethanol, and o.d. was measured at 590 nm.

Table 2. Changes in wettability (contact angles) (A), and in electroendosmotic potentials and derived net wall charge densities (B), on treated polystyrene

<table>
<thead>
<tr>
<th>Duration of acid pretreatment</th>
<th>Plates</th>
<th>Beads</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>30 s</td>
</tr>
<tr>
<td>A Contact angle (θ)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Advancing</td>
<td>93–94</td>
<td>71–72</td>
</tr>
<tr>
<td>Receding</td>
<td>86–87</td>
<td>~10–13</td>
</tr>
<tr>
<td>B Zeta potential (mV)</td>
<td>-35</td>
<td>-52</td>
</tr>
<tr>
<td>A²/unit electron charge</td>
<td>1800</td>
<td>1100</td>
</tr>
<tr>
<td>Charge density, σ (μC/cm²)</td>
<td>-2.6</td>
<td>-4.3</td>
</tr>
<tr>
<td>Δσ</td>
<td>0</td>
<td>-1.7</td>
</tr>
</tbody>
</table>

settled beads in a pendant drop and the analysis of Mingins & Scheludko (1979). This method gives only the receding angle (θR) and is accurate to ±2°. The results are shown in Table 2.

Treatment with sulphuric acid has a marked effect on the contact angle for both beads and plates: the surfaces became increasingly wettable. There is also a marked increase in hysteresis (θA–θR is not zero), which could arise from chemical or topographical heterogeneities of the surface. Scanning electron microscopy of the surfaces of untreated beads and of beads treated for 5 min with acid revealed no obvious change in the otherwise smooth surfaces of the beads, so it is more likely that acid treatment is causing chemical changes in the surface.

The changes in the zeta potential are less substantial than those in the contact angle. The net charge densities at the wall can be calculated from Gouy–Chapman double-layer theory (Verwey & Overbeek, 1947), if it is assumed that the zeta potential corresponds to the diffuse-layer potential. The net charge densities on untreated polystyrene, thus calculated, are low and negative, typically approximately 2–3 μcoulomb/cm² in 10⁻² M-sodium chloride solution.
Table 3. *Cells adhering per chamber, as % of total number of cells administered*  

<table>
<thead>
<tr>
<th>Duration of acid treatment</th>
<th>Periplaneta</th>
<th>Schistocerca</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (BG)</td>
<td>25±2±7.3</td>
<td>22.8±3.6</td>
</tr>
<tr>
<td>5s</td>
<td>33±5±7.5</td>
<td>24.3±3.2</td>
</tr>
<tr>
<td>30s</td>
<td>39±5±15.3</td>
<td>25.6±1.8</td>
</tr>
<tr>
<td>1 min</td>
<td>44±1±12.6</td>
<td>24.0±2.3</td>
</tr>
<tr>
<td>5 min</td>
<td>62±3±8.7</td>
<td>25.3±4.3</td>
</tr>
</tbody>
</table>

Values represent mean ± S.D. of results from three experiments (see Materials and Methods).

Treatment with sulphuric acid for 30 s and 5 min increases the net negative charge to 4.3 and 5.6 μC/cm², respectively. Although this represents a doubling of the charge density of the wall, the charge densities themselves are relatively low.

In *vitro* adhesion of haemocytes to sulphuric acid-treated polystyrene plates

The results, expressed as percentage of administered cells that adhere, are shown in Table 3. It is immediately apparent that there are marked differences between the two insect species in the way in which their haemocytes behave on the different surfaces. On untreated plates, *Periplaneta* cells tended to clump and adhere to each other, in preference to the polystyrene surface, and very little spreading was noted. In contrast, on the plates that had been acid-treated for 5 min, the cells were more evenly distributed and the majority were well spread.

Locust cells appeared to spread well on all of the surfaces tested and the same proportion of the administered cells adhered irrespective of the duration of the pretreatment of the surface with acid.

In *vivo* encapsulation of acid-treated polystyrene beads

Although all beads were initially washed in detergent and distilled water, otherwise untreated beads tended to clump and trap air-bubbles when suspended in Carlson's saline for injection. Apart from the difficulty of injecting these beads as a suspension, it has been found that air bubbles are themselves encapsulated by insect haemocytes *in vitro* (personal observation; and Salt, 1970); consequently, untreated beads were not used in this series of experiments.

The thickness of the haemocytic capsules around the acid-treated beads, 24–30 h after implantation into the insects, is shown in Fig. 1.

In *Periplaneta*, capsules were significantly thicker around the beads that had been acid-treated for a longer time. In *Schistocerca*, however, the different surface properties of the two classes of bead had no apparent effect on the subsequent cellular encapsulation: approximately 10–15 % of the beads were found to be unencapsulated at the end of the experiment (30 h) and there were only patchy clusters of cells around approximately half of the beads.
In vivo encapsulation of Sepharose beads

Not only do the differently charged beads provoke different degrees of encapsulation within one species, but the ranking of the response is different between the species. Data from each experiment were compared and, since the differences between the types of bead were of the same order and significance, all data were pooled for analysis (Fig. 2).

In locusts, the weakly negative beads remained unencapsulated even at 9 days post-injection.

The weakly positive beads were thickly encapsulated within cockroaches, and by 24 h were easily recognizable due to a dark brown melanized core of necrotic haemocytes next to the bead surface. However, a small proportion of these beads remained unencapsulated by cells but turned dark brown themselves, presumably due to the deposition and infiltration of melanin into the bead.
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Periplaneta

Capsule thickness (μm)
median (95% confidence limits)

DEAE (positive)

n = 52
39.6 (36.0–48.0)

U = 8.68
P < 0.001

CM (negative)

n = 155
32.4 (24.0–36.0)

U = 7.55
P < 0.001

Schistocerca

n = 125
12.0 (9.0–12.0)

U = 8.14
P < 0.001

6B (neutral)

n = 180
21.6 (16.8–25.2)

U = 7.55
P < 0.001

n = 71
21.6 (20.4–24.0)

n = 92
0

Fig. 2. The thickness of haemocytic capsules formed around Sepharose beads after 24 h in vivo (pooled results from three experiments for Periplaneta and from two experiments for Schistocerca).

DISCUSSION

It is immediately apparent that the haemocytes of Periplaneta and Schistocerca respond very differently, both in vitro and in vivo, to the surfaces tested. The question is, to what are they responding?

Maroudas (1975) detected anionic changes in acid-treated polystyrene using the dye crystal violet, and interpreted these changes as being due to sulphonation; increased dye-binding was also found in this investigation. However, measurements of the surface zeta potential by Gingell & Vince (1982) for pure polystyrene, and by Drs Gregory and Mingins for the commercial polystyrene used here, show that the changes in negativity are relatively small, indicating that little or no sulphonation has taken place. Crystal violet, being a triphenolmethane dye, is also capable of hydrogen bonding (Coulson, 1959), and results of recent work by Curtis (personal communication) suggest that acid treatment may instead increase the number of hydroxyl groups on the surface. The increase in contact-angle hysteresis with acid treatment reported here and by Gingell & Vince (1982) may be due to an increase in roughness of the surface, which would increase the available surface area to which dye could bind.
However, scanning electron microscopy revealed no obvious changes in the surfaces of acid-treated compared with untreated beads. It is thus apparent that the changes induced in polystyrene surfaces by sulphuric acid are fairly complex; they have been investigated in greater detail by Curtis, Forrester, McInnes & Lawrie (1983). For the purposes of this investigation, it is sufficient to note the increase in wettability and the small increase in negative-charge density of the acid-treated polystyrene.

In their unmodified form, Sepharose beads may contain a very small number of sulphate and carboxyl groups; the negative and positive charges on the CM and DEAE beads are conferred by carboxyl and amino groups, respectively. Sepharose beads are prepared from agarose, and since the serum agglutinins of insects are frequently lectin-like, it might be argued that haemocyte adhesion to the bead could be mediated through the putative opsonic activity of the agglutinins. However, the agglutinating activities of Periplaneta and Schistocerca sera are not inhibited by D-glucose or D-galactose (Lackie, 1981b), so the influence of agglutinins on encapsulation of unmodified Sepharose is unlikely to be important.

It is apparent that there is a close correspondence between the in vivo and the in vitro 'attractiveness' of acid-treated polystyrene for haemocytes, but can the difference in capsule thickness in vivo be equated to the differences in the proportion of cells adhering in vitro? It was argued earlier that the interaction between the haemocytes and foreign substratum in vivo must influence the subsequent recruitment of haemocytes to the capsule; if the first haemocytes to contact the foreign surface were maximally stimulated they might release greater quantities of 'recruiting factors' than if they were only slightly stimulated. The nature of this stimulation is unknown, but it is a plausible hypothesis that a surface to which cells can more readily adhere could induce greater changes in the adhering cells, leading to greater recruitment (Lackie, 1981a). Certainly, the proportion of adherent Periplaneta haemocytes that were widely spread increased with the wettability of the polystyrene substratum in vitro.

Haemocytes are not a homogeneous population; in cockroaches and locusts they comprise three major cell types: the plasmatocytes and granulocytes, which are involved in capsule formation in vivo and of which the plasmatocytes, in particular, adhere to glass in vitro; and the coagulocytes, which lyse and cause local plasma coagulation when in contact with foreign surfaces in vivo (Ratcliffe & Rowley, 1979) or when blood is extracted without the use of chelating agents such as EDTA. It has been suggested, on the basis of transmission electron microscopy, that coagulocyte lysis upon contact with foreign surfaces in vivo stimulates recruitment of plasmatocytes and granulocytes to the capsule (Schmit & Ratcliffe, 1977). These three cell types are certainly found in capsules around Sepharose beads injected into Periplaneta and Schistocerca (Lackie, Tetley & Takle, unpublished data), although it is by no means certain in which sequence they are recruited in vivo. In the in vitro assays for adhesion, not only is there some loss of coagulocytes by lysis during preparation of the cell suspension, but the cells contact the substratum in the sequence in which they settle out of suspension; the predominant adherent cell is the plasmatocyte.
Despite these difficulties in comparing the results of the in vivo and in vitro experiments, it is readily apparent that both the charge and the wettability of the substratum influence the extent of the cellular responses. The plasmatocytes, which are highly phagocytic, adhere more readily in vitro to more hydrophilic substrata, an observation that is in direct contrast to that of van Oss & Gillman (1972) for mammalian phagocytic leucocytes, but in agreement with results for chick heart fibroblasts (Maroudas, 1975) and results for Dictyostelium amoebae (Gingell & Vince, 1982).

The good response of cockroach haemocytes to negatively charged beads in vivo contrasts with the situation in caterpillars of the boll-worm Heliothis (Vinson, 1974); Vinson found that – as reported here for Schistocerca – weakly positive and neutral Sephadex beads were encapsulated but weakly negative beads were not. Negatively charged beads were not adhered to in vitro by haemocytes of silkworm larvae (Walters & Williams, 1967) or of spruce budworm larvae (Choristoneura fumiferana) (Dunphy & Nolan, 1982).

The absence of cellular adhesion to the negative CM-Sepharose beads and the very weak response to the acid-treated polystyrene beads by haemocytes of the locust could have several explanations: the beads may adsorb certain plasma components so that they become disguised as 'self'; or, the surfaces of these beads already appear similar to 'self' and are thus not recognized. Obviously, in this latter case, discrimination between self and not-self must be based on rather crude comparisons between the range of charge and wettability of self tissue surfaces and that of the object. The locust is particularly interesting in this respect because its haemocytes apparently do not recognize a wide range of biotic and abiotic materials (reviewed by Lackie, 1981a) and therefore the range of surfaces that are considered as self, and therefore not reacted against, is obviously quite wide. It should be possible theoretically to find a surface whose negative charge lies somewhere between that of Sepharose-6B and CM-Sepharose, which 'switches on' the encapsulation response. The surface properties of the acid-treated polystyrene beads clearly lie somewhere close to this threshold.

Whether the insect's immune response is stimulated by direct recognition of the physicochemical properties of the foreign surface by the haemocytes, or by recognition of sterically altered plasma proteins that have attached to the surface, is not yet understood. What is apparent, however, is that abiotic surfaces with relatively simple – compared to living material – modifications in the surface properties can influence the degree of haemocyte adhesion and the extent of the cellular response.

I am very grateful to Dr D. Gregory and Dr J. Mingins at Unilever's Port Sunlight Laboratory for their measurements of wettability, zeta potential and calculations of surface charge of the polystyrene beads and plates. I am also grateful to Professor A. S. G. Curtis of the Department of Cell Biology in this University for discussions about the effects of treating polystyrene with acid. I thank Dr J. M. Lackie, Department of Cell Biology, for carrying out the assay for Table 1.

REFERENCES


A. M. Lackie


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