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Flow cytometric comparison of haemocytes from three species of bivalve molluscs

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Haemocyte subpopulations from three bivalve species (the clams *Ruditapes philippinarum* and *Mercenaria mercenaria* and the oyster, *Crassostrea virginica*) were characterised using light-scatter flow cytometry and a standard set of methods. Two parameter (forward and side scatter) plots for the three species were very similar and resembled plots for mammalian white blood cells. Two haemocyte groups (granulocytes and agranulocytes) were found in both the haemolymph and the extrapallial fluid of the clams while those two groups and an additional third group were found in the haemolymph of the oyster. All subpopulations were sorted on to glass slides, identified, photographed, and measured microscopically. Sorting of the bivalve granulocyte and agranulocyte groups indicated varying degrees of heterogeneity within each population in terms of either size or granularity, or both. However, subsorting of selected regions within the major groupings produced highly pure haemocyte populations. The comparison showed both similarities and differences among species. For instance, a distinct subpopulation of small granulocytes was present only in oysters and a subpopulation of spindle-shaped haemocytes, only in *M. mercenaria*. The haemocyte subpopulations delineated by light-scatter flow cytometry underscore questions about cell lineages, but the instrument also offers a powerful technique for answering them.

Key words: cell sorting, light-scatter, haemocyte subpopulations, granulocyte, hyalinocyte.

I. Introduction

Some of the reported differences in number and proportions of haemocyte types in bivalves are likely due to the subjective nature of the microscopic methods generally used to differentiate among cells in whole haemolymph samples. Techniques such as differential centrifugation are capable of physically separating different haemocyte types [1–3], but they are time-consuming and require several steps, including the immersion of cells in a separation medium, a process that stresses cells and limits the ability to study the role of the separated cells. Light-scatter flow cytometry, which was first introduced to

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study blood-cell composition in mammals [4], can perform precise and rapid measurements of cellular characteristics and allow the physical sorting of live cells with minimum handling [5] and relative objectivity compared to the human eye.

The earliest report of flow cytometric characterisation of bivalve haemocytes were contradictory. For instance, Friedl [6] found a single population of haemocytes in the American oyster *Crassostrea virginica*, whereas Fisher [7] reported four subpopulations in the same species. Meanwhile, a unimodal distribution of haemocytes was described in the hard clam *Mercenaria mercenaria* [8]. Later flow cytometric investigations identified three haemocyte groups in the oyster *C. virginica* [9], and two in the mussel *Mytilus galloprovincialis* [10]. Each one of these studies involved only a single species, which raises the possibility that differences in sample handling and analysis contributed to the disparate results. Further, with the exception of the work of Ashton-Alcox and Ford [9], none of these confirmed the suspected identity of the subpopulations by physically sorting and viewing them microscopically. Visual verification is an essential precursor to the standard use of flow cytometry for blood cell discrimination.

The present study describes the separation and characterisation of haemocytes from three commercially important bivalve species using flow cytometry: the clams *Ruditapes philippinarum* and *Mercenaria mercenaria*, and the oyster *Crassostrea virginica*. A standard set of procedures was employed on all species. Haemocyte populations were examined microscopically for size and appearance, so that the identity of each cell grouping determined by flow cytometry was confirmed by microscopic observation. Both similarities and differences among the species were noted and quantified.

II. Materials and Methods

ANIMALS

R. philippinarum ($41 \pm 0{\cdot}5$ mm, mean ± S.E.M.) from Puget Sound, WA, U.S.A. were shipped to the Haskin Shellfish Research Laboratory (HSRL) overnight. *M. mercenaria* (57 ± 2 mm) were harvested from Great Bay, NJ, U.S.A. *C. virginica* (78 ± 3 mm) were collected from oyster beds in the Delaware Bay. Animals were maintained in aerated marine aquaria at 14° C and fed daily using cultured algae. Sea water salinity was adjusted to 34 ppt for *R. philippinarum* and 28 ppt for *M. mercenaria* and *C. virginica*.

HAEMOLYMPH AND EXTRAPALLIAL FLUID COLLECTION

Haemolymph (typically 500 μl) was withdrawn from the adductor muscle of each animal using a syringe containing 500 μl cold 6% formalin in sterile sea water (SSW). Samples were then held on ice for no more than 2 h, until analysis by flow cytometry. In addition, extrapallial fluid was collected from the clams (*R. philippinarum* and *M. mercenaria*) for flow cytometric comparison with haemolymph. Extrapallial fluid was withdrawn through a hole made

in the central part of the shell as previously described [11] and was treated in the same manner as haemolymph samples.

FLOW CYTOMETRY

Flow cytometric analyses were performed as previously described by Ashton-Alcox and Ford [9] using a Coulter Epics C flow cytometer (Coulter Electronics, Hialeah, FL, U.S.A.) with an argon ion laser (488 nm). Filtered (0·2 μm) sea water was used as sheath fluid. Forward light-scatter (FS) and log side-scatter (SS) signals were collected for at least 10 000 particles from each individual and stored as list mode data files. Electronic gains and photomultiplier high voltages were adjusted to include all detected events on FS and SS plots. FS and SS gains were set at 5 for all species. Photomultiplier values of SS were 300 and 250 for clams and oysters, respectively. The FS noise discriminator was adjusted beforehand to discard debris. The flow cytometer operates by passing a stream of cells single-file through a focused laser beam. The cells scatter light at all angles from the incident beam. FS gives a relative indication of cell size, while SS is an indication of complexity, texture or granularity of cells. Signals were recorded on two dimensional (SS × FS) plots and discernible groups were gated electronically using bitmaps. The ratio of the particles within the bitmap to the whole haemocyte population was calculated. The bitmaps were also used to define subpopulations before physically sorting them on to glass slides. Bitmaps were adjusted for each individual as necessary [12]. Sorted haemocytes were used for microscopic identification, counting, and size measurements.

MICROSCOPIC CELL COUNTS AND SIZE MEASUREMENTS

Aliquots of whole fixed haemolymph and extrapallial fluids were discharged on to clean glass slides and allowed to settle. A minimum of 200 haemocytes per individual was identified and counted by phase contrast microscopy (Zeiss inverted ICM 405 microscope, × 400). Preliminary assays showed that microscopic identification of unstained cells was similar to identification from stained samples (Surestain™ Wright–Giemsa, Fisher Scientific, Pittsburgh, PA, U.S.A.). Differential cell counts were also performed on flow-cytometrically sorted subpopulations. The diameters of at least 50 cells of each type per sorted sample were determined with an ocular micrometer.

STATISTICS

Differential haemocyte counts determined microscopically were compared with those obtained with the flow cytometer using linear correlation analysis. Mean percentages from the two methods were compared using a Student's *t*-test. Within each species, differences in size and percentage of each cell type between haemocyte groups were tested using a one-way general linear model ANOVA followed by a Fisher's PLSD post-hoc test. All proportional data were arcsin-transformed before analysis. Differences were considered significant at $\alpha = 0{\cdot}05$.

III. Results

FLOW CYTOMETRIC PATTERNS

Two-parameter plots of the light-scatter signals of cells from both haemolymph and extrapallial fluid displayed two recognisable groupings in the two clam species (Fig. 1). Three groupings were found in the haemolymph of *C. virginica* (Fig. 1). In both the oysters and clams, the first group had high FS and SS signals (Fig. 2, Group A), indicating relatively large size and granularity, while the second (Fig. 2, Group B) had low FS and SS signals, indicating relatively small size and granularity. A minor third grouping (Fig. 2, Group C) was arbitrarily defined as the few intermediate cells located between Groups A and B. Oysters had an additional group (Fig. 2, Group D) of cells with low FS and high SS signals, indicating small, highly granular cells. Group B was subsorted (Fig. 2, B1 and B2) in all species; Group A was subsorted only in the clams (Fig. 2, A1, A2, and A3) because Ashton-Alcox and Ford [9] had demonstrated that this group is composed almost solely of granulocytes in *C. virginica*.

WHOLE HAEMOLYMPH AND EXTRAPALLIAL FLUID (MICROSCOPY)

Two cell types were distinguished microscopically in whole haemolymph and extrapallial fluid of both clam species and in the haemolymph of the oyster: granulocytes and agranular haemocytes. Granulocytes from all species were relatively large, contained highly refringent cytoplasmic granules, and a low nucleus:cytoplasm ratio. Some cells that also have a low nucleus: cytoplasm ratio (similar to that of granulocytes) were characterised by the presence of a few, highly refringent granules. In our analysis, they were called 'slightly granulated granulocytes' and are included in the 'granulocyte' group (Tables 1 and 2). The agranular haemocytes were also of two classes. The first, which we call hyalinocytes, were recognisable because of their small size, high nucleus:cytoplasm ratio, and cytoplasm containing few or no granules. These are morphologically similar to mammalian lymphocytes. The second type were larger cells having a nucleus:cytoplasm ratio somewhat lower than hyalinocytes but higher than granulocytes and containing some vacuoles and relatively dark granules. The latter were readily distinguishable from the bright, highly refringent granules present in granulocytes. The size and shape of intracellular inclusions were used to differentiate vacuoles (relatively large, often having oval shape) from granules (small round inclusions).

Both granulocytes and agranular haemocytes in *R. philippinarum* were smaller than those in the other two species (granulocytes: 10·5–10·8 μm *v.* 8·2 μm in diameter; agranular haemocytes: 7·5–7·7 μm *v.* 6·8 μm) and cell sizes were the same in the haemolymph and extrapallial fluid of the clams (Table 1). Granulocytes comprised 63–65% of all haemocytes in the clams, but only 33% in the oysters (Table 2). In *R. philippinarum*, the percentage of granulocytes in the extrapallial fluid (69%) was statistically the same as in the haemolymph; in *M. mercenaria*, it was higher (81%, P=0·03). A few elongated agranular haemocytes were also observed in the haemolymph and extrapallial fluid of *M. mercenaria*. They were relatively homogeneous in size ranging from 8·1 to

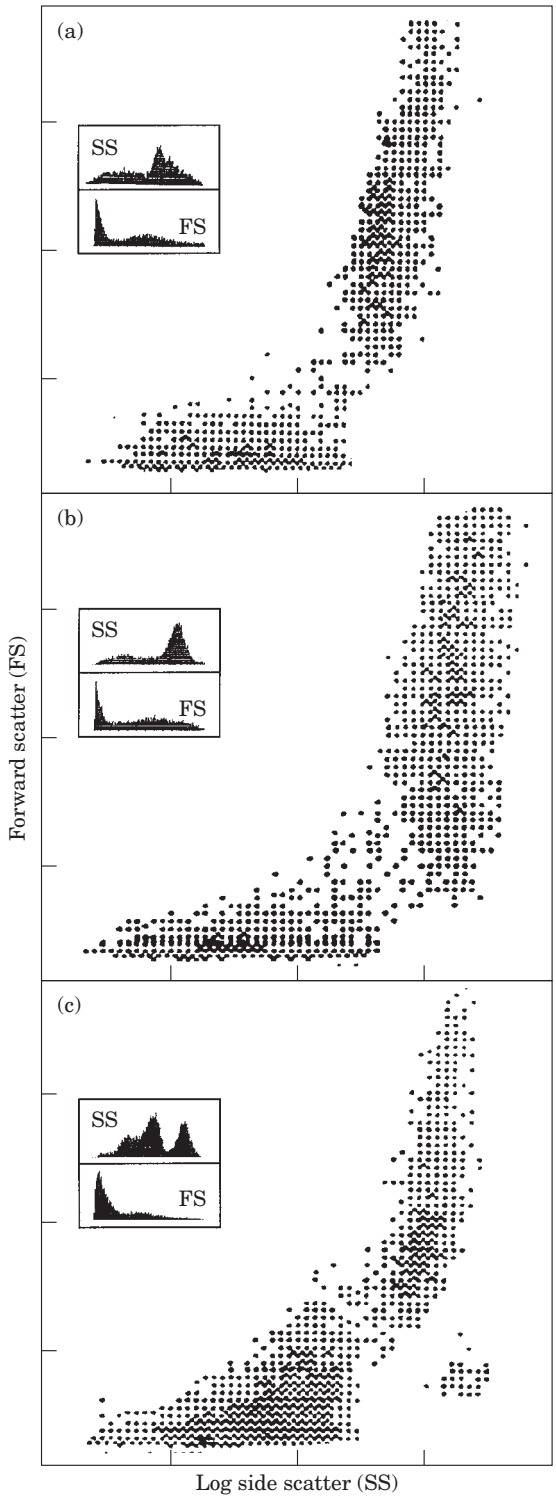

Fig. 1. Flow cytometry bivariate plots showing distributions of log side scatter (SS) and forward scatter (FS) of haemocytes in clams and oysters. (a) *R. philippinarum*, (b) *M. mercenaria*, (c) *C. virginica*.

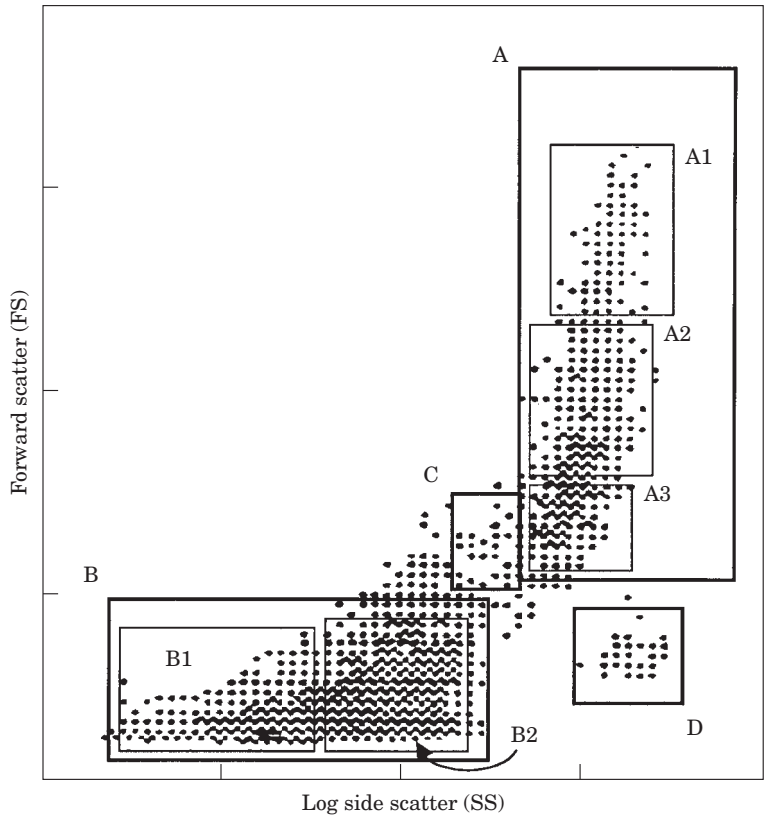

Fig. 2. Haemocyte groupings used for sorting and subsequent analysis in the Eastern oyster *Crassostrea virginica*. The same groupings were used for the Manila clam, *Ruditapes philippinarum*, and the hard clam, *Mercenaria mercenaria*, except for Group D, which was not present in either clam species.

10·4 μm in diameter (mean of the largest and smallest dimensions). The oysters had an additional and distinct third group of haemocytes called 'small granulocytes' [9] which averaged 6·7 ± 0·2 μm in diameter.

SORTED SUBPOPULATIONS (FLOW CYTOMETRY AND MICROSCOPY)

Group A

In the haemolymph of all species, granulocytes comprised 92% to 96% of Group A (Figs 3A, 4A1 and 5A, Table 2). These sorted granulocytes had essentially the same mean diameter as those found in whole haemolymph (Table 1). Similar results were found in the extrapallial fluid of *R. philippinarum* and *M. mercenaria* where Group A averaged over 93% granulocytes of about the same size as those described from whole haemolymph (Table 1).

Subsorting of Group A from haemolymph of both clam species revealed that cells located in the highest FS channels (Group A1) were almost exclusively (99%, Table 2) composed of granulocytes that were significantly larger than the average granulocyte in the whole haemolymph (Table 1) and contained

Table 1. Cell diameter (mean ± S.E. in μm) within each haemocyte grouping in clams and oysters. Numbers within parentheses are the range. NP: not present; NA: not assayed. $n=3$ individuals/species. A minimum of 50 cells was measured in each haemocyte grouping. Letters (a, b, c, d, e and f) represent differences among haemocyte subpopulations within each column (ANOVA, $P<0{\cdot}05$)

	Hemolymph			Extrapallial fluid	
	R. philippinarum	*M. mercenaria*	*C. virginica*	*R. philippinarum*	*M. mercenaria*
Whole population					
Granulocytes	$8{\cdot}2 \pm 0{\cdot}7$ (6–13)^{a,c}	$10{\cdot}8 \pm 0{\cdot}6$ (7–15)^{a}	$10{\cdot}5 \pm 0{\cdot}6$ (8–14)^{a}	$8{\cdot}8 \pm 0{\cdot}4$ (7–12)^{a}	$11{\cdot}1 \pm 0{\cdot}4$ (8–15)^{a}
Hyalinocytes	$6{\cdot}8 \pm 0{\cdot}3$ (5–11)^{b}	$7{\cdot}7 \pm 0{\cdot}4$ (6–11)^{b}	$7{\cdot}5 \pm 0{\cdot}4$ (6–9)^{b}	$7{\cdot}3 \pm 0{\cdot}3$ (6–11)^{b,c}	$7{\cdot}9 \pm 0{\cdot}6$ (7–11)^{b}
Small granulocytes	NP	NP	$6{\cdot}7 \pm 0{\cdot}2$ (6–7)^{c}	NP	NP
Sorted subpopulations					
Group A	$8{\cdot}4 \pm 0{\cdot}1$ (7–13)^{c}	$11{\cdot}3 \pm 0{\cdot}2$ (9–17)^{a}	$10{\cdot}9 \pm 0{\cdot}3$ (8–16)^{a}	$8{\cdot}8 \pm 0{\cdot}4$ (8–15)^{a}	$11{\cdot}6 \pm 0{\cdot}3$ (9–16)^{a}
Group A1	$10{\cdot}1 \pm 0{\cdot}2$ (9–15)^{d}	$13{\cdot}0 \pm 0{\cdot}2$ (11–18)^{c}	NA	NA	NA
Group A2	$7{\cdot}7 \pm 0{\cdot}1$ (6–14)^{a,e}	$9{\cdot}1 \pm 0{\cdot}2$ (8–15)^{d}	NA	NA	NA
Group A3	$7{\cdot}6 \pm 0{\cdot}2$ (6–13)^{a,e}	$9{\cdot}1 \pm 0{\cdot}2$ (7–14)^{d}	NA	NA	NA
Group B	$6{\cdot}7 \pm 0{\cdot}2$ (4–11)^{b}	$8{\cdot}0 \pm 0{\cdot}2$ (6–12)^{b}	$7{\cdot}5 \pm 0{\cdot}2$ (5–11)^{b}	$6{\cdot}9 \pm 0{\cdot}3$ (5–10)^{c}	$8{\cdot}2 \pm 0{\cdot}3$ (7–11)^{b}
Group B1	$6{\cdot}0 \pm 0{\cdot}1$ (4–9)^{f}	$6{\cdot}9 \pm 0{\cdot}2$ (5–10)^{e}	$5{\cdot}5 \pm 0{\cdot}1$ (4–8)^{d}	NA	NA
Group B2	$7{\cdot}3 \pm 0{\cdot}2$ (6–12)^{a,b,e}	$8{\cdot}6 \pm 0{\cdot}2$ (6–13)^{f}	$8{\cdot}2 \pm 0{\cdot}2$ (6–13)^{e}	NA	NA
Group C	$7{\cdot}4 \pm 0{\cdot}2$ (6–13)^{a,b,e}	$8{\cdot}3 \pm 0{\cdot}2$ (7–14)^{b,f}	$8{\cdot}6 \pm 0{\cdot}2$ (6–13)^{e}	$7{\cdot}8 \pm 0{\cdot}4$ (6–11)^{b}	$8{\cdot}7 \pm 0{\cdot}5$ (7–12)^{b}
Group D	NP	NP	$6{\cdot}8 \pm 0{\cdot}1$ (5–8)^{c}	NP	NP

Table 2. Percentage of granulocytes and hyalinocytes (mean ± S.E.) within each haemocyte grouping in haemolymph of clams and oysters. Numbers within parentheses are the range (lower–upper). NP: not present; NA: not assayed. n=6 individuals/species. Letters (a, b, c, d, e, f, g and h) represent differences among haemocyte subpopulations within each column (ANOVA, P<0·05)

	R. philippinarum		*M. mercenaria*		*C. virginica*	
	Granulocytes	Agranulocytes	Granulocytes	Agranulocytes	Granulocytes	Agranulocytes
Whole population	63·1 ± 8·1[a] (49–73)	36·9 ± 8·1[a] (27–51)	65·4 ± 9·8[a] (55–80)	34·6 ± 9·8[a] (20–45)	33·4 ± 6·3[a] (25–47)	66·6 ± 6·3[a] (53–75)
Sorted subpopulations						
Group A	91·9 ± 0·8[b] (88–95)	8·1 ± 0·8[b] (5–12)	94·7 ± 1·0[b] (91–97)	5·3 ± 1·0[b] (3–9)	96·1 ± 1·0[b] (93–99)	3·9 ± 1·0[b] (1–7)
Group A1	99·0 ± 0·5[c] (98–100)	1·0 ± 0·5[c] (0–2)	99·3 ± 0·4[c] (99–100)	0·7 ± 0·4[c] (0–1)	NA	NA
Group A2	97·1 ± 0·5[d] (97–98)	2·9 ± 0·5[d] (2–3)	95·5 ± 1·7[b] (92–98)	4·5 ± 1·7[b] (2–8)	NA	NA
Group A3	79·4 ± 2·2[e] (76–83)	20·6 ± 2·2[e] (17–24)	83·1 ± 3·4[d] (77–89)	16·9 ± 3·4[d] (11–23)	NA	NA
Group B	1·6 ± 0·2[f] (0–4)	98·4 ± 0·2[f] (96–100)	2·8 ± 0·6[e,f] (1–5)	97·2 ± 0·6[e,f] (95–99)	5·9 ± 0·8[c] (2–8)	94·1 ± 0·8[c] (92–98)
Group B1	0·6 ± 0·4[g] (0–1)	99·4 ± 0·4[g] (99–100)	1·3 ± 0·9[f] (0–3)	98·7 ± 0·9[f] (97–100)	2·2 ± 0·5[d] (1–3)	97·8 ± 0·5[d] (97–99)
Group B2	2·6 ± 0·9[f] (1–4)	97·4 ± 0·9[f] (96–99)	4·4 ± 1·6[e,g] (2–7)	95·6 ± 1·6[e,g] (93–98)	8·1 ± 2·1[c] (4–11)	91·9 ± 2·1[c] (89–96)
Group C	17·1 ± 2·4[h] (11–25)	82·9 ± 2·4[h] (75–89)	5·6 ± 1·9[g] (3–9)	94·4 ± 1·9[g] (91–97)	38·7 ± 3·0[a] (33–47)	61·3 ± 3·0[a] (53–67)
Group D	NP	NP	NP	NP	98·4 ± 0·6[e] (97–100)	1·6 ± 0·6[e] (0–3)

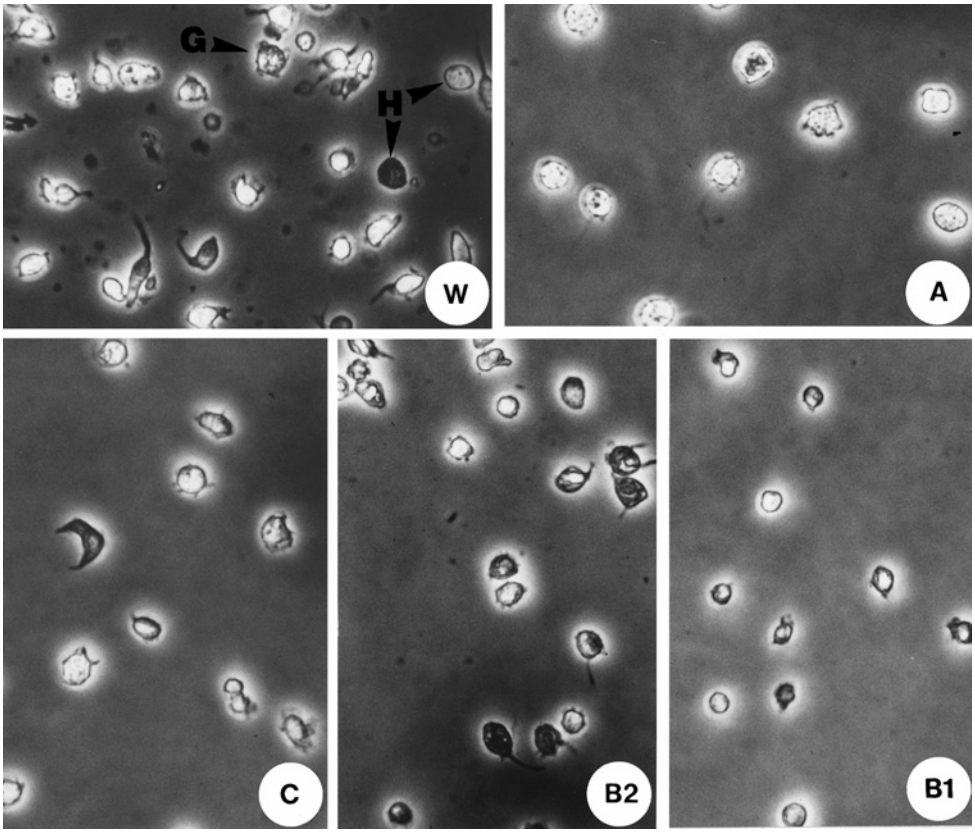

Fig. 3. Haemocytes of the Manila clam, *Ruditapes philippinarum*. W: whole haemocyte population; A, C, B2 and B1: haemocytes present within the groups A, C, B2 and B1, respectively. G: granulocyte; H: hyalinocyte. White circle=18 μm in diameter.

abundant refractive granules (Figs 3 and 4). Group A2 also contained primarily granular haemocytes (96–97%) that were significantly smaller than those in A1 (Table 1). The lower FS channels (Group A3) contained about 80% granulocytes and 20% large and vacuolated agranular haemocytes (Table 2). In *M. mercenaria*, Group A3 also contained spindle-shaped, slightly granulated cells (15%, $9{\cdot}3 \pm 0{\cdot}3\,\mu$m in diameter) particularly at the lower FS channels (Fig. 4). These probably correspond to the 'fibrocytes' described by Foley and Cheng [13], who concluded that these cells resulted from a degranulation of granulocytes [14, 15].

Group B

In all species, Group B was composed mainly of agranular haemocytes (Figs 3, 4 and 5). In the clams, this was over 97% both in haemolymph (Table 2) and extrapallial fluid (97·9 and 97·3% in *R. philippinarum* and *M. mercenaria*, respectively), whereas in *C. virginica*, Group B contained approximately 94% agranular, and up to 6% slightly granulated, haemocytes. The cells in Group B of all species had the same mean diameters as the agranulocytes measured in whole haemolymph (Table 1).

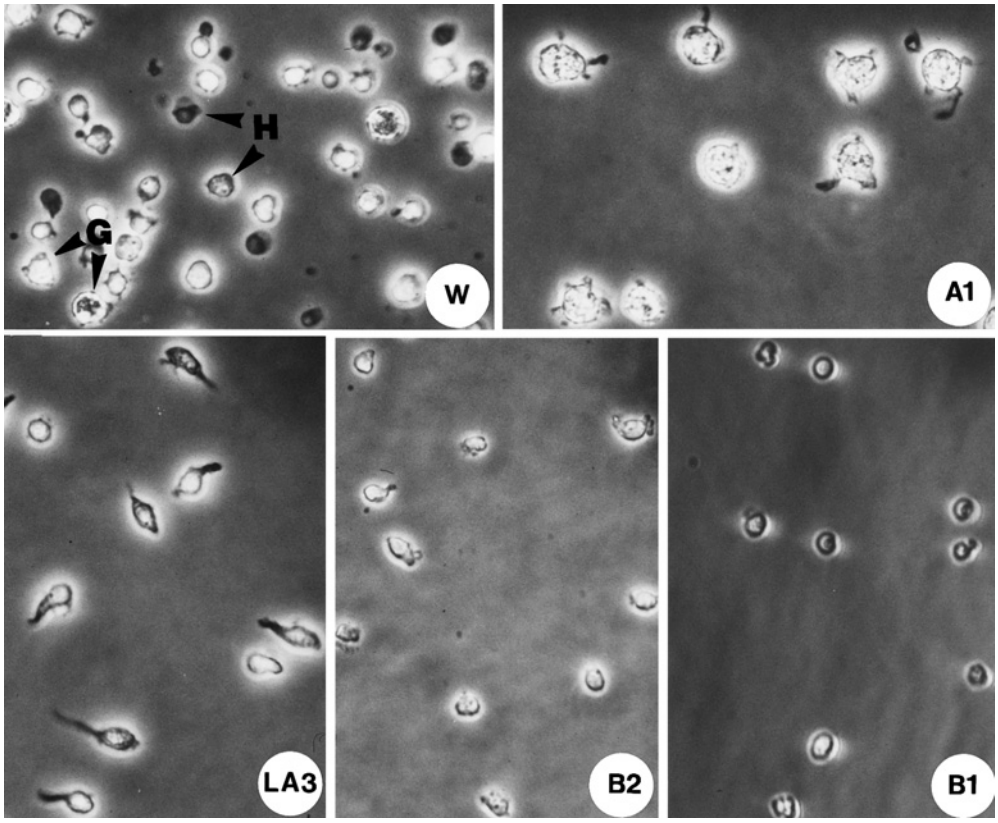

Fig. 4. Haemocytes of the hard clam, *Mercenaria mercenaria*. W: whole haemocyte population; A1, B2 and B1: haemocytes present within groups A1, B2 and B1, respectively; LA3: haemocytes present at low forward light-scatter channels within the Group A3. G: granulocyte; H: hyalinocyte. White circle = 18 μm in diameter.

Subsorting of Group B showed that Subgroup B1 was dominated by agranular haemocytes (98–99% in all species, Table 2). These cells were somewhat smaller on average than the agranular haemocytes from whole haemolymph, possessed a relatively large nucleus (Figs 3 and 4; Table 1), and met the definition of hyalinocytes. Group B2 was also dominated by agranular haemocytes (over 92%, Table 2), which were relatively larger than those measured from whole haemolymph (Figs 3 and 4; Table 1) and possessed a smaller nucleus:cytoplasm ratio than hyalinocytes observed in B1. Some of these cells contained dark granules and light vacuoles. The remaining cells in Group B2 (3–8%, Table 2) were made up of slightly granulated granulocytes.

Group C

Cells present in the intermediate Group C in all three species were morphologically heterogeneous (Figs 3 and 5). They were mostly non-hyalinocyte agranular haemocytes containing small numbers of vacuoles and/or dark granules. The rest of the cells in this group were haemocytes with low numbers of refractive granules compared to granulocytes in Group A. We

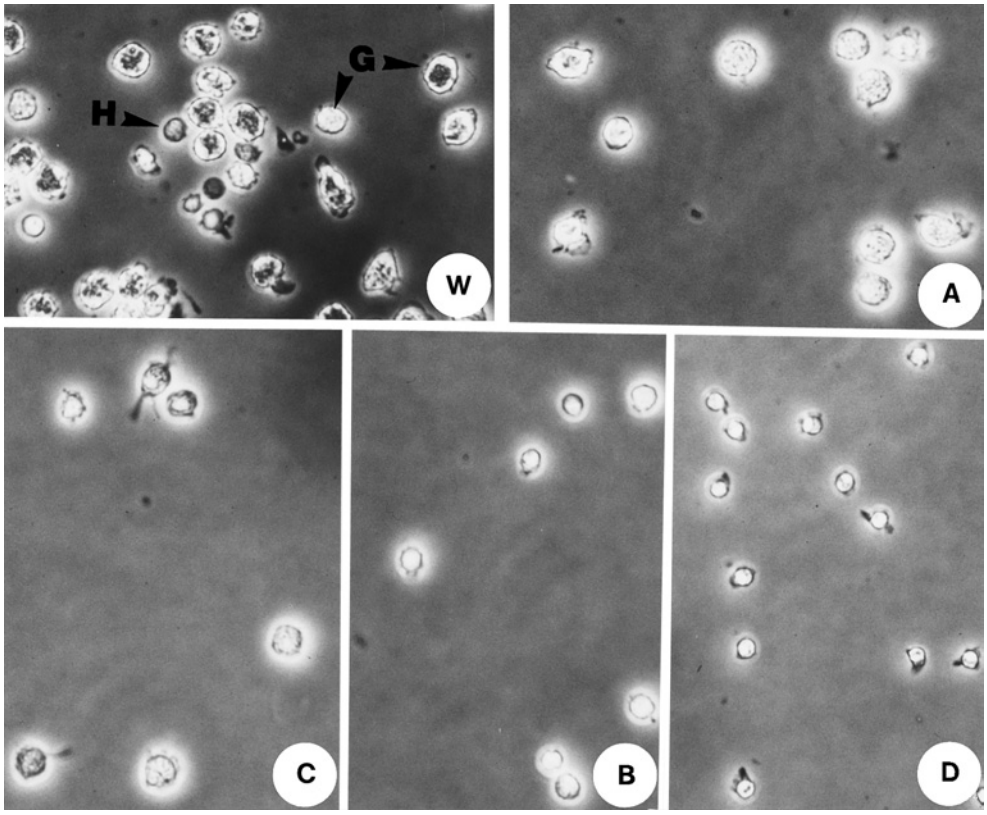

Fig. 5. Haemocytes of the American oyster, *Crassostrea virginica*. W: whole haemocyte population; A, C, B and D: haemocytes present within groups A, C, B and D, respectively. G: granulocyte; H: hyalinocyte. White circle=18 μm in diameter.

considered these as slightly granulated granulocytes. The percentage of granulocytes in Group C was quite variable among species, ranging from $5{\cdot}6 \pm 1{\cdot}9$ in *M. mercenaria* to $38{\cdot}7 \pm 3{\cdot}0$ in *C. virginica* (Table 2). In the extrapallial fluid of the two clam species, the proportion of granulocytes was statistically similar to that in the haemolymph ($21{\cdot}1 \pm 4{\cdot}4$ and $8{\cdot}7 \pm 3{\cdot}5$% in *R. philippinarum* and *M. mercenaria*, respectively; $P>0{\cdot}05$). Cells present in Group C of all species had the same diameter as those found within Group B2 (Table 1).

Group D

This group was present only in *C. virginica* and was composed of small ($6{\cdot}8 \pm 0{\cdot}1$ μm, Table 1), highly refractive cells (Fig. 5D) that were previously identified as small granulocytes [9]. The percentage of small granular cells in this group was 98·4%, while the other 1·6% consisted of agranular cells (Table 2).

FLOW CYTOMETRY *V.* MICROSCOPY

In all species, the percentage of granulocytes in whole haemolymph calculated by flow cytometry (cells present within Group A) was significantly

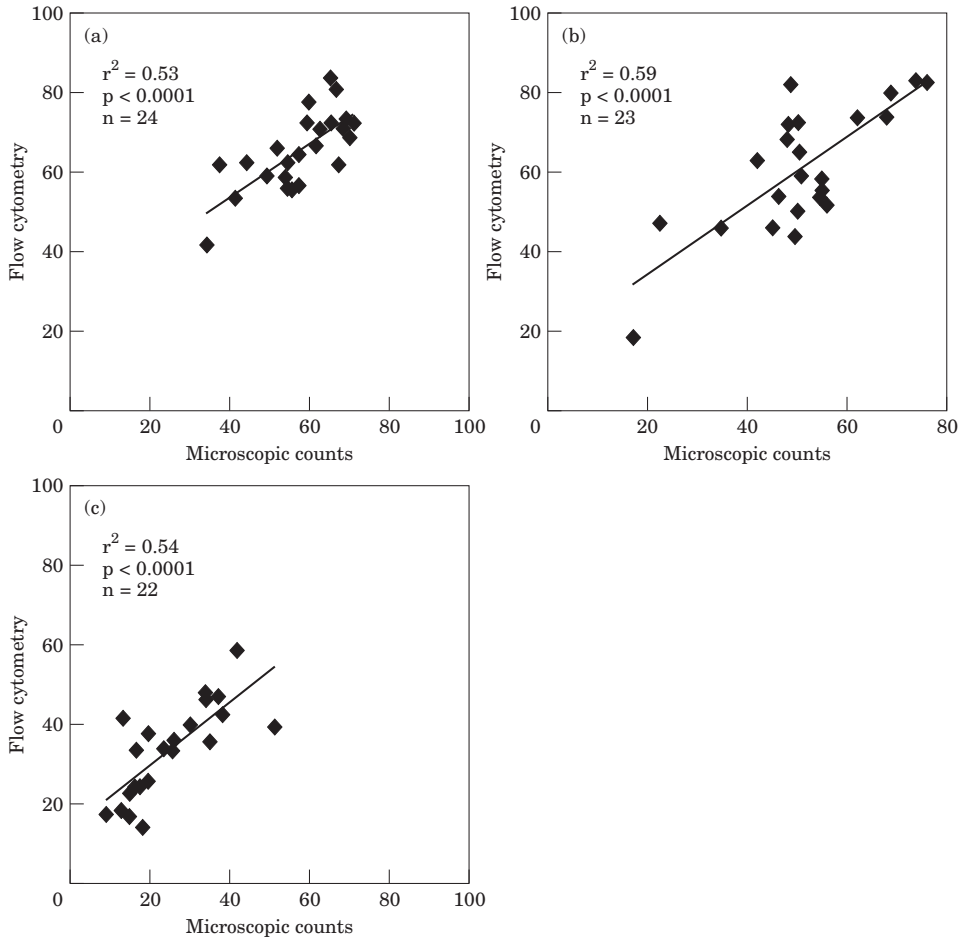

Fig. 6. Correlation between granulocyte percentages measured both flow cytometrically and microscopically in clams and oysters. (a) *R. philippinarum*, (b) *M. mercenaria*, (c) *C. virginica*.

correlated (P<0·0001) with that calculated microscopically and r^2 values ranged from 0·53 to 0·59 (Fig. 6). However, the flow cytometric results provided a significantly higher percentage of granulocytes than did microscopic counts for each of the species studied (Table 3). Similarly, in *C. virginica*, the percentage of small granulocytes estimated by flow cytometry (1·26 ± 0·31) was significantly higher than that calculated by microscopy (0·50 ± 0·14), but results obtained using both methods were highly correlated (r^2=0·72, P<0·0001, n=22).

IV. Discussion

Although much use is now made of fluorescent probes to distinguish specific mammalian cell types by flow cytometry, the preliminary differentiation of peripheral leukocytes, which resemble bivalve haemocytes in certain ways, is

Table 3. Comparison of granulocyte percentages (mean ± S.E.) measured flow cytometrically or microscopically in clams and oysters. *Significant differences between both methods (Student's *t*-test, $P<0{\cdot}05$). n=number of individuals

	R. philippinarum $n=24$	*M. mercenaria* $n=23$	*C. virginica* $n=22$
Microscopy	57·3 ± 2·1	51·1 ± 2·8	24·7 ± 2·3
Flow cytometry	65·9 ± 2·0*	61·3 ± 3·2*	33·7 ± 2·4*

made using light-scatter measurements, FS *v*. SS [5, 12]. Thus, it is appropriate to gather comparative baseline measurements for bivalve haemocytes using the same parameters. Our work clearly demonstrates a high degree of similarity in light-scatter patterns for haemocytes of three marine bivalves, and that they are similar to those of mammalian white blood cells. The use of flow cytometry also enabled us to find subtle differences among the bivalve species that would have been difficult to document using more labour intensive and subjective methods.

In general, two major haemocyte types have been noted in bivalves: (1) granulocytes, which contain cytoplasmic granules of various numbers, sizes and staining qualities, and usually have a low nucleus:cytoplasm ratio; and (2) hyalinocytes which lack such granules and generally have a high nucleus: cytoplasm ratio [16–19]. Hyalinocytes, as we define them, are a subset of agranular haemocytes. Other authors use the term more loosely for any cell with few or no granules, many of which may be degranulated granulocytes [17, 18, 20, 21]. Several observations suggest that granulocytes are the major effector cells for internal defense in bivalve molluscs. They are the most phagocytic [22–24], migrate in response to chemoattractants [25], and produce a wide array of enzymes and antimicrobial substances [19, 21], including antibacterial peptides [26]. The role of hyalinocytes is unclear. They aggregate in lesions caused by the oyster parasite *Haplosporidium nelsoni* (MSX) [27, 28], but their function at the infection site is not known. Several other authors have reported that 'agranular' haemocytes participate in aggregation and encapsulation; however, they do not differentiate between hyalinocytes and other agranular cells [29–31].

The relative position on the scatterplot of the hyalinocytes was the same as that of mammalian lymphocytes, which are also characterised by their small size, large nucleus and few granules [12]. Similarly, mammalian polymorphonuclear leukocytes (PMLs), which are large granular cells, appear in the same relative position as granulocytes. Monocytes, which are large cells with relatively few granules fall into a group between PMLs and lymphocytes, approximately at the same location of our intermediate Group C. These similarities are not surprising given that the light-scatter plots are based on morphological characteristics, but it is dubious whether this implies functional similarities. Like bivalve granulocytes, the primary function of polymorphonuclear leukocytes is phagocytosis, and the production of hydrolytic enzymes and antibacterial substances [32, 33]. On the other hand, the

similarity between hyalinocytes and lymphocytes may be just morphological. At the very least, mammalian lymphocytes are the primary effectors of immunological memory, which bivalves do not have. Further, monocytes are a unique cell type, whereas our intermediate Group C is a mixture of different cells.

The origin, life cycle, and life span of bivalve haemocytes are still largely unknown and the role of each cell type has not been completely elucidated. The flow cytometer is likely to be an important tool for investigating these critical issues. For instance, the small granulocytes found in *C. virginica* had been described from microscope observations [1, 34, 35], but they are relatively very scarce. The large number of cells that we were able to analyse made this small subgroup stand out as being characteristic of the oyster. For now, their role is unknown, but Cheng [17] proposed that they are immature granulocytes (progranulocytes) that become intermediate-sized, then large, granulocytes. Our results do indicate a progression between intermediate-sized and large granulocytes (Groups A1 and A2), but none between the overall granulocyte group and the small granulocytes. Instead, the flow cytometer has repeatedly shown that the small granulocytes form a clearly separate group, which does not grade into that of the granulocytes [7, 9, 18, this study]. The absence of obvious intermediate forms may indicate that the small and 'large' granulocytes are not part of the same cell line. On the other hand, the relatively low number of small granulocytes may mean that intermediate stages are even more rare and difficult to detect among the population of 'large' granulocytes. Fortunately, the distinctiveness of the small granulocyte group and the ability to obtain a very homogeneous population of 'large' granulocytes will facilitate the recovery of pure populations for assays that should help answer questions about the relationship between these two cell types.

The light-scatter patterns are less clear in answering the question of whether granulocytes and hyalinocytes represent two different life stages of the same cell line, with hyalinocytes being the immature form, as suggested by some authors [10, 36]. A transformation from hyalinocytes to granulocytes might appear in flow cytometer plots as a gradual transition of particles between the two major cell categories. In fact, an 'intermediate' cell group is present (Fig. 2, Group C), which appears to be a combination of slightly granulated granulocytes and relatively large agranular cells with vacuoles. The latter were also present in some of the small size classes of granulocytes (Groups A2 and A3). This would be consistent with a transition from hyalinocytes to granulocytes. A bimodal distribution of light-scatter parameters has been demonstrated in the mussel *Mytilus galloprovincialis* as has the presence of numerous intermediate cells [10]. Based on the cytometric pattern, and similarities in the binding of mammalian-cell antibodies, enzyme presence, adherence to slides, and phagocytosis, they suggested that type I haemocytes in *M. galloprovincialis* (=hyalinocytes) represent 'young cells' that subsequently acquire cytoplasmic complexity (first becoming vacuolated and later acquiring cytoplasmic granules) and become type II haemocytes (=granulocytes). However, we believe that our results could equally well be interpreted to show that granulocytes become degranulated and are identified as

'agranular'. Phagocytic granulocytes may also contain phagolysosomes, which would appear as vacuoles in largely degranulated haemocytes [37]. A second lineage was proposed by Cheng [17], who considered that hyalinocytes and granulocytes represent distinct cell lines. In support of this scheme, he noted differences between the two cell types in lectin binding characteristics [1]. Both systems rely largely on inference, however, and at present there is no conclusive evidence supporting either hypothesis.

Recently, Allam *et al.* [11] documented the existence of functional haemocytes in the extrapallial fluid of *R. philippinarum*. They proposed that the extrapallial fluid be considered a 'pseudo' internal compartment where haemocytes act against pathogens invading the shell cavity. Whether differential counts are similar between the two compartments [11, this study for *R. philippinarum*] or whether granulocytes are relatively more abundant in the extrapallial fluid [38, this study for *M. mercenaria*], is not clear. What is clear is that there is migration across epithelial borders of both types of haemocytes. It is not yet known if this migration occurs equally in both directions, but preliminary evidence indicates that haemocytes that ingest particles in the pallial and extrapallial fluids do traverse external epithelia into mantle and gill tissue [39].

The sorting methods described here provided high purity subpopulations, particularly in outlying groups (98–99% in Group A1 and B1 in all three species) and for the small granulocytes in oysters. Nevertheless, some cell types were present in more than one grouping. The drawing of bitmaps around cell groupings is always somewhat subjective, even for mammalian blood cells [12], and can lead to variability in the inclusion or exclusion of cell types from a particular group. Variation among individuals or between species is particularly likely when the cell grouping falls between two other populations, such as Group C in our analysis. Thus, it is not surprising that differential counts were most variable in this group and in the adjacent regions of the other populations (see Table 2). The fact that flow cytometry generated higher percentages of granulocytes than did microscopic counts (see Table 3) probably results from the inclusion by the flow cytometer of vacuolated agranular cells in Group A (mainly within Subgroup A3), which is primarily a granular subpopulation. Intracellular complexity, which is measured by side scatter, would be provided by both vacuoles and granules and would lead to the inclusion of both types in the same group.

Despite the variability in cell granularity and size, flow cytometric measurements were significantly correlated with microscopic counts, with r^2 values ranging from 0·53 to 0·72. These coefficients were within the range of values found in vertebrates such as mammals (0·05 for human monocytes to 0·74 for lymphocytes [12, 40]), fish (0·57 for neutrophils and 0·68 for lymphocytes [41]), and in bivalves (0·50 for granulocytes [9]). Using specific fluorescent tags in conjunction with light-scatter flow cytometry should improve identification and subsorting of haemocytes, particularly within bitmaps where mixed cell types were noted.

Analysis of haemocytes from several species and two body fluids under the same conditions, as we have done, largely eliminates uncertainties associated with differences in handling and analytical methods that could produce

different results in different laboratories. For instance, our results show that the presence of the small granulocyte population only in oysters, and the fibrocyte population only in *M. mercenaria*, is not an artifact, because the same methodology and flow cytometer was used for all species. On the other hand, we cannot be sure that previous findings of unimodal distributions in clams and oysters from Florida [6, 8] were due to differences in handling because microscopic examination of haemocytes in other studies showed both site and seasonal differences in cell types that could account for the different cytometer readings [35, 42, 43]. The haemocyte subpopulations delineated by light-scatter flow cytometry underscore persistent questions about cell lineages, but the instrument also offers a powerful technique for answering them.

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