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Journal of Insect Physiology 52 (2006) 417–429
Journal of Insect Physiology
www.elsevier.com/locate/jinsphys

Review

Insect haemocytes: What type of cell is that?

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Received 13 January 2006; received in revised form 17 January 2006; accepted 17 January 2006

Abstract

Classification of insect larvae circulating haemocytes is the subject of controversy, and the terminology used to designate each cellular type is often different from one species to another. However, a survey of the literature on insect haemocytes suggests that there are resemblances for most of the cell types and functions, in different insect species. In this review paper, we compare the structure and functions of circulating haemocytes in those insect species that are, by far, the most often used species for insect physiology studies, i.e. lepidopteran species and *Drosophila*. We show that there is high degree of homology of haemocyte types and suggest possible synonymies in terminology among species from these taxa.

Keywords: Lepidopteran; *Drosophila*; Hemocyte

Contents

1. Introduction

Like *Arabidopsis thaliana*, *Caenorhabditis elegans* or *Danio rerio* (zebra fish), *Drosophila melanogaster* is a model system that is extensively used for studies on development of metazoans. *Drosophila* is especially used to investigate the mechanisms of innate immunity as ''most of the genes involved in this host defence are homologous or very similar to genes implicated in mammalian innate immune defences'' (Hoffmann, 2003). Depending on the final effector that attacks the foreign body, reactions of innate immunity are divided into cellular and humoral reactions. Until now, most studies have been devoted to the production of antimicrobial peptides, and reactions of humoral immunity have began to be well understood. On the other hand, cellular immune reactions have not been explored so extensively. Nevertheless, more and more numerous teams are now beginning to investigate these cellular reactions. This means that comparisons between

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doi:10.1016/j.jinsphys.2006.01.005

different works on *Drosophila* and other insect species will be necessary. Most, if not all, other studies on insect cellular immune reactions have been performed on species of the Lepidoptera Order. Unfortunately, while the bulk of haemocyte types (Plasmatocytes, Granular cells, Oenocytoids and Spherule Cells) are recognized in most insect species (review in Lavine and Strand, 2002), *Drosophila* appears as a peculiarity in the class Insecta concerning the cellular components of its haemolymph. Among the three main *Drosophila* haemocyte types recognized by all investigators, the names of two of them, Lamellocytes and Crystal Cells (review in Meister and Lagueux, 2003), suggest that these cell types are present only in *Drosophila* species. Furthermore, the third *Drosophila* haemocyte type, called Plasmatocyte, seems to be very different from the plasmatocytes observed in species belonging to other insect orders, especially in Lepidoptera. For these reasons, it appears necessary to compare *Drosophila* haemocyte types to those described in Lepidoptera in order to allow easy comparisons of results gained on cellular immune reactions in all these insect species.

The aim of this review is not to give a complete view of the classification and rôles of circulating haemocytes in the insect class. For this, the reader can refer to the recent review by Lavine and Strand (2002). Other reviews, excellent although sometimes old, are for example those by Rizki (1957), Price and Ratcliffe (1974), Ratcliffe and Rowley (1979), Rowley and Ratcliffe (1981), Brehélin and Zachary (1986), Lackie (1988), Ratcliffe (1993), Lanot et al. (2001). Here, we focus on lepidopteran species and *Drosophila*, and suggest possible homologies of circulating haemocyte types in larvae and synonymies in their terminology.

2. Typical haemocyte types in Lepidoptera and their corresponding types in *Drosophila*

Four main circulating haemocyte types have been recovered in all the lepidopteran species already studied. They are called Plasmatocytes, Granular Haemocytes, Oenocytoids and Spherule Cells. Three of them seem to have homologous types in *Drosophila* and some of their functions, especially in cellular defence reactions, are known.

2.1. Plasmatocytes

In Lepidoptera: After immediate fixation at the time of blood removal (i.e. they have probably the same shape in haemolymph in vivo), plasmatocytes are spherical or oval cells (up to 20 μm long) with a regular shape, sometimes spindle-shaped (Fig. 1A). In TEM, few pseudopods, pinocytotic vesicles and clear vacuoles are sometimes observed (Fig. 2A) as well as numerous polyribosomes. According to most authors, the cytoplasm of plasmatocytes encloses a moderate amount of rough endoplasmic reticulum (RER) (Rowley and Ratcliffe, 1981), sometimes developed in long and narrow cisternae (Brehélin and

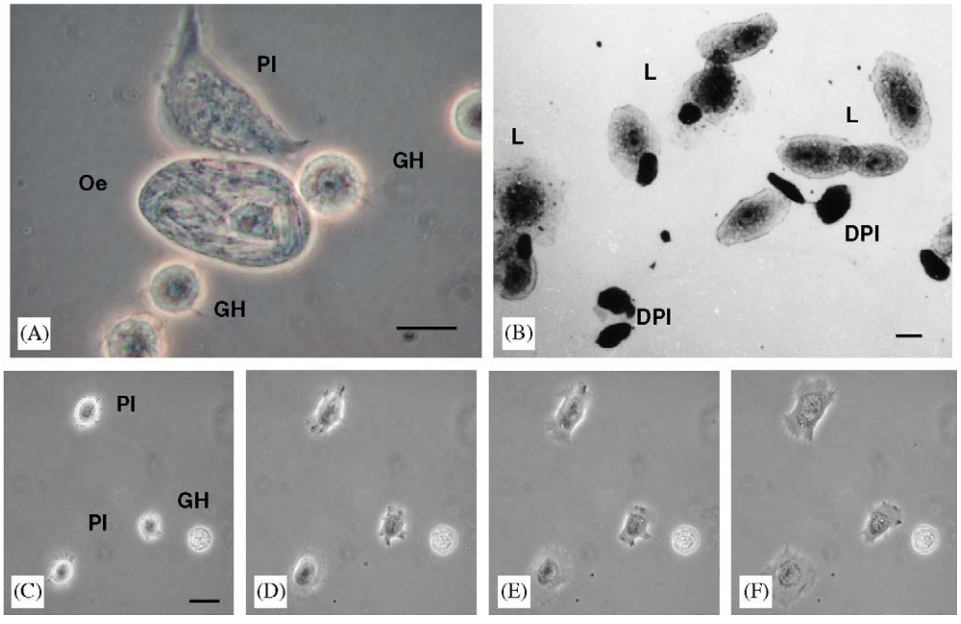

Fig. 1. Haemocytes observed in light microscopy, using different techniques. Bars = 10 μm. (A) Haemocytes of *S. littoralis*, after fixation at blood collection. Each haemocyte shows the typical morphology of its type. GH, granular haemocyte: spherical and very refractive cells; Oe, oenocytoid: large cell with a low nuclear to cytoplasmic ratio and eccentric nucleus; Pl, plasmatocyte: here it is spindle-shaped. Phase contrast microscopy. (B) Monolayer of haemocytes from third instar larva *D. melanogaster* parasitized by *Leptopilina boulardi* (Hymenoptera), after fixation at 30 s post-collection and toluidine blue staining. DPl, drosophila plasmatocyte; L, lamellocyte. Note the faint staining of lamellocytes compared to drosophila plasmatocytes, due to their flattened shape. (C)–(F) Shape modifications of plasmatocytes (Pl) and granular haemocytes (GH) of *S. littoralis* in monolayers at different times after blood removal (C: 30 s, D: 3 min, E: 6 min, F: 10 min). Note the importance of plasmatocyte spreading, compared to granular haemocytes. Note also the small scattered chromatin masses in plasmatocyte nuclei. Phase contrast microscopy.

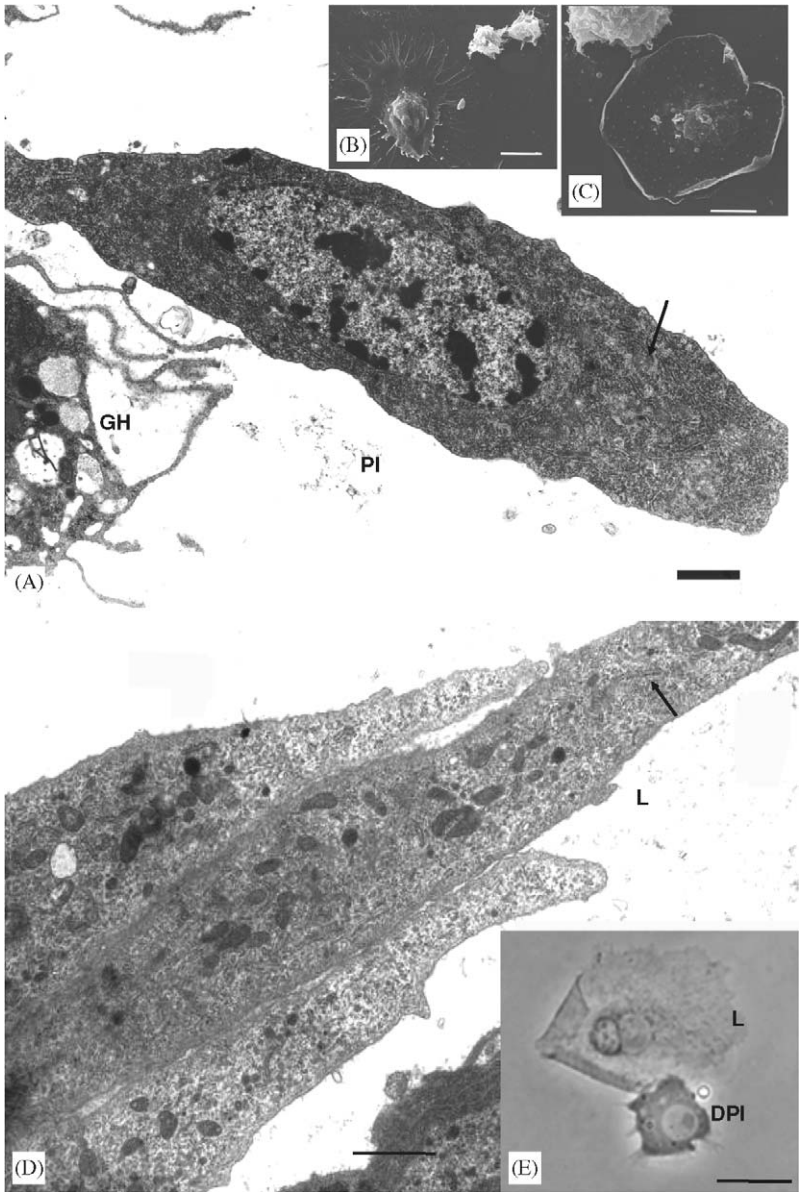

Fig. 2. Plasmatocytes of Lepidoptera and lamellocytes of *Drosophila*. (A) Plasmatocyte (Pl) from *M. unipuncta*: cell with rare pinocytotic vesicles and pseudopods (compare with granular haemocyte, GH, on the left), no granular inclusion, rough endoplasmic reticulum (RER) in narrow cisternae (arrows). TEM, bar = 1 μm. (B) and (C) SEM of the same type of cell as the plasmatocyte in (A), fixed 5 min (B) and 10 min (C) after monolayer formation. Bars = 10 μm. (D) Three lamellocytes (L) from unparasitized 80-h-old larva of *D. yakuba*. These cells have similar features to the lepidopteran plasmatocyte shown in (A). Arrow: narrow cisternae of the RER. TEM, bar = 1 μm. (E) Haemocytes of *D. melanogaster*, 5 min after blood collection. Note the flattened shape of lamellocyte (L) compared to drosophila plasmatocyte (DPl). Phase contrast microscopy, bar = 10 μm.

Zachary, 1986). The Golgi apparatus is present but often little developed. In most lepidopteran species, plasmatocytes are devoid of granules (Neuwirth, 1973; Akai and Sato, 1973; Raina, 1976; Beaulaton and Monpeyssin, 1976; Rowley and Ratcliffe, 1981; Essawy et al., 1985; Strand, 1994; Ribeiro et al., 1996; Butt and Shields, 1996), but in *Galleria mellonella*, they may contain granules (Schmit and Ratcliffe, 1977).

In monolayers, after a few minutes incubation, plasmatocytes are easy to recognize as they spread rapidly on contact with the coverslip (Figs. 1C–F) and become large and thin cells with a characteristic fibroblast-like morphology that has been described by all authors. They develop numerous pseudopodia and long and wide lamellipodia (Fig. 2B). They show a large rounded nucleus with small scattered chromatin masses (Fig. 1F). They sometimes look like the hyper-spreading cells described in *Manduca sexta* by Dean et al. (2004) but with a much smaller size (Fig. 2C).

Functions: All authors agree that plasmatocytes form the bulk of capsules around foreign bodies too large to be phagocytosed, or nodules around masses of bacteria and

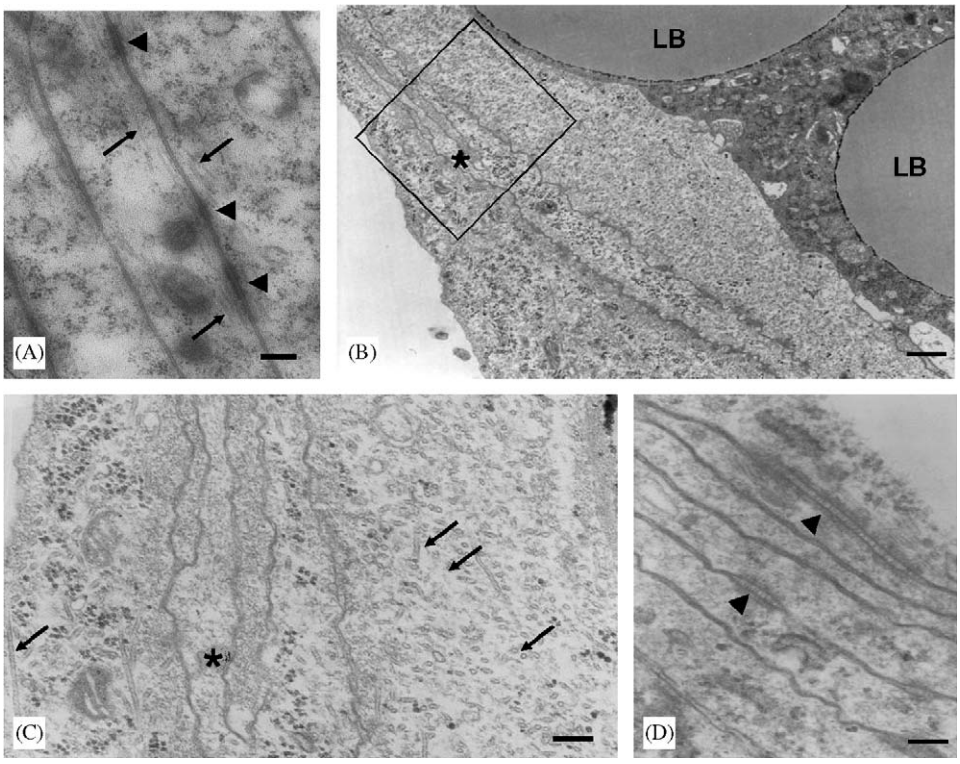

Fig. 3. TEM of capsules and nodules. Note the presence of desmosome-like junctions (arrowheads) and of numerous microtubules (arrows). (A) *S. littoralis*: nodule built by plasmatocytes, 12 h after injection of *Photorhabdus luminescens* (Enterobacteriaceae) in last instar larva. Bar = 1 μm. (B) and (C) *D. melanogaster* capsule built by lamellocytes, 48 h after injection of latex beads (LB) in third instar larva. C: enlargement of B (asterisk); B: bars = 2 μm; C: bar = 0.5 μm. (D) *D. melanogaster* capsule built around a parasitic *L. boulardi* egg, 20 h after parasitization. Bar = 1 μm.

necrotic melanised material, in vivo. Capsule and nodule formations look identical at the cytological level (Ratcliffe and Gagen 1976, 1977; Lavine and Strand, 2002). In these formations, plasmatocytes synthesize numerous desmosomes and contain large amounts of microtubules in their cytoplasm (Fig. 3A) (reviewed by Götz, 1986). The role of plasmatocytes in phagocytosis is disputed. For some authors, they are phagocytes (Ratcliffe and Rowley, 1975; Tojo et al., 2000; Ling and Yu, 2006) but for other authors they are clearly not phagocytic cells (Akai and Sato, 1973; Neuwirth, 1974; Beaulaton, 1979). Using the methods described in our papers, we have never observed phagocytosis other than in low amounts, by plasmatocytes of lepidoptera, both in vivo and in vitro (Costa et al., 2005), even in *G. mellonella*. (Figs. 4A, B; see also Brehélin and Hoffmann, 1980). The reason for this discrepancy must probably be found in the conditions of the experiments that could change the amount of material engulfed by these cells (see Section 3).

In Drosophila: The haemocytes called plasmatocytes in *Drosophila* species are not the equivalent of lepidopteran plasmatocytes. They have different histological and cytological features, different behaviour in monolayers and different functions. In *Drosophila* species, haemocytes that show the characteristics of lepidopteran plasmatocytes are called lamellocytes (Fig. 1B). These cells have a regular shape, with very rare pseudopodia and no cytoplasmic inclusions. Polyribosomes are numerous and their RER is little developed, in narrow cisternae never enlarged (Fig. 2D). Lamellocytes appear as flat and thin cells in monolayers (Fig. 2E). As in the case of lepidopteran plasmatocytes, *Drosophila* lamellocytes form the bulk of capsules and nodules in which they synthesize numerous desmosome-like junctions (Russo et al., 1996) and microtubules (Figs. 3B–D; see also Rizki and Rizki, 1979). As for lepidopteran plasmatocytes, injected particulate material is phagocytosed in very low amounts, if at all, by *Drosophila* lamellocytes (Brehélin, 1982; Lanot et al., 2001). Finally, Lanot et al. (2001) strongly suggest that, in opposition to the hypothesis of Rizki and Rizki (1980) already questioned by Brehélin (1982), lamellocytes do not originate from *Drosophila* plasmatocytes but are derived from stem cells through a specific lineage of differentiation in lymph gland. Transformation of stem cells into *Drosophila* plasmatocytes is under the control of Glial-cell-missing factors whereas lamellocyte differentiation from stem cells seems to be controlled by the JAK/STAT pathway (review in Meister and Lagueux, 2003; see also Agaisse and Perimon, 2004). For many authors, the large majority of lamellocytes in *Drosophila* (Lanot et al., 2001; Holz et al., 2003), and of plasmatocytes in Lepidoptera (Nittono, 1964; Hinks and Arnold, 1977; Gardiner and Strand, 2000; Nardi

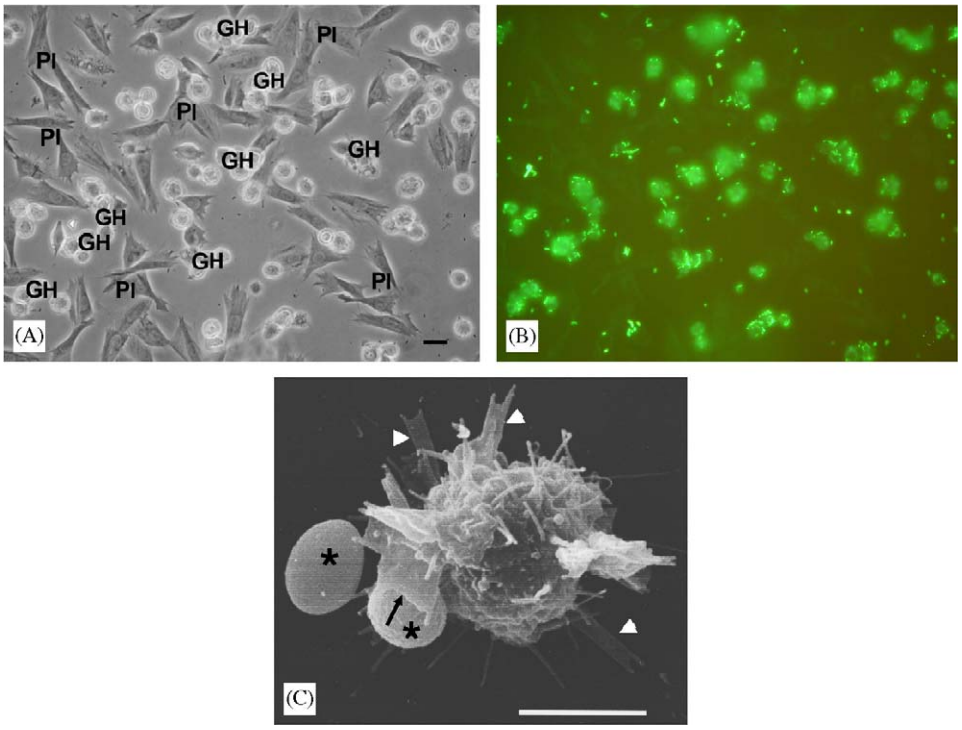

Fig. 4. Phagocytosis by lepidopteran haemocytes. (A) and (B) Monolayer of *G. mellonella* haemocytes, after 30 min incubation with FITC-labelled heat-killed *Escherichia coli*. A: phase contrast microscopy; B: epifluorescence of the same picture. Note that bacteria are fixed in very large amounts on granular haemocytes (GH) and are very few (if any) on plasmatocytes (Pl). Bar = 10 μm. (C) Mode of engulfment (arrow) of yeast (asterisk) by granular haemocyte of *M. unipuncta*, 7 min after addition of the yeast cells to the previously formed monolayer. Arrowheads: short lamellipodia allowing the cell to adhere to the coverslip. SEM, bar = 10 μm.

et al., 2003), originate from haematopoietic organs and not from divisions of circulating cells produced in course of embryogenesis before lymph gland formation.

There are, however, two major differences between plasmatocytes in Lepidoptera and lamellocytes in *Drosophila*. The first difference bears on the number of circulating cells. Numerous circulating plasmatocytes are always present in lepidopteran larvae whereas lamellocytes are very few in circulating haemolymph of healthy larvae of *D. melanogaster*. Their number increases drastically in parasitized larvae. But in some *Drosophila* species other than *D. melanogaster*, for example in *Drosophila yakuba* (Brehélin, 1982) or *Drosophila sechellia* (Eslin and Prévost, 1998), the number of circulating lamellocytes in healthy larvae is much higher than in *D. melanogaster*. Another difference between lepidopteran plasmatocytes and *Drosophila* lamellocytes concerns the modifications of the cell shape. Lamellocytes are flattened cells not only in monolayers but even when they are fixed at blood removal. This means that they are flattened, circulating cells, whereas lepidopteran plasmatocytes are rather ovoid, circulating cells that become flattened only when they participate in capsule/nodule formation or in monolayers.

2.2. Granular haemocytes

In Lepidoptera: After fixation at blood removal, lepidopteran granular haemocytes appear as spherical (diameter from 5 to 8 μm) and very refractive cells in phase contrast (Fig. 1A). In TEM, they show a developed RER in enlarged cisternae filled with flocculent material, numerous pinocytotic vesicles and most often numerous thin and long pseudopodia (Fig. 5A). Three different kinds of membrane bound inclusions have been described in these cells (see Brehélin and Zachary, 1986). Inclusions of one type, highly irregular in shape and filled with heterogeneous material, look like phagolysosomes (Fig. 5A). They originate from cytoplasmic areas in autolysis or mainly from a fusion between phagosomes and lysosomes containing hydrolases synthesized by the Golgi complex (Ribeiro et al., 1996). Inclusions of this type can become very numerous and of large size (Raina, 1976) for instance at the end of larval development (Essawy et al., 1985; Ribeiro et al., 1996; Nardi et al., 2001), and granular haemocytes overloaded with large phagolysosomes could be confused with spherule cells (see below Fig. 9A–B) in light microscopy. Inclusions of the two other types, the dense granules and the structured granules, have a rather regular rounded shape and are filled only with material synthesized by the Golgi apparatus. They represent the typical granules of this cell type. In each species studied, some granular haemocytes can release their typical granules into the medium (Beaulaton and Monpeyssin, 1976; see also Fig. 5B), for instance, at the beginning of capsule or nodule formation (review in Rowley and Ratcliffe, 1981; and see "Functions", below).

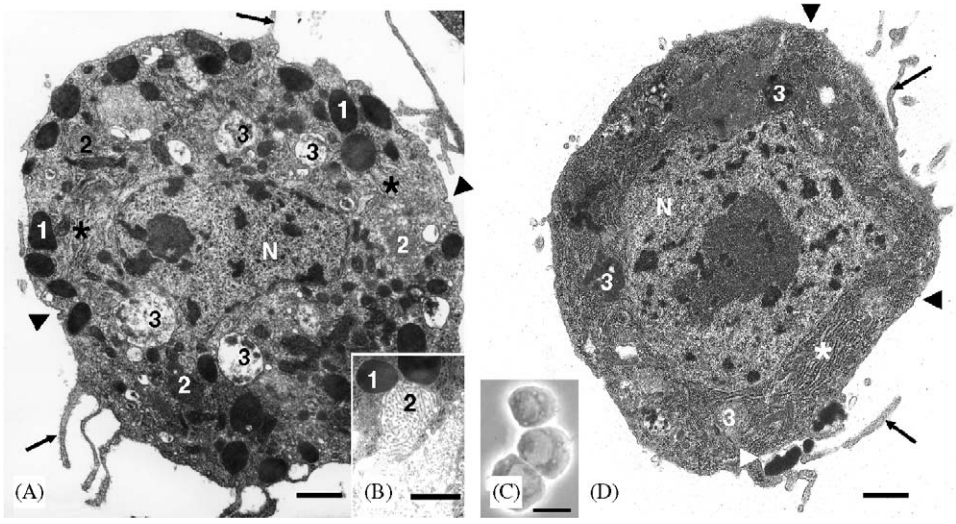

Fig. 5. Phagocytes: granular haemocytes in Lepidoptera (A and B) and drosophila plasmatocytes (C and D). (A) Granular haemocyte of *M. unipuncta*. Note the characteristic features of this haemocyte type: numerous pseudopods (arrows) and pinocytotic vesicles (arrowheads), development of the RER (asterisk), presence of three kinds of granular inclusions (1: dense granules; 2: structured granules; 3: heterogeneous bodies looking like secondary lysosomes). TEM, bar = 1 μm. (B) Exocytosis of structured granules (2) by *S. littoralis* granular haemocyte. TEM, bar = 0.5 μm. (C) Monolayers of drosophila plasmatocytes (*D. yakuba*), 10 min after blood removal. Phase contrast microscopy, bar = 10 μm. (D) Drosophila plasmatocyte of *D. yakuba*, engaged in phagocytic process. Note that all the typical features of lepidopteran granular haemocytes (see above) are observed in this cell, with exception of the dense and structured granules. But the secondary lysosome-like bodies (3) are present. TEM, bar = 1 μm.

In monolayers, most granular haemocytes remain refractive cells. They do not spread extensively but are fixed on glass by pseudopodia and lamellipodia (Fig. 4C) which sometimes encircle the cell. In incubation experiments of long duration (6 h and more), most of the typical granules of granular haemocytes are lost and the cells become much less refractive. So these granular haemocytes, deprived of granules, could be confused with plasmatocytes in light microscopy but not in TEM.

Functions: One of the main functions of granular haemocytes is phagocytosis. This has been shown by numerous authors both in vivo and in vitro (Akai and Sato, 1973, in *Bombyx mori*; Neuwirth, 1974, in *Calpodes ethlius*; Raina, 1976, in *Pectyinophora gossypiella*; Beaulaton, 1979, in *Antheraea pernyi*; Brehélin and Hoffmann, 1980, in *G. mellonella*; Wago,1980, in *B. mori*; Horohov and Dunn, 1983, in *M. sexta*; Essawy et al., 1985, in *Heliothis armigera*; Mazet et al., 1994, in *Spodoptera exigua*; Pech et al., 1994, in *Pseudoplusia includens*; Dunphy, 1995, in *G. mellonella*; Yokoo et al., 1995, in *Agrotis segetum* and *G. mellonella*; Ribeiro et al., 1996, in *Mythimna unipuncta*; Pendland and Boucias, 1996, in *S. exigua*; Butt and Shields, 1996, in *Lymantria dispar*; Tojo et al., 2000, in *G. mellonella*; Nardi et al., 2001, in *M. sexta*; Costa et al., 2005, in *Spodoptera littoralis*). Granular haemocytes have also been shown to be the first cells to come into contact, in small numbers, with a foreign body at the beginning of capsule/nodule formation. When in contact with the foreign body, they release their granular content (Akai and Sato, 1973; Ratcliffe and Gagen, 1977; Schmit and Ratcliffe, 1977). According to most authors, this exocytosis of typical inclusions by granular haemocytes serves to attract plasmatocytes (Gillespie et al., 1997) or at least help plasmatocytes to build the capsule or nodule (Pech and Strand, 1996). This exocytosis of opsonin-like material is another main function of granular haemocytes.

In Drosophila: There is no true equivalent of granular haemocytes in the haemolymph of *Drosophila* species. But we must underline that the cells called plasmatocyte in *Drosophila* look more like granular haemocytes than they look like lepidopteran plasmatocytes. Like lepidopteran granular haemocytes, *Drosophila* plasmatocytes are spherical cells (Fig. 5C) (diameter 5–8 μm) with a developed Golgi apparatus and RER in enlarged cisternae filled with flocculent material, numerous pinocytotic vesicles, thin pseudopodia and the presence of more or less numerous phagolysosome-like inclusions (Fig. 5D; see also Brehélin, 1982; Lanot et al., 2001). The major difference is the absence, in *Drosophila* plasmatocytes, of the two types of inclusions that originate from the Golgi apparatus in lepidopteran granular haemocytes. But granules of the third type described in lepidopteran granular haemocytes (see above), the phagolysosome-like bodies, are sometimes numerous in *Drosophila* plasmatocytes. In other words, the ultrastructure of *Drosophila* plasmatocytes looks like that of lepidopteran granular haemocytes deprived of two (among three) types of granular inclusions (compare Figs. 5A and D). In monolayers, *Drosophila* plasmatocytes do not spread extensively as do plasmatocytes of Lepidoptera but remain more or less spherical, adhering to the coverslip with short filopodia and lamellipodia, like lepidopteran granular haemocytes. But at the time of puparium formation, the spreading capabilities of

Drosophila plasmatocytes seem to increase (Lanot et al., 2001). In all studies published so far, *Drosophila* plasmatocytes are the main phagocytes in *Drosophila* as are granular haemocytes in Lepidoptera. Like lepidopteran granular haemocytes, at the time of larval–pupal metamorphosis, *Drosophila* plasmatocytes are overloaded with phagocytosed material (Brehélin, 1982; Lanot et al., 2001) and at this time these cells could be mistaken for Granular Cells (see Yu et al., 1976) or Spherule Cells (see below). Unlike lepidopteran plasmatocytes, *Drosophila* plasmatocytes do not build the bulk of capsules and nodules but like lepidopteran granular haemocytes they are the first cells observed, in small number, at the surface of a foreign body just at the beginning of capsule formation (Russo et al., 1996). Although granular haemocytes and *Drosophila* plasmatocytes have been observed in the respective haematopoietic organs of Lepidoptera and *Drosophila*, the bulk of these circulating cells in larvae seem to originate from outside the lymph glands (Nittono, 1964; Hinks and Arnold, 1977; Gardiner and Strand, 2000; Nardi et al., 2003; Holz et al., 2003). For all these reasons, supported by histological, cytological, behavioural and functional features, we consider that *Drosophila* plasmatocytes should be considered to be equivalent to lepidopteran granular haemocytes rather than lepidopteran plasmatocytes. A strong resemblance between lepidopteran granular haemocytes and *Drosophila* plasmatocytes was also emphasized by Nardi et al. (2003) and by Ling et al. (2005). As they are deprived of typical inclusions, the dense and structured granules, these cells cannot be called granular haemocytes as in lepidopteran species. But to avoid any confusion, in *Drosophila* we propose to call these cells "drosophila plasmatocytes" rather than just "plasmatocytes".

2.3. Oenocytoids

In Lepidoptera: After fixation, oenocytoids appear as large cells (diameter up to 25 μm) rather regular in shape (Fig. 1A), with a variable refractivity in phase contrast microscopy and a low nuclear to cytoplasmic ratio. The nucleus of oenocytoids often has an eccentric location. In TEM, the cytoplasm is filled with numerous free ribosomes but other typical cytoplasmic organelles are little developed, especially the Golgi apparatus and the cisternae of the RER which are rare, very short but often enlarged (Fig. 6A). In some species, the cytoplasm is homogenous as in *A. pernyi* (Beaulaton and Monpeyssin, 1977). In other species, areas with variable electron density are often observed in the cytoplasm of lepidopteran oenocytoids. These are rounded areas with a low density to electrons as in *M. unipuncta* (Fig. 6A) or in *H. armigera* (Essawy et al., 1985), or long areas with an internal structure in parallel fibres as in *S. littoralis* (Fig. 6B; see also Harpaz et al., 1969) or in non-mature oenocytoids of *B. mori* (Akai and Sato, 1973). These areas are never observed to be membrane bound in any lepidopteran species.

In monolayers prepared from most lepidopteran species, these cells are very fragile and rapidly lyse in vitro (Strand and Noda, 1991), but they seem to be more stable in other species such as in *G. mellonella* (Rowley and Ratcliffe, 1981). In *M. unipuncta* and in *S. littoralis*, for example, if not fixed at blood removal, oenocytoids remain visible only for a short time and literally blow up after few minutes of incubation on glass slides (Figs. 7A–C).

Functions: In Lepidoptera, one of the phenoloxidases (PO), which is responsible for haemolymph darkening (melanin synthesis), is present in these haemocytes (Neuwirth,

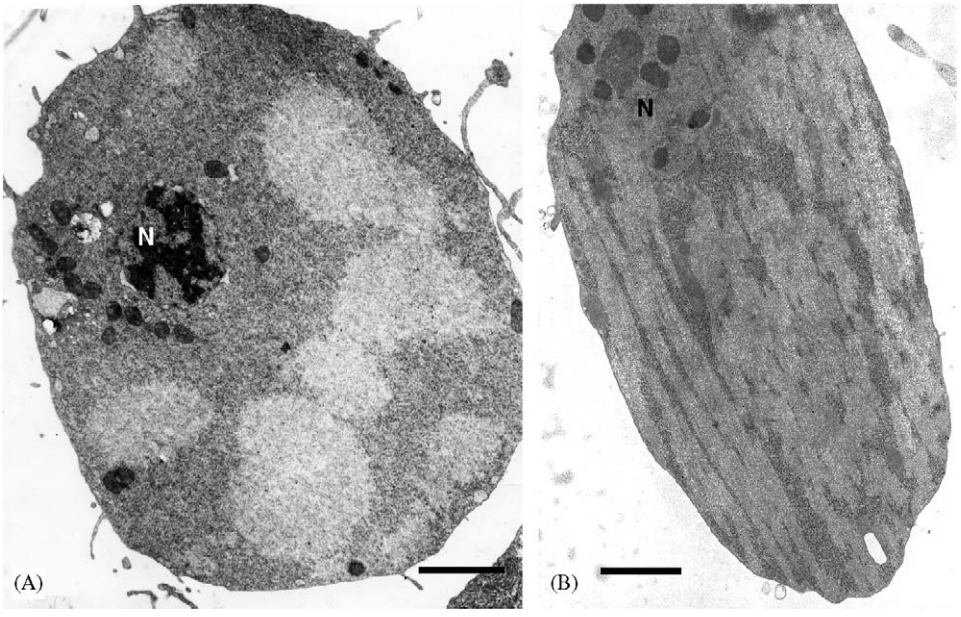

Fig. 6. Lepidopteran oenocytoids. (A) From *M. unipuncta* and (B) from *S. littoralis*. Note the regular shape of these cells, their eccentric nucleus (N) and their paucity in cytoplasmic organelles with exception of free ribosomes. TEM, bar = 2 μm.

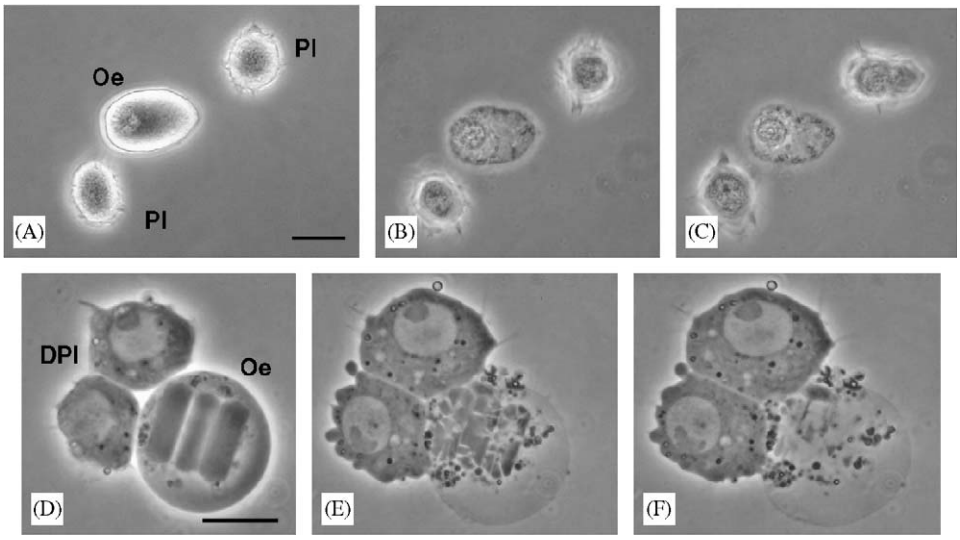

Fig. 7. Oenocytoid lysis in monolayers: comparative observation between lepidopteran and *Drosophila melanogaster*, in phase contrast microscopy. Bars = 10 μm. (A)–(C) Haemocytes were collected from *S. littoralis* larva, directly on a coverslip (A) and were observed in phase contrast microscopy. One oenocytoid (Oe) and two plasmatocytes (Pl) are seen in the field of view. After 2 min incubation (B), the oenocytoid has lost its refringency and was lysed after 3 min (C), releasing its content in the surrounding medium, whereas the two Pl began to spread on glass. (D)–(F) Haemocytes were collected from third instar larvae of *D. melanogaster* and observed as above. One oenocytoid (= crystal cell) (Oe) and two drosophila plasmatocytes (DPl) are seen in this field of view. D shows the cells at the time of collection. After 5 min incubation (E) the crystal-like inclusions begin to dissolve and after 8 min (F), the oenocytoid has released almost all its cytoplasmic content in the medium. Note that the two drosophila plasmatocytes do not spread intensively.

1973; Essawy et al., 1985; Ribeiro et al., 1996). Ashida and co-workers (Iwama and Ashida, 1986; Ashida et al., 1988) conclusively demonstrated that PO is synthesised in oenocytoids of *B. mori* and is released into the plasma when these cells lyse.

In Drosophila: No *Drosophila* haemocyte type is called oenocytoid. But a recognized haemocyte type, the crystal cell (Rizki, 1957), shows high similarities with the oenocytoid both in structure and functions. In phase contrast microscopy, crystal cells are rather large cells with a regular shape, an eccentric nucleus and irregular cytoplasmic inclusions. In *D. melanogaster*, the shape of these inclusions show sharp contour-like crystalline inclusions (Fig. 8A), but in *Drosophila teissieri*, *Drosophila willistoni*, *Drosophila pseudoobscura*, *Drosophila novamexicana* and *Drosophila nebulosa* (Rizki and Rizki, 1980) or in *D. yakuba* (Brehélin, 1982), these inclusions are rounded and do not look like crystals (Fig. 8C). In TEM, an internal paracrystalline organization is observed in the inclusions of *D. melanogaster* (Fig. 8B; see also Yu et al., 1976; Brehélin et al., 1978; Rizki and Rizki, 1980) but in *D. yakuba* these inclusions are amorphous (Fig. 8D; see also Brehélin, 1982). As in oenocytoids of Lepidoptera, the inclusions observed in the cytoplasm of crystal cells in *Drosophila* species are never membrane bound (Rizki and Rizki, 1980; Brehélin, 1982). Other cytoplasmic organelles of crystal cells show the same characteristics as those of lepidopteran oenocytoids. Especially, free ribosomes are numerous and cisternae of the RER are short and often enlarged (Figs. 8B and D). As lepidopteran oenocytoids, *Drosophila* crystal cells contain large amounts of PO (Rizki and Rizki, 1959; Shrestha and Gateff, 1982). *Drosophila* crystal cells are very fragile cells that lyse shortly after haemolymph collection in absence of fixative (Figs. 7D–F), as do lepidopteran oenocytoids.

As shown above, in different *Drosophila* species other than *D. melanogaster*, the inclusions of cells of this type do not look like crystals, so in these species, the name of "crystal cells" is inappropriate. Further, due to their histological and cytological features, their behaviour in vitro and the presence of PO, the crystal cells of *D. melanogaster* have been interpreted as a type of oenocytoid by several different authors (Rizki and Rizki, 1959; Brehélin et al., 1978; Rowley and Ratcliffe, 1981; Lavine and Strand, 2002) and we therefore propose to call them oenocytoids in place of crystal cells. In addition, the bulk of oenocytoids in Lepidoptera (Hinks and Arnold, 1977) and in *Drosophila* (Lebestky et al., 2000; Lanot et al., 2001; Duvic et al., 2002) seem to originate from haematopoietic organs.

2.4. Spherule cells

In Lepidoptera: These are rounded cells, relatively stable in monolayers, containing a small number of large inclusions (the spherules) that cause the cell to adopt an irregular shape (Fig. 9A). In TEM, these inclusions show an internal structure of either lamellated concentric layers or a crystal-like lattice of dense particles, depending on the section (see, for instance, Ribeiro et al., 1996) These cells are quite different from granular haemocytes overloaded with phagocytosed material (Fig. 9B). The functions of spherule cells are totally unknown.

In Drosophila: Although spherule cells have been observed in all Lepidopteran species studied so far, there

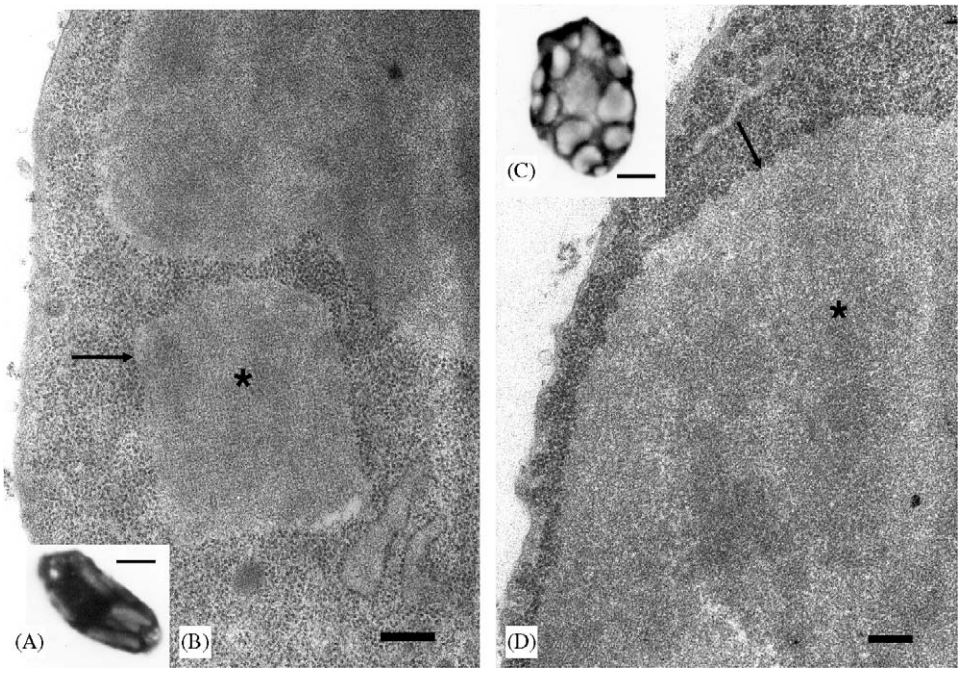

Fig. 8. *Drosophila* oenocytoids. (A) and (B) *D. melanogaster*, (C) and (D) *D. yakuba*. (A) and (C): light microscopy after fixation and toluidine blue staining. Note that the crystal-like shape of the *D. melanogaster* oenocytoid inclusions (A) is not visible in the oenocytoid from *D. yakuba* (C). Bar = 5 μm. (B) and (D) TEM. The paracrystalline lattice of inclusions (asterisk) in *D. melanogaster* oenocytoid is absent in oenocytoid from *D. yakuba*. None of these inclusions are membrane bound (arrows). Numerous free ribosomes are present in cytoplasm of both cells. Bar = 0.5 μm.

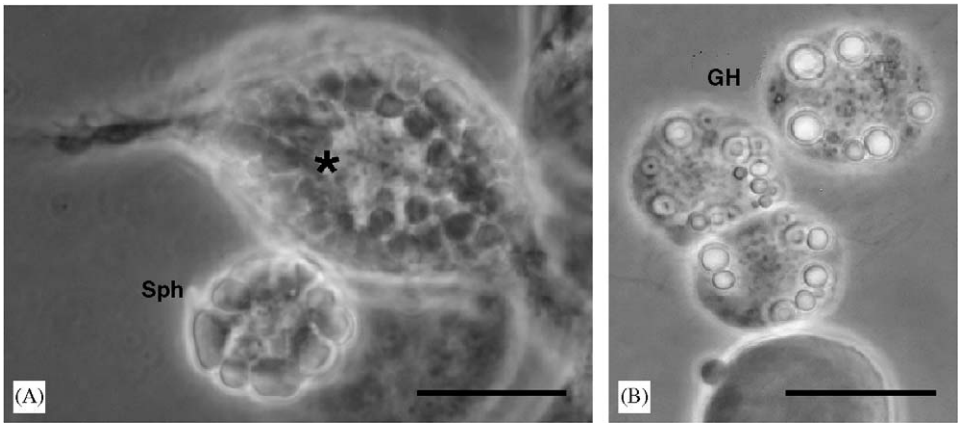

Fig. 9. Other granular haemocytes of Lepidoptera. (A) Spherule cells (Sph) are present in all lepidopteran species studied so far, but we have observed the larger cell type above (asterisk) only in *S. littoralis* haemolymph. (B) granular haemocytes (GH) of *S. littoralis* overloaded with phagocytised material at the time of metamorphosis. These cells, also observed in *Drosophila* at the end of the larval life, must not be confused with true spherule cells from which they clearly differ. Phase contrast microscopy, bar = 10 μm.

is no equivalent haemocyte of the spherule cell type in *Drosophila*. A description of "spherule cells" in different *Drosophila* species by Srdic and Gloor (1979) was a mistaken interpretation of crystal cells as has been demonstrated by Rizki and Rizki (1980).

2.5. Other circulating haemocyte types

In Lepidoptera: In all the species studied, rare small haemocytes, regular in shape and with a high nuclear to cytoplasmic ratio, have been described and called pro-haemocytes because they are believed to be precursors to differentiated haemocyte types. This interpretation is supported mostly by cytological features, and further work is needed before this hypothesis can be confirmed. In a number of species, very long and thin haemocytes have often been observed in circulating haemolymph (Fig. 10A, B). In TEM, they show numerous small (less than 0.25 μm) membrane-bound cytoplasmic inclusions. These cells, called vermiform cells or podocytes, were regarded as a kind of plasmatocytes by different authors. But as they do not appear engaged in capsule/nodule formation, and

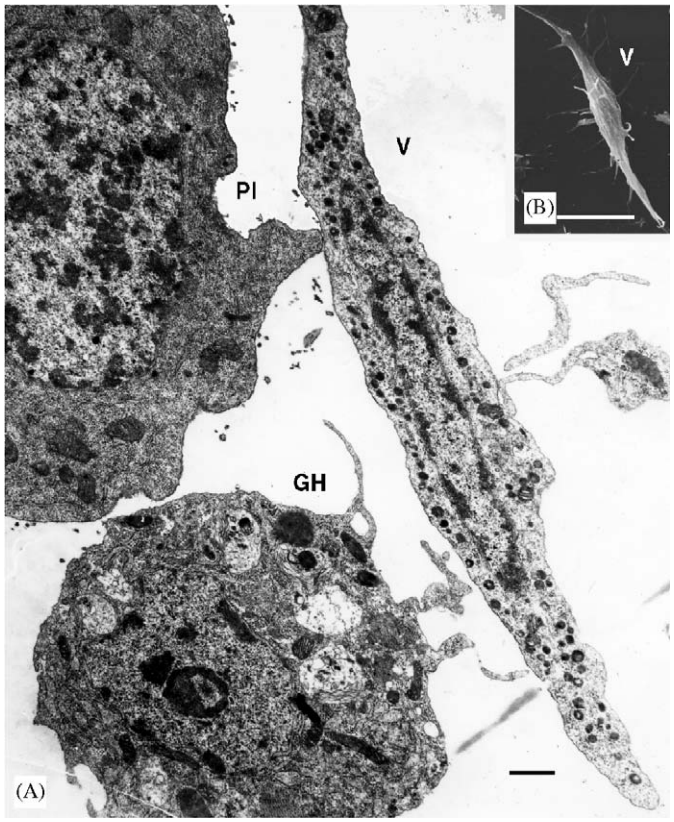

Fig. 10. Vermiform cells of Lepidoptera. These cells (V), here observed in *M. unipuncta*, are filled with small dense granules and belong to a cell type different from granular haemocytes (GH) and plasmatocytes (Pl). They are never observed in *Drosophila* species. (A) TEM, bar = 1 μm and (B) SEM, bar = 10 μm.

never spread in monolayers, we suggest that their classification as plasmatocytes remains to be proved.

Other peculiar cell types have sometimes been observed in one or a small number of Lepidopteran species. For example, large cells filled with numerous dense granules, different from both granular haemocytes and spherule cells, are observed in *S. littoralis* (Fig. 9A) but not in *Spodoptera frugiperda*.

The functions of all these cell types are unknown.

In Drosophila: Circulating cells, looking like prohaemocytes, are rare but present in *Drosophila* larvae (Brehélin, 1982; Lanot et al., 2001). There is no description of circulating haemocyte types other than lamellocyte, drosophila plasmatocyte or oenocytoid. But haemocytes belonging to one of the above three types (lamellocyte, drosophila plasmatocyte and oenocytoid), sometimes show slight differences with the features that allow the definition of each type. For instance, Lanot et al. (2001) described, at the time of prepupal stage, haemocytes that developed veil-like extensions which were not visible earlier in larval development. For Lanot et al., these haemocytes are true drosophila plasmatocytes. These authors show that the drosophila plasmatocytes with veil-like extensions sometimes have been confused with lamellocytes or with "podocytes", an hypothetical circulating intermediate form between drosophila plasmatocyte and lamellocyte (Rizki and Rizki, 1980; Shrestha and Gateff, 1982).

3. Discussion

When studying circulating cells in insects, there is no single technique allowing an exact determination of haemocyte types, and different methods in combination must be used. This means that it is necessary to know the limits of each of the methods used. As ultrastructural features are easy to observe, they are less subject to dispute than other criteria for cell type determination. We propose first to characterize each haemocyte type by their ultrastructural features in TEM, provided there are enough cells and enough thin sections observed by cell. In a second step, the different types can be found again in semithin sections, then in light microscopy after fixation of haemocytes at blood removal. In a last step, studies of cell behaviour in monolayers can also give useful criteria for haemocyte type characterization. We must keep in mind that the ultrastructure of a haemocyte, as in the case of other cells, can change depending on the physiological state of the cell and of the whole organism. So it is necessary to study the structure of haemocytes taken from uninfected insects of the same species and at different steps of its development. Monolayers can be easily observed in phase contrast microscopy and in SEM, but we must stress that the behaviour of a cell in vitro also depends on the conditions of the experiment. For example, the presence of LPS on the slides could allow a better spreading of cells than in the absence of endotoxin. Another pool of criteria come from study of the haemocyte functions. But here again we must keep in mind that the same cell type can exhibit different functions and that the same function can be achieved by different cell types. Furthermore, the conditions of experiments can also modify the function itself. For example, phagocytosis depends on numerous biological, chemical and physical factors (see below). The study of function can be used in the characterization of a cell type but this must be correlated with ultrastructure, morphology and biochemical content of the cell. This is the case, for example, for granular haemocytes and drosophila plasmatocytes, where the presence of secondary lysosomes, numerous pseudopods, acid phosphatase in vesicles from Golgi apparatus correlate with their function as phagocytes. Concerning the dispute on phagocytosis by lepidopteran plasmatocytes, Yokoo et al. (1995) and Tojo et al. (2000) have clearly shown that in *G. mellonella*, plasmatocytes did not engulf small particles (Indian ink: less than 1 μm) but were active phagocytes against particles of a large size (silica beads: 5 μm). We can hypothesize that the phagocytosis of large beads by plasmatocytes in *G. mellonella* could be in fact a beginning of encapsulation process which is one of the functions of plasmatocytes in lepidopteran insects. Ling and Yu (2006) have shown that both inert and biological particles are engulfed by granular haemocytes but only inert beads were phagocytosed by

plasmatocytes in *M. sexta*. It is evident that haemocytes belonging to two different types have the capabilities to phagocytose particulate material. One type is the granular haemocyte and drosophila plasmatocyte which have been described to engulf foreign bodies, most often in very large amounts, whatever the experimental conditions in vivo and in vitro. They could be called "insect macrophages" as they appear, in some aspects, as the functional equivalent to vertebrate macrophages. The other haemocyte type is the plasmatocyte but the capabilities of these haemocytes to phagocytose largely depend on size and nature of the particles to be engulfed and also depend on different other experimental conditions which sometimes are unknown.

It is evident that none of the different methods listed above are sufficient to characterize an haemocyte type, but we believe that a combination of different techniques (histology, cytology, cytochemistry, behaviour in monolayers, functions) can lead to the correct determination of insect haemocyte type.

Monoclonal antibodies (MAb) could also be used as tools for identifying circulating haemocyte types. Different MAbs specific for a cell type in one insect species have been raised by different workers in order to be used as tools in the same insect species (Chain et al., 1992; Mullett et al., 1993; Willott et al., 1995; Strand and Johnson, 1996; Hori et al., 1997; Gardiner and Strand, 2000). But such studies are few in number and the use of MAbs is much less conclusive when haemocytes from different species have to be compared. And even in a single species, MAb labelling is not easy to use. For instance, in *M. sexta*, Dean et al. (2004) tried to compare the hyper-spreading haemocytes to plasmatocytes using MS13, a MAb known to serve as marker of plasmatocytes in this species. Although MS13 labelled both plasmatocytes and hyper-spreading haemocytes, the authors logically concluded that "it cannot be assumed that hyper-spreading haemocytes are well-spread plasmatocytes but only that they share a particular cell-surface epitope". To use MAbs as specific markers of an haemocyte type, it is necessary to use MAbs raised against antigens shown to be specific of an haemocyte type after studying this type in course of differentiation from stem cells. In fact, identification of the signal pathways activated during haemocyte differentiation (Lebestky et al., 2000; Alfonso and Jones, 2002; Duvic et al., 2002) could be the best criterion to distinguish one cell type from another. But this technique cannot be used easily for characterization of the haemocyte types in each insect species.

Lectin labelling is subject to the same limitations as labelling by MAbs.

In conclusion, the names used in this classification of circulating haemocytes are those given to lepidopteran haemocytes in the literature and we have focused on the haemocyte types that have corresponding types in *Drosophila* larvae. Concerning circulating haemocytes of larvae in *Drosophila* species, we propose to retain the name of lamellocytes. But for the reasons discussed above, we propose to use the names drosophila plasmatocytes and oenocytoids, respectively, for the cells that are currently called plasmatocytes and crystal cells in the literature. Using this terminology, the haemocyte populations in *Drosophil*a species no longer appear to be dissimilar from those of other insects; the new nomenclature also allows easier comparison with studies of haemocytes of other species.

Acknowledgements

We thank the Editor, Prof. S. Reynolds and the referees who have helped us to improve this review. We also thank Jorge Medeiros and Pierre-Alain Girard for technical assistance. Carlos Ribeiro was funded by grants from CRUP-Portugal and OECD Co-operative Research Programme Fellowship. This work was supported by Grant from INRA (2004 1133 01).

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