LIMPET HAEMOCYTES
I. STUDIES ON AGGREGATION AND SPIKE FORMATION

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SUMMARY
In this paper a promising new spontaneously aggregating system of amoebocytes from the blood of limpets is described. The system has the advantage of being readily available without cell culture and of being operational at room temperature.

The blood of the limpet *Patella* contains 2 types of haemocytes, amoebocytes and macrophages, the latter accounting for less than 1% of the cell population. The concentration of cells in suspension varies with the body temperature of the animal, from about $1 \times 10^6$ cells/ml at $5 \, ^\circ\text{C}$ to $9 \times 10^6$ cells/ml at $25 \, ^\circ\text{C}$.

Following blood withdrawal the amoebocytes are approximately spherical in shape with superficial convoluted lamellae, but over a period of 5 min develop stout marginal spikes. This development is temperature dependent and independent of divalent cations in the medium.

Most interestingly, and apparently associated with the stimulus of blood withdrawal, the cells become spontaneously adhesive and commence an extremely rapid aggregation. With a shaking speed of 700 strokes/min the half time of aggregation varies from 15 s at an initial cell density of $4.7 \times 10^6$ cells/ml to 60 s at a density of $1.4 \times 10^6$ cells/ml. The aggregates so formed are very resistant to mechanical dispersion and tight junctions have been observed between aggregated cells in electron micrographs. Complete aggregation requires the presence of divalent cations and another unidentified plasma component. Aggregation will occur at $0 \, ^\circ\text{C}$ but the process is slower and less complete than at $20 \, ^\circ\text{C}$. It is suggested that aggregation involves the triggering or activation of a pre-synthesized adhesive system and that the adhesiveness per unit area increases with increase in temperature.

INTRODUCTION
This paper is the first in a series, describing the general features of a new rapid aggregating system of amoebocytes from an invertebrate source. Our interest in the haemocytes of the limpet *Patella vulgata* was aroused by 2 observations: (1) Blood removed from limpets kept at $5 \, ^\circ\text{C}$ contained about $1 \times 10^6$ cells/ml but 4 h after transfer of the animal to water at $25 \, ^\circ\text{C}$, the cell population had risen to about $9 \times 10^6$ cells/ml. The haemocyte population fell as rapidly when the animals were returned to $5 \, ^\circ\text{C}$. (2) Immediately following removal from the animal, the haemocytes became spontaneously adhesive and commenced rapid aggregation. With gentle agitation, aggregation appeared to be complete in under 1 min.

Remarkably little appears to be known of invertebrate cell behaviour and this study was undertaken to obtain the necessary background data for a study in depth on what appears to be a promising new system for the study of aggregation, and cell movement.

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One of the advantages of this system is that the material is readily obtainable in reasonable quantity without the necessity for cell culture and sterile technique, and observations on cell movements may be made at normal room temperatures.

In this paper we describe the aggregation of the cells in suspension, and in subsequent communications the characteristics of movement when spread on a glass surface (Partridge & Davies, in preparation), and cell ultrastructure will be described.

**MATERIALS AND METHODS**

Limpets were obtained from the rocky shores of the Firth of Clyde and kept for up to a week in aerated seawater at 10 °C. In order to obtain a high cell density in the blood sample, the animals were kept overnight in aerated seawater at 20 °C before sampling their blood. The blood was aspirated directly from the pallial vein at the point where it curved around the edge of the foot at the anterior end, using a 21- or 25-gauge hypodermic needle connected to glass tubing (Fig. 3). Up to 3 ml of blood could be obtained from very large specimens whilst medium-sized animals of 30-40 mm shell length yielded 1 ml or more. The blood does not clot, and the suspension of cells obtained at withdrawal may be used directly.

Work by Webber & Dehnel (1968) has shown that in the related genus *Acmaea*, the ionic composition of the blood is not very different from seawater, and we have therefore used membrane-filtered seawater as a suspending medium in some experiments. Calcium- and magnesium-free seawater (CMF) was made with the composition, in g per l.: KCl, 0.725; NaCl, 27; Na₂SO₄, 3.9 (Robertson & Webb, 1939). In addition, EDTA seawater comprising, in g per l.: KCl, 0.725; NaCl, 18.22; Na₂SO₄, 3.9; and disodium EDTA, 37.24 (0.1 M), adjusted to pH 8.2 was made, to chelate all calcium and magnesium ions upon addition to an equal volume of blood.

**RESULTS**

**Cell structure**

Examination by phase-contrast microscopy of a drop of blood from which the cells have been allowed to settle on a glass slide, reveals the presence of 2 cell types.

The majority of the cells are 10–20 × 25–35 µm in transverse dimensions with a typical ‘holly leaf’ outline (Fig. 4). Following observations on their locomotion (Partridge & Davies, in preparation) we have termed these amoebocytes. Spikes of various lengths and thicknesses project radially in the plane of the substrate from deep in the cytoplasm. They are united along much of their proximal portions into one or more groups by a thin lamellar extension of the cell. The cell margin is thus disposed in a series of concave curves between the spikes. There is a small clear nucleus containing a variable number of nucleoli, a dense juxtanuclear Golgi region, occasionally a few small clear spherical vacuoles and a variable number of smaller dark vesicles, some of which show the lysosomal property of concentrating acridine orange to fluoresce red under ultraviolet irradiation. Gomori staining reveals a trace of acid phosphatase which is probably located in these dark vesicles. Most of the cytoplasm is of even density and large quantities of glycogen have been identified in electron micrographs.

A second form, which we have termed macrophages because of superficial resemblance to vertebrate macrophages, constitute 1% or less of the blood cell population.
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They are more or less circular when spread with a diameter of 30–50 μm. The cell margin is usually relatively smooth and these cells lack the long spikes typical of amoebocytes even when the margin is fimbriate (Fig. 5). They contain groups of large highly refractile vacuoles or granules placed around or to one side of the nucleus, and these stain strongly by the Gomori technique and concentrate acridine orange. This, however, is the only evidence available indicating probable phagocytic or pinocytotic activity on their part. The nuclei are usually larger and denser than those of amoebocytes.

It is possible that these 2 forms represent different stages or functional responses of a single cell type, but no evidence in support of this view was found and no intermediate cell types were seen. However, since they exhibit differences in behaviour as well as in appearance, they will be treated as 2 distinct cell types.

Characteristics of amoebocytes in suspension

Spike formation. Immediately after withdrawal from the limpet, the amoebocytes are roughly circular in outline, and about 10 μm in diameter. Within 1 min the cells develop large surface spikes, presumably in response to some stimulus associated with withdrawal of the blood. In order to follow the sequence involved, a sample of blood was withdrawn and 1 drop immediately expressed into glutaraldehyde fixative; further drops were expressed into fixative at intervals of 10 s. Those cells fixed immediately on withdrawal are devoid of spikes but bear smooth-edged flattened projections which sometimes appear convoluted (Fig. 6). The presence of convoluted lamellae has been confirmed by electron microscopy (Elder & Davies, unpublished). By 30 s the first signs of spikes are apparent at the edges of the projections (Fig. 6) and they then grow progressively to a final maximum length of 10–15 μm over the next 3 min (Fig. 8). There is also an increase in number of spikes over this period. Some appear as single structures and some united with their neighbours by a membranous web. If the formation of the spikes as observed, is a result of metabolic activity or assembly, it may be expected that the rate of appearance following blood withdrawal would be temperature sensitive. To test this a limpet was cooled in seawater at 0 °C for 5 min. Following withdrawal, the blood was divided into 2 batches, and one maintained at 0 °C whilst the other was rapidly warmed to 20 °C. Aliquots were withdrawn at intervals and fixed as before. It was found that the 20 °C series corresponded well with the sequence described above. The 0 °C series on the other hand, displayed a significant retardation of the rate of spike appearance and 8–12 min elapsed before these reached a final size and appearance corresponding to the 20 °C series. From examination of micrographs of cells fixed at intervals at intermediate temperatures, the Q₁₀ for spike formation is approximately 2.

Development and maintenance of the spikes and associated membranes is not dependent on the presence of divalent cations, since they develop normally in blood withdrawn into an equal quantity of EDTA seawater, and persist for several hours in this medium. Attempts to inhibit or reverse spike formation by addition of colchicine at concentrations of 50 μg/ml to freshly drawn blood, also gave negative results.

Aggregation. On withdrawal from a limpet which has been kept at 20 °C, the blood
is more or less opalescent due to the concentration of cells. If, however, the blood is allowed to stand for a few minutes, or if it is agitated for a shorter period, the suspension flocculates to form large white aggregates in clear plasma.

A qualitatively similar process occurs if the fresh blood is added to an equal quantity of seawater, but is completely inhibited if it is mixed with an equal quantity of EDTA seawater. Such EDTA-blood suspensions consist entirely of single cells and show no sign of flocculation over several hours. The cell pellet obtained by centrifugation of EDTA-blood suspensions can be redispersed in CMF seawater as a stable single-cell suspension. Redispersion in normal seawater also gives a single cell suspension initially, followed by flocculation to form small 2-6 cell aggregates and a number of single cells.

Redispersion of the pellet in cell-free plasma results in the same aggregation pattern as in freshly drawn blood: large aggregates are formed with few single cells remaining. In an attempt to evaluate the roles of divalent cations and plasma in the aggregation process, the experiment scheduled in Fig. 1 was carried out.

Five samples were then removed from each tube to a haemocytometer and the number of particles (Curtis & Greaves, 1965) counted. The mean counts (0.01-μl sample) ± standard error (with EDTA seawater tube corrected for dilution) were: plasma, 4.0 ± 0.9; 50% plasma-50% seawater, 6.8 ± 1.1; seawater, 12.7 ± 1.2; EDTA seawater, 117.5 ± 7.2 (single cells).

It is thus evident that divalent cations play a critical role in aggregation. However, the greatly diminished number of particles present in the plasma tube indicates that there is a further aggregation-stimulating factor present in the blood.

Since the surface of the amoebocytes is thrown out in spike formation shortly after withdrawal, it is likely that initial contact between cells will occur at the distal end of the spikes. We have shown earlier that the rate of appearance of the spikes is tempera-
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Fig. 2. Effect of temperature and initial cell density on the adhesion of amoebocytes in suspension in blood, immediately after withdrawal. The tubes containing the suspension were shaken at 700 strokes/min. A, Initial density $4.7 \times 10^6$ cells/ml. B, Initial density $1.4 \times 10^6$ cells/ml. •, at 5 °C; ■, at 20 °C; at arrow the tubes at 5 °C were transferred to and shaken in a waterbath at 20 °C.

ature dependent and it was of interest therefore to determine the effect of temperature upon rate of aggregation.

A limpet was cooled to 0 °C by immersion for 5 min in seawater at 0 °C. Approximately 1 ml of blood was withdrawn into a chilled pipette and equal volumes were added to each of 2 test tubes, clamped in a shaker so that one was immersed in a 0 °C waterbath and the other in a 20 °C waterbath. Zero-time samples were taken as quickly as possible from each tube with a 10-μl Ependorf pipette, and the shaker started with a speed of 700 strokes/min. Further samples were taken from each tube alternately at 15-s intervals for 3 min and thereafter at 1-min intervals. The 10-μl samples were added immediately to 20 μl glutaraldehyde fixative and counted in a haemocytometer for the total particle concentration (Curtis & Greaves, 1965). Using this procedure, it was found that the particle concentration in the 20 °C tube fell considerably in the initial few seconds between adding the blood and taking the zero-time sample. The procedure was changed, therefore, so that both tubes were kept at 0 °C until the zero-time samples were taken, when one was transferred to the 20 °C
bath and shaking begun. Samples were removed at the same intervals as before, and after 5 min the 0 °C tube was transferred to the 20 °C bath and samples removed from it at intervals of 1 min. Fig. 2 shows the progress of aggregation, as indicated by the decline in total particle concentration, in 2 experiments with different initial cell densities. These 2 graphs are representative of a number (8) obtained for this experiment and the main points are consistent within the series.

It is remarkable that even at low initial cell densities the aggregation process has virtually stopped at both temperatures within 2 min. Aggregation is both more rapid and more complete at the higher temperature, but the half times for flocculation at the 2 temperatures are very similar at each initial density: about 15 s from a starting density of 4.7 x 10^6 cells/ml and 60 s at 1.4 x 10^6 cells/ml. This indicates that difference in collision rate at the 2 temperatures is not a significant factor in determining the difference in rate of aggregation. The faster aggregation at 20 °C and the more complete final state of aggregation at this temperature are, therefore, probably attributable to a higher adhesiveness between contacting cells, leading to a higher collision efficiency and a greater resistance of aggregates to disruption by shear forces.

At 0 °C the curves plateau at about half the initial particle density, which represents the formation of small aggregates of from 2 to 4 cells. This may be the largest size able to resist disruption in the shaker system. Certainly the limitation of aggregate size at low temperatures is a property of the whole shaker system rather than of 0 °C blood, which will eventually form very large aggregates if not agitated.

The high initial particle density situation in Fig. 2 A emphasizes the rapidity of the response of cells to increased temperature; the higher rate of aggregation is evident within 1.5 min of transference to the 20 °C bath, when the temperature of the blood can have risen by only a few degrees.

From the graph (Fig. 2 A) there appears to be a gradual increase in the particle number in the 0 °C tube following the initial decline, presumably reflecting a break up of aggregates. This was not always an obvious feature of these experiments, but when it occurred it was a real phenomenon.

Within 1—1.5 min of moving the 0 °C tube to the 20 °C bath, maximal aggregation had occurred, demonstrating that no irreversible change has occurred to prevent aggregation at 0 °C.

Aggregates formed at 20 °C are very resistant to mechanical dispersion: an hour of rapid shaking, or prolonged vigorous pipetting does not cause significant visible breakdown of aggregates or release of single cells. Excess of EDTA seawater causes rapid reversal of the early stages of aggregation but permits redispersion of large aggregates only very slowly, 5—10 min of vigorous pipetting being required. This resistance may be due to the limited access of EDTA to the extensive areas of cell—cell contact bounding 20-nm gaps in these aggregates (Elder & Davies, unpublished), or to the development, with prolonged contact, of a less divalent cation-dependent adhesive structure, e.g. a tight junction (Benedetti & Emmelot, 1967). Large aggregates formed at 0 °C also seem to be resistant to mechanical dispersion, which, in view of their apparent early instability, also suggest that stronger adhesions are formed with prolongation of contact.
DISCUSSION

The spikes which the amoebocytes develop appear to be a result of a stimulus associated with the removal of blood from the haemocoel. Amoebocytes fixed in situ within the mantle tissue of limpets do not bear them and appear roughly spherical, bearing convoluted smooth-edged lamellae. However, the nature of the stimulus remains completely obscure because we were not able to vary the treatment or mode of withdrawal of the blood so as to prevent spike formation.

The stiff and extensive nature of the spikes and their role in cell spreading and movement (Partridge & Davies, in preparation) suggest that they are particularly well developed versions of the microspikes or filopodia which may be observed on a number of cells (Follett & Goldman, 1970). It was found that spikes were developed and maintained at 0 °C and also in a medium containing 50 μg/ml of colchicine. This argues against the participation of microtubular structures (see Taylor, 1966) but does not entirely preclude them. Further investigations using electron microscopy are being conducted into the nature of these structures.

Aggregation is a second consequence of the withdrawal of blood from the haemocoel and, as with the formation of spikes, the nature of the stimulus is not known. The dependence of aggregation on divalent cations is common to many cell aggregation systems, and the importance of a plasma factor in the process is perhaps comparable to the role of ADP in mammalian platelet aggregation (Born, 1962) or of other homologous serum factors in some vertebrate thrombocyte systems (Belamerick, Shepro, Kien & Fulton, 1967), except that the limpet amoebocytes appear to be more spontaneously adhesive.

Spiky projections of the cells have been shown to precede aggregation initiated by some stimulatory agents in the platelet system (Born, 1962) and in the aggregating slime mould system (Garrod & Born, 1971). However, in the limpet amoebocyte system there is no evidence that spike production and aggregation are causally related. At 20 °C, aggregation is well advanced within 15-30 s and spike formation is barely evident by the end of this period. Moreover, the slow development of spikes at 0 °C shows no correspondence to the pattern of aggregation at this temperature, in which the plateau level is attained within the first minute or two. As with platelets (Born, 1970) and slime mould cells (Garrod & Born, 1971) aggregation is not an inevitable consequence of spike formation, as demonstrated by the fact that spikes develop normally in the presence of concentrations of EDTA which completely inhibit aggregation.

The speed of the aggregation response of amoebocytes to a rise in temperature is interesting. It seems unlikely that intercellular cement, as proposed for chick cells by Moscona (1961a, b) and for sponge cells by Moscona (1963, 1968) and Humphreys (1963), could be synthesized in significant amounts during the 15-30 s in which marked differences are developed between the states of aggregation of cells at 0 °C and 20 °C. The situation is more easily explained in terms of the triggering or activation of a pre-synthesized system. A similar conclusion is reached by Garrod & Born (1971) with respect to aggregating slime mould cells, which form weak adhesions in the cold.
and strong ones at 24 °C. These authors suggest that the increased adhesiveness between warm cells can be attributed, at least in part, to their increased motile activity and propose that contraction of the microspikes which form the initial intercellular contacts results in increased area of contact between adhesive cell surfaces. Under the microscope we have observed the process of pulling together of limpet amoebocytes, after they have made initial contact by their spikes, both during the aggregation of spread cells from freshly drawn blood and also on unspread cells following replacement of EDTA seawater by normal seawater (Partridge & Davies, in preparation).

The time taken was from 1 to 3 min. Obviously this process could not effectively stabilize the adhesions which develop within a few seconds of collision as observed in the aggregation preparation which was shaken at 20 °C. This would suggest that warm cells may possess a higher adhesiveness per unit area than cold cells, in addition to increased mobility. On the other hand, the lamellar web between the spikes seems to play a major role in the spreading and movement of these cells (Partridge & Davies, in preparation) and activity of this structure could increase the area of contact between cells before the cell bodies are seen to move together, so evidence on this matter is far from conclusive.

The functional importance of the amoebocytes is still a matter for conjecture. The aggregation behaviour may be important in wound healing, in a manner analogous to that of mammalian platelets and the amoebocytes of the snail Lymnaea (Stang-Voss, 1970). This suggestion is strengthened by the observation that in vitro the aggregated limpet amoebocytes move out, when placed on a glass substrate, to form a loose epithelioid monolayer. The rapid change in the numbers of cells in suspension in the blood, with change in body temperature, is more difficult to explain. The absence of mitotic figures and the rapidity and reversibility of the response suggests that cell division is not involved. Examination of sectioned limpet material shows the presence of amoebocyte-like cells lining the haemocoel. It is suggested therefore that the adhesiveness of these cells to the tissues of the limpet may be reduced at higher temperatures, causing them to round up and drop off into the blood circulation. This is worthy of further investigation.

REFERENCES


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Fig. 3. Method of withdrawal of blood. The pipette is inserted into the pallial vein (pv) at the point at which it runs inward to the heart. The head is held down by the left hand, using a spatula.

Fig. 4. Amoebocytes after spreading on to a glass surface, displaying rigid spikes supporting a fine cytoplasmic web at the periphery. Phase-contrast micrograph. n, nucleus; s, spikes. × 900.

Fig. 5. Single macrophage (centre, m) spread to glass surface showing centrally located vesicles. The dark peripheral patches are probably areas of thickened membrane. Phase-contrast micrograph. × 900.
Figs. 6–8. Amoebocytes in suspension, to show the development of spikes. The stimulus for this appears to be associated with the act of blood withdrawal. Phase-contrast micrographs, ×1500.

Fig. 6. Cells fixed immediately after withdrawal. No spikes but marginal convoluted lamellae (cl) present.

Fig. 7. Fixed 30 s after withdrawal. Spike initiation (si) has begun.

Fig. 8. Fixed 3 min after withdrawal. Marginal spikes (mt) now fully developed.