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Review of haemocyte count, response to chemicals, phagocytosis, encapsulation and metamorphosis in insects

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Abstract
Cellular defences are accomplished by haemocyte-mediated responses such as phagocytosis and encapsulation. This review describes the current knowledge regarding cellular immune responses of insects of different orders at developmental stages of larvae, pupae and adults, emphasizing studies on different phylogenic groups of insects. Insect haemocytes originally evolved from mesodermally-derived stem cells that differentiated into specific cell lines, which are identified based on their forms, functions and molecular markers. In insects, most cellular defence responses involve granular cells and plasmatocytes, whereas in *Drosophila* they primarily involve plasmatocytes and lamellocytes. Insect haemocytes recognise a variety of foreign bodies as well as altered self components. Their cell-surface receptors are involved in these specific recognition events. Once a target has been identified as a foreign body, haemocyte-mediated defence responses are evoked by signaling factors and effector molecules that control cell adhesion and cytotoxicity. Several lines of evidence indicate that humoral and cellular defence responses are well coordinated with one another. Coordination between the immune system and the nervous system may also play a role in regulating inflammatory-like responses in insects during infection. The total haemocyte counts and the differential haemocyte counts vary in the different life stages. Foreign bodies and chemicals have also been reported to be factors that affect the number of haemocytes.

Keywords: *Haemocytes, blood volume, phagocytosis, encapsulation, metabolic contents*

Introduction

Haemocytes are a morphologically distinct cell type (Price & Ratcliffe 1974; Mead et al. 1986), comparable to vertebrate leucocytes (Jones 1977), that constitute important and crucial components of the haemolymph in the open circulatory system of insects (hexapods) as well as in other arthropods and invertebrates (Wigglesworth 1933, 1939, 1955, 1956, 1979; Jones 1962, 1975, 1977; Gupta 1970, 1979). These cells were first discovered by Swammerdam in 1669 (Swammerdam 1669; Jones 1962; Gupta & Sutherland 1967; Ribeiro & Brehlin 2006), and their versatile features within a species and the duplication of similar and compatible shapes amongst different species encouraged Cuenot (1896) to be the first to classify them, dividing them into four different categories (Millara 1947). The classification has been revised several times (Yeager 1945; Wigglesworth 1956; Jones 1962; Yu 1976; Gupta 1979; Al-Khalifa & Siddiqui 1979, 1985, 1994, 1999; Dean et al. 2004; Ribeiro & Brehlin 2006; Qamar & Jamal 2009; Siddiqui & Al-Khalifa 2012).

Functionally, haemocytes are the generally accepted cellular defence units in insects and are partially responsible for their immune system (Gupta & Sutherland 1967; Ratcliffe & Rowley 1979; Al-Khalifa & Siddiqui 1985, 1994; Ribeiro & Brehlin 2006; Wood & Jacinto 2007). In fact, insects and other arthropods have various physico-chemical methods to combat, check or attack the challenges from their biological enemies, which include viruses, bacteria, protozoans, fungi and cestods (Ratcliffe et al. 1976; Mead et al. 1986). Trespassing of these biological agents into the haemocoel usually elicits cellular responses, such as phagocytosis, nodule formation and encapsulation by haemocytes (Ratcliffe & Rowley 1975; Gupta 1979). The contribution of

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haemocytes to the prophenoloxidase system through the involvement of granular and/or plasmatocytes prompted Gupta (1985), Yokoo et al. (1995), Tojo et al. (2000) and Ling and Yu (2006) to label these two categories of haemocytes as immunocytes. However, the type of immunocytes present and their role in insects are debatable and more information on the subject is needed for clarity (Siddiqui & Khan 1979; Al-Khalifa & Siddiqui 1986; Hazarika & Gupta 1987; Han & Gupta 1989; Alfonso & Jones 2002). In addition to biological agents, fungi, protozoans and other pathogenic and nonpathogenic agents are known inducers of haemocytic responses such as a variation in total haemocyte counts (THC) and differential haemocyte counts (DHC) (Shapiro 1979; Christensen et al. 1989).

While considering the importance of insect haemocytes in intermediary metabolism and the role of ecdysones in regulating growth and moulting in insects, the application of exogenous ecdysones including natural (β-ecdysone) and its analogues was performed to study the haemocyte response.

However, lacunae, pitfalls and controversies regarding fundamental knowledge about insect haemocytes currently exist; thus, various aspects of insect haematology demand more investigation. This review represents an attempt to present a unified classification of haemocytes and a description of their phagocytic and encapsulation activities, variations in THC and DHC, and variations in blood volume and localisation of lipids and glycogen in haemocytes.

Therefore, the present review may certainly be useful for future research on insect haemocytes in general and in particular for phylogenic studies of haemocytes.

Haematological techniques

Separation of haemocytes

Attempts have been made to separate different types of haemocytes of a few species of insects. Jones (1977) pioneered the isolation of plasmatocytes (PLs) and granular haemocytes (GRs) in *Leucophaea maderae* L., 1758 by taking advantage of their ability to adhere to a glass surface (glass cover slips). Peake (1979) used Ficol gradients to separate *Calliphora vicinea* Robineau-Desvoidy, 1830 haemocytes according to their densities. Chain and Anderson (1982) attempted to separate of haemocyte types of *Galleria mellonella* L., 1758 by injecting *Bacillus cereus* and obtained a relatively pure population of GRs. However, the method of Mead et al. (1986) for separating different types of haemocytes by centrifugation through a Percoll gradient was demonstrated to be successful with cells from *Menduca sexta* Butler, 1875, *G. mellonella* and *Blaberus craniifer* Burmeister, 1834, and they could attain a purity up to 93 ± 1.5% for *M. sexta* and 94% for *G. mellonella*.

Total haemocyte count (THC)

In insects, most of the haemocytes or blood cells normally rest on the surface of various organs of the haemocoel although some cells circulate freely in the haemolymph. The number of these cells varies enormously in the developmental stages as well as in different physiological states (Wigglesworth 1973) in the same species.

The number of haemocytes in the haemolymph of unfixed insects is always lower in comparison to that in the haemolymph of a fixed insect (Tauber & Yeager 1935). The reason for such variation is the adhesion and coagulation of the haemocytes at the site of withdrawal. Wigglesworth (1956) and Jones and Liu (1961) counted a lower number of cells in the haemolymph of unfixed *Rhodnius prolixus* Stal, 1859, compared to the haemolymph of a heat-fixed *R. prolixus*. According to Wigglesworth (1956) the first drop of haemolymph that oozed out of *R. prolixus* had a higher number of haemocytes than the second and subsequent drops. Matsumoto and Sakurai (1956) explained that the reason for the presence of a higher number of haemocytes in the first drop of blood from *Bombyx mori* L., 1758 larvae than in subsequent drops was adhesion of the haemocytes to body tissue near the withdrawal site. He further estimated that, due to adhesion, there was a decrease of approximately 883 cells between the first and second drops of *Bombyx* larvae haemolymph, and decrease of approximately 587 cells between the second and third drop.

The THC for the haemolymph drawn from nymphs, larvae or adults of the same insect vary (Webley 1951). However, there appear to be no general pattern of larvae having more haemocytes than adults or vice versa.

In many hemimetabolous insects, the THC is highly variable throughout an individual's development (Han & Gupta 1989). Generally, the haemocytes increase in number at a relatively constant rate during the growth of larvae of holometabolous insects and reach a maximum in the pre-pupae. The THC declines very rapidly at pupation and eventually falls to a minimum level during the pupal stage. However, there is a slight increase in

the THC at adult emergence. The average THC is markedly lower in adults compared to that in larvae. In both hemi- and holometabolous insects, the THC tends to increase prior to each ecdysis, then decreases sharply at ecdysis and increases again shortly afterwards. Furthermore, the THC variations reported may be dependent on different techniques. Tauber and Yeager (1934) initiated an investigation of THC in insects belonging to Orthoptera, Odonata, Hemiptera and Homoptera (Tauber & Yeager 1935) as well as Neuroptera, Coleoptera, Lepidoptera and Hymenoptera (Tauber & Yeager 1936). According to them, the THC in the nymphs of hemimetabolous insects was lower compared to those of the respective adults. In contrast, the larval THCs were higher than those of the respective imaginal stages in holometabolous insects. They also suggested that certain physiological and pathological states of the insects such as parasitism, oviposition (or carrying ootheca) and ecdysis may be associated with excessively high total counts. The THC variation in insects has been reviewed and discussed by Munson (1953), Jones (1962, 1964, 1970, 1977), Wigglesworth (1959, 1965), and Gupta (1970, 1979).

THC in hemimetabolous insects

In normal insects

In hemimetabolous insects such as *Schistocerca gregaria* Forskall, 1775 larvae, Mathur and Soni (1937) found lower THC values than those in adults. Smith (1938), Arvy et al. (1948), and Ogel (1955) recorded that in some species of Orthoptera, the nymphs have higher THC than do the adults.

THC may also vary between the sexes of the same species (Arvy et al. 1949). In some species, the males show higher THC than the females, whereas in other insects, the reverse is true. Smith (1938) reported that male *Periplaneta americana* L., 1758 showed a higher THC than females. Arvy et al. (1949) studied the THC in mantids and found that the females had a higher THC than the males. In *Locusta migratoria migratoroides* L., 1758 (Webley 1951) and *P. americana* (Wigglesworth 1955; Jones 1964), the THC decreased at each molt and then increased again during the nymphal period. However, according to Wheeler (1963), the reason for the decrease of THC in *P. americana* at moulting may be the increase in the blood volume at this stage. The mean THC of the third stage of *Melolontha* Fabricius, 1775 larvae was 400 mm^{-3} (Collin 1963). Feir and O'Conner (1969) found that the THC of *Oncopeltus fasciatus* Dallas, 1852, increased slightly during the first 3 days after ecdysis (35,500 mm^{-3}–40,500 mm^{-3}). Bahadur and Pathak (1971) observed variations in the THC in relation to ecdysis, development and sex in the *Halys dentata* Fabricius, 1775 bug. Zaidi and Khan (1975) studied the THC of *Dysdercus cingulatus* Fabricius, 1775 and reported that the THC reached a peak level during the intermoult period and prior to metamorphosis in 5th instar nymphs. Further, the THC pattern was based on dimorphism, so that the females showed a significantly higher THC than the males of the corresponding age. Furthermore, the number of cells was maximal before mating in the males, and prior to the laying of each batch of eggs in the females. Bharvaga et al. (1980) confirmed higher THC in females than in the males of *D. cingulatus*. Furthermore, they also verified the earlier observations that there was a gradual rise in counts from the beginning of each stage in both sexes until before the next moult, when there was a sharp decrease. The number of haemocytes generally increased gradually from the 3rd nymphal instar stage to the mature adult stage. The THC in adult *Chrotogonus trachypterus* Blanchard, 1836 and *Acrida exaltata* L., 1775 varied from 1110–3020 mm^{-3} haemocytes and from 1900 to 3326 mm^{-3} haemocytes respectively (Sharma & Dutta 1979). Islam and Roy (1982) noticed that in *Schizodactylus monstrosus* Drury, 1773, THC appeared to be much lower during the day than at night.

In experimental insects

Gupta and Sutherland (1968) reported that chlordane treatment caused an apparent increase in THC of the American cockroach, *P. americana* L. Furthermore, in *Halys dentata* F., the THC decreased after extirpation of corpora allata in the early adult stage, while an abrupt increase was noted after the 7th day. When both the corpora allata and the corpora cardiaca were extirpated, the THC was significantly decreased throughout the life span of the adults. However, when only the corpora cardiaca was removed, there was no significant variation up to the 7th day, but later the THC gradually decreased. After the implantation of brain, corpora cardiaca and corpora allata, there was a significant rise in the THC throughout the life span. Implantation of the corpora cardiaca of 1-day-old adult into 1-day old-adult caused a significant rise in the THC. Thus, the corpora cardiaca influences the THC after a critical period (Pathak 1983). According to Ahmad (1986), following the injection of different doses of Makisterone A (a phytoecdysone) into the 5th instar hoppers (48 hrs old) of *Hieroglyphus nigrorepletus*

Bolivar, 1912, the THC of the female hoppers was reduced more than that of the male hoppers. Likewise, after treated hoppers moulted, the THC of the adult females was much lower than that of the males of a corresponding age. The effect on THC was dose-dependent. Qamar (1990) and Qamar & Jamal (2009) studied the comparative effects of sublethal concentrations of dichlorodiphenyltrichloroethane (DDT) (5000, 2500, 1250, 625 and 312.5 ppm), Cythion-a 90% malathion (80, 60, 40, 20 and 10 ppm) and Furadan (10, 8, 6, 4 and 2 ppm), following topical application of each concentration of insecticide to 4th and 5th instar nymphs (24 hrs old) of *Dysdercus cingulatus* on the THC of the subsequent stages as well as that of the nymphs of F1 generation. She found that the THC of the nymphs of the Fl generation did not vary compared to that of the control.

THC in holometabolous insects

In normal insects

Arvy et al. (1948) suggested that there was no significant difference in the THC of the adults, pupae and first stage larvae of *Leptinotarsa decimlineata* Say, 1824. Jones (1956) estimated the THC in *Sarcophaga bullata* Parker, 1916 and found higher values in the larvae than in the pupae. Arnold (1952a) and Nittono (1960) observed that the THC was definitely higher in larvae than in the other developmental stages of the *Anagasta* sp. Heinrich, 1956. THC variation was observed in normal as well as in different experimental conditions in *Galleria mellonella* L. (Stephens 1963; Srivastava & Richards 1964; Shapiro 1966; Jones 1967, 1979; Jones & Liu 1968). In the 5th, 6th and 7th instar larvae of *G. mellonella* L., the THC increased during larval development in both heat-fixed and unfixed larvae but the counts from heat-fixed insects were higher than those of the unfixed larvae (Shapiro 1966). The THC in the army worm, *Pseudaletia unipuncta* Haworth, 1809 were high in the moulting 5th instar larvae, but after moulting the haemocyte population decreased and continued to fall until prepupation (Wittig 1956). According to Shapiro et al. (1969) in a change in the haemocyte population occurred from the age of 6–10 days (between 23,000 and 25,000 mm^{-3}) in *Heliothes zea* Boddie, 1850 larvae. Kitano (1969) noticed that in *Pieris rapae crucivora* L., 1857 the haemocyte cell population from early 5th instar larvae appeared to be higher than that of late 5th instar larvae. Raina and Bell (1974) found that the THC of diapausing larvae of *Pectinophora gossypiella* Saunder, 1818 was significantly reduced with respect to all types of haemocytes. On termination of diapause, the changes in the THC of the haemolymph in the larval and early pupal stages of *Papillio demoleus* L., 1857 were associated with metamorphosis. According to Arnold and Hinks (1976), the cell number increased from 6000 mm^3 to 20,000 mm^3 from the 2nd to the 6th instar larval stage of the noctuid, *Euxoa declarata* Walker, 1865. Wago and Ichikawa (1979) found that there was a gradual increase in the THC from the 1st to the 3rd instar and then there was remarkable increase from the 4th to the 5th instar of *Bombyx mori* larvae. Kaaya and Otieno (1981) noticed that THC of *Glossina morsitans* Wiedimann, 1830 and *G. pallidipes* W. significantly dropped during the first 48 hrs following emergence of the adult and the values subsequently leveled off with only minor fluctuations. The sudden drop in THC resulted primarily from a remarkable decrease in the number of spindle cells in the haemolymph. Nishi (1982) recorded a gradual enhancement of the THC of *Spodoptera litura* Fabricius, 1775 from the 5th instar to the late 6th instar stage (pre-pupae) and a subsequent decrease at the pupal stage.

In experimental insects

Jones and Warner (1969) observed that when *Galleria* larvae were injected with saline, they showed lower THC (13630–21950 mm^{-3} as compared to those of normal larvae (27053–33774 mm^{-3}). Furthermore, Jones and Liu (1968) found that the THC of heat-fixed *G. mellonella* larvae was not significantly reduced over a period of 7 days after a ligature was tied behind the head, whereas the THC rapidly decreased by over 50% in the larvae that were ligatured in the thorax. However, when the larvae were ligatured in the middle of the abdomen, THC was significantly lower in the blood from both halves of the body than in control larvae. In all cases of ligatured insects, the front half of the body had significantly more haemocytes than did the posterior part. Hinks and Arnold (1977) examined 15 species of Lepidoptera and concluded that the THC was reduced in the posterior section of larvae that were ligatured in the middle. Nishi (1982) observed the effect of different doses (0.5 µg, 1.0 µg, 2.0 µg 4.0 µg and 6.0 µg) of β-ecdysone (moulting hormone) on the THC of the 6th instar larvae, pre-pupae and pupae of *Spodoptera litura*. She reported that the THC was increased in the 6th instar larvae which moulted from 5^{th} instar larvae treated with 0.5 µg and 0.1 µg doses in the pre-pupae that moulted from treated 6th instar larvae although the increases were statistically insignificant compared to the control.

However, the reduction of the THC in 6th instar larvae, pre-pupae and pupae was significant when treated with 4.0 μg and 6.0 μg β-ecdysone in their earlier stage. Rao et al. (1984) observed the effect of ligation and ecdysone on the THC in *S. litura*. Ligation of 5th instar larvae behind the thorax initially resulted in a decrease in THC in both the posterior and the anterior halves. By 48 hrs after ligation, the cell number had increased in the anterior half. They explained this by the possibility of the presence of prothoracic glands and haemopoietic organs in the anterior half of the body. Ignoffo et al. (1983) injected *Trichoplusia* sp. McDunnough, 1944 with blastopores of *Nomuraea rillevi* and noticed that the THC decreased approximately 6–7 times from an average of 8990 μL^{-1} (2 days post-injection) to a low of 1350 μL^{-1} (6 days post-injection). Ahmad (1986) further examined THC in the larvae of *S. litura* following ingestion of different doses of β-ecdysone (0.5 μg, 1.0 μg 2.0 μg, 4.0 μg and 6.0 μg) by 5th and 6th instar larvae and registered a significant drop in the THC of the larvae of the next stage. Subsequently, the THC in pre-pupae and pupae were negligible. Furthermore, injecting different doses of Makisterone A into 5th instar larvae of *S. litura* (24 hrs old) resulted in a significant drop in the THC following their moulting to the next instar stage and in the prepupal stage.

Differential haemocyte counts (DHC)

The proportion of different types of haemocytes (DHC) in the haemolymph changes during the developmental stages and life cycle of the insects. The data on the variation of DHC in insects are meager compared to those for THC (Jones 1962).

DHC in hemimetabolous insects

In normal insects

Hrdy (1958) investigated the DHC in *Acheta domesticus* L., 1758 and found that in the last moulting cycle the number of PLs decreased at ecdysis, and the number of "degenerating cells" [cystocytes (CYs)] increased while the number of other cell types fluctuated. Wheeler (1963) observed that the CYs in *Periplanata americana* increased in proportion to other cells before ecdysis, reached a maximum level at ecdysis and then decreased. Jones and Liu (1961) observed that *R. prolixus* prohaemocytes (PRs) and adipohaemocytes (ADs) increased prior to ecdysis. At the time of ecdysis, the PRs and GRs increased, whereas PLs, oenocytoids (OEs) and ADs decreased. After ecdysis, the PLs and OEs were greater in number but GRs were fewer. During a period of 9 days, *R. prolixus* took its blood meal, and the PLs increased and GRs strikingly decreased. Arnold (1969) studied the differential haemocyte counts in serial blood samples taken before and after dark from individual *Blaberus giganteus* L., 1758 adults to determine whether the haemocyte number changed regularly along with the cycle. He observed that there was a substantial increase in the relative percentage of SPs (Sherulocytes) and a somewhat proportional decrease in GRs after dark in four of the 16 individuals tested. In contrast, Roy and Bagchi (1973) reported that the percentage of PLs, PRs, GRs, SPs and spindle-shaped PLs occurred in respective descending order in *P. americana*. Zaidi and Khan (1975) stated that in adult *D. cingulatus* the percentage of PRs was very small in comparision to that of the PLs and ADs. The OEs and GRs were also poorly represented. Furthermore, the percentage of PLs was higher in the newly emerged females than in males of the same age. However, the percentage of PLs in both sexes increased with ageing, especially after the first reproductive cycle, whereas the percentage of ADs was the highest of all the cell types and was significantly higher in the males than in females following emergence. Contrary to PLs, the percentage of ADs decreased with advancing age and was low in both sexes, especially after the first reproductive cycle. In *Chrotoqonus trachypterus* L., the percentage of PLs ranged from 52 to 57%, the percentage of PRs from 26 to 32%, the percentage of GRs and CYs from 11 to 14%, and the percentage of rest of the other cell types ranged from 1 to 4% of the total free haemocytes in circulation (Sharma & Dutta 1979). Islam and Roy (1982) noted that at night the percentages of PRs and SPs in *Schizodactylus monstrosus* increased substantially over those of GRs, PLs and ADs.

In experimental insects

Jones (1967) determined the DHC in *R. prolixus* and concluded that the percentage of PLs increased and that of GRs decreased during the fasting period following each moult. After a blood meal during the 4th and 5th stage, the number of PLs was reduced and that of GRs increased. In contrast, the percentage of PLs increased and that of GRs was enhanced in adults that had fed. PRs in the mitotic phase were abundant during the first half of the period following feeding in both 4th and 5th instar larvae. Gupta and Sutherland (1968) reported that there was a decrease in the number of PLs, GRs and SPs in *P. americana* after treatment with chlordane. When 5th instar hoppers (48 hrs old) of *Hieroglyphus nigrorepletus* Bolivar,

1912 were injected with different doses of Makisterone A, all the haemocytes were pathologically affected, and after 3 days the haemocyte counts revealed that GRs were more affected than were other cell types. The PLs were the next most affected cells. Because the OEs were the most resistant cells, their percentage was eventually higher in the emerged adults (Ahmad 1986). Experiments on the topical application of sub-lethal concentrations of DDT (5000, 2500, 1250, 625 and 312.5 ppm), Cithion-a (80, 60, 40 and 20 ppm) and analogues were made to study the response of haemocytes (pathology, variations in THC and DHC) in *D. cingulatus* (Qamar & Jamal 2009), in *Spodoptera litura*, *Spilosoma obligua* Walker, 1855 and *Hieroglyphus nigrorepletus* (Khan et al. 1984) and in *Dysdercus cingulatus* and *Dicrisia obligua* (Khan et al. 1990) in projects sanctioned and financially supported by the Indian Council of Agricultural Research, Government of India, New Delhi. The comparative and extensive data obtained by a team of workers has revealed new information with regard to the action of exogenous ecdysones on insect haemocytes.

DHC in holometabolous insects

In normal insects

Jones (1967) found that during the active feeding phase, PRs and PLs were the predominant haemocytes (95%) in *G. mellonella*. During the non-feeding and pre-cocoon spinning periods, ADs have also been reported. As the spinning continued, the proportion of ADs increased and reached a peak in highly cocooned larvae and then declined. The mature ADs increased to a maximum of 57% in newly formed pupae. The SPs decreased during prepupation and were not observed in pupae. As the larvae transformed into pupae, the percentage of PLs decreased to approximately 40%. Shapiro et al. (1969) noted the increase of SPs from 38% in 5-day-old larvae to 59% in 8-day-old larvae, and then there was a decrease in the number of these cells. The PRs and PLs initially decreased from 5 to 8 days and then increased until pupation. However, the OEs remained stable at 1–2%. Nappi (1970) observed that PLs were the most numerous type of haemocytes during the development of *Drosophila euronotus* Petterson and Ward, 1952, whereas POs and lamellocytes, both variant forms of PLs, increased in percentage during larval development. OEs that first appeared in 2nd stage larvae increased in percentage up to the 3rd and final larval stage and then decreased markedly during the prepupal stage. Furthermore, Takada and Kitano (1971) recorded PLs at 4%, GRs at 54%, PRs at 1% and OEs at 4.4% of the haemocytes in 5th instar *Pieris rapae crucivora* L. 1758 larvae. Nishi (1982) observed that the percentage of PLs in *S. litura* increased from the 5th instar to late 6th instar larval stage (pre-pupal), reaching the maximal percentage of these cells which then declined in 2-day-old pupae. In contrast, the percentage of PRs decreased at the prepupal stage as compared to those of PLs and PRs, which reached their maximal percentage in the pupal stage. The cell density of ADs was maximal at the prepupal stage. The percentage of SPs was higher in 6th instar larvae than in 2-day-old 5th instar and late 6th instar larvae.

In experimental insects

Jones (1957) found a low percentage of phagocytic cells, PLs in DDT-treated meal worm larvae. Takada and Kitano (1971) observed changes in the DHC in *Pieris rapae crucivora* following the injection of India ink, with an increase in GRs and a proportionate decrease in PLs. The number of PRs increased, reaching a peak 24 hrs post-injection, and then decreased. Raina and Bell (1974) found that during the diapause of *Pectinophora gossypiella*, the percentage of each haemocyte cell type was lower in the diapausing larvae than in the non-diapausing larvae. Later, Hinks and Arnold (1977) examined 15 species of Lepidoptera and recorded a more marked decline in PRs and PLs than in other types of haemocytes, when larvae were ligatured in the middle of the body. Larvae with two or more ligatures usually had a higher percentage of PRs and PLs in the parts of the body that included the haemopoietic organs. The inclusion of the head and thorax in a section resulted in still higher counts of PRs and PLs as well as higher mitotic indices. Nishi (1982) observed the effect of different doses (0.5 μg, 1.0 μg, 4.0 μg and 6.0 μg) of β-ecdysone (moulting hormone) on the DHC of *S. litura* and reported that following application of these doses on 5th instar larvae and after they had moulted, the percentage of PLs increased in 2-day-old 6th instar larvae and pre-pupae, whereas after injection of 0.5 μg and 1.0 μg doses, the percentage of PRs was enhanced in 6th instar larvae and pre-pupae but declined in 2-day-old pupae. The percentage of ADs decreased subsequent to the application of a 0.5-μg dose to 6th instar larvae, pre-pupae and pupae. The percentage of POs increased in 6th instar larvae but declined to a negligible number in the pre-pupae and pupae. The OEs were the only cells resistant to β-ecdysone because their percentage was not much affected even by the

injection of the strongest dose (6% μg β-ecdysone larva^{-1}).

Ahmad (1986) reported that when different doses of β-ecdysone were ingested by the 5th instar larvae (24 hrs old) of *S. litura*, the percentage of PRs increased because there was less damage to these cells and more destruction of other cells in the stage following moulting. Further intensive damage occurred to ADs, CYs and SPs, and their percentage became negligible in pre-pupae. The changes were dose-dependent. When the treated larvae reached the pre-pupal or pupal stage, they showed a very high percentage of PRs compared to PLs, whereas other cell types were almost absent. Similarly, when 5th instar larvae of *S. litura* were injected with different doses of Makisterone A, following their moulting into 6th instar larvae, the percentages of ADs, CYs and SPs were zero even with the weakest dose (0.5 μg larva^{-1}). PRs were more abundant than PLs but OEs still existed at a higher percentage due to their resistance to this chemical (Ahmad 1986).

Metabolic contents of haemocytes

Occurrence of protein, lipid and carbohydrates

Haemocytes are the known sites of intermediary metabolism and storage of certain metabolites. Various metabolites have been histochemically detected in the haemocytes of a number of insect species. Carbohydrates are stored in the haemocytes as mucin (neutral glycoprotein) or in an acidic form with sulphate or sialic acid residues (Ashhurst 1979). The presence of lipids in insect haemocytes was reported in only a few species. Babers (1941) assayed the distribution of glycogen between the haemocytes and plasma of *Prodenia* larvae following feeding on a glucose-starch paste and concluded that most of the glycogen was deposited in the haemocytes. Yeager and Munson (1941) histochemically detected the presence of glycogen in the PLs and CYs of *Prodenia eridenia*. They stated that blood cell glycogen increased during normal larval development until it attained a maximum in the pre-pupae. The blood-cell glycogen then rapidly decreased, remained at a low level during most of the pupal period and tended to disappear towards the end of this period. In the 1st instar and most likely in the adult, blood-cell glycogen occurred very infrequently. In the same species, administration of poisons such as arsenicals, fluorides and mercuric chloride resulted in a decrease or loss of blood-cell glycogen (Yeager & Munson 1942). Glycogen was reported in the haemocytes of *G. mellonella* by Ashhurst and Richards (1964), who used the Periodic Acid Schiff's Reagent (PAS) method, but they failed to detect glycogen in the haemocytes of *Blaberus giganteus*. Similarly, Costin (1975) could not find glycogen in the haemocytes of the 5th instar hoppers of *Locusta migratoria* using the PAS diastase method. In contrast, Brehelin et al. (1975) detected glycogen in abundance in the GRs of 5th instar hoppers of *Locusta* sp. using electron microscopy; in this case glycogen was undetectable by the histochemical technique due to the presence of abundant polysaccharide or mucoproteins that masked it. Crossley (1975) was of the opinion that glycogen, especially in its B-form, has a distinctive ultrastructure. Furthermore, glycogen was observed in the haemocytes of *R. prolixus* (Lai-Fook 1968), *Calliphora erythrocephala* (Crossley 1968), *Blaberus giganteus* (Moran 1971), and *Antheraea pernyi* (Beaulaton & Monpeyssin 1976). However, glycogen could not be detected in the SPs of *G. mellonella* using the PAS test (Ashhurst 1982). Ahmad (1986) selected 17 species of insects belonging to Orthoptera, Dictyoptera, Hemiptera, Lepidoptera, Diptera, Hymenoptera and Coleoptera to investigate the occurrence of glycogen with a histochemical method (PAS test) but found it only in the haemocytes of the larvae of the lepidopterous species *S. obligua* and *S. litura* from 3rd instar to the early pupal stage. In the former species, glycogen was observed in the form of fine granules as well as small to large magenta-coloured inclusions of different shapes, whereas in *S. litura* larvae the glycogen deposits were in fewer cells and occurred only in the form of inclusions. After topical application of sublethal concentrations of DDT and Furadan to 3rd instar larvae of both species, glycogen deposits in their haemocytes tended to disintegrate and progressively decreased in the subsequent developmental stages, and the effect was dose-dependent.

Phagocytosis

One of the functions of the haemocytes is to engulf foreign particles of the blood, i.e. "phagocytosis", which has been experimentally demonstrated by injecting India ink or small biological materials such as fungi into the haemocoel. Metalnikov and Chorine (1929) were the first to demonstrate phagocytosis by haemocytes (using *G. mellonella*), and since then a number of reports on this aspect of insect haematology have been published (Robinovitch & DeStefano 1970; Ratcliffe & Rowley 1975, 1979). However, the phagocytic activity of insect haemocytes after the injection of fungi into the haemolymph has been studied in only a few insects. Bioczkowska (1935) demonstrated the phagocytosis of *Meterrhizium anisopliae* fungal spores of the haemocytes

of *Galleria mellonella*. Sirotina (1961) studied the phagocytosis of *Beauveria bassiana* Bals-Criv by the haemocytes of *Leptinotarsa decemlineata* Say, 1824, which eventually caused a reduction of the PL population. In contrast, injection of *Aspergillus flavus* and *Aspergillus niger* into the haemolymph of *Cecropia* sp. was followed by an increase in the total haemocyte counts. Sussman (1952) recorded phagocytosis of *Aspergillus flavus* by the haemocytes in *Hyalophora* sp. and mentioned the disintegration of cell membrane in the phagocytic cells. However, Lea and Gilbert (1966) observed an increase in the number of circulating cells in the same species of wax moth due to a fungus infection and on this basis they asserted that the blood profile of an insect may reflect a healthy or morbid condition.

Encapsulation and effect of Protozoa

Apart from phagocytosis, insect haemocytes are also known for a defence mechanism in which blood-borne foreign biological bodies that are generally larger than those engulfed by phagocytosis are encapsulated (Ratcliffe & Rowley 1979; Wigglesworth 1979). According to Salt (1970), encapsulation involves the recognition of the foreign nature of a material through the release of chemicals by the haemocytes and subsequent sensing of the interaction with the foreign object. Nappi and Stoffolano (1972) studied *Musca domestica autumnalis* L., 1758 and supported the idea of Salt (1970), which was further confirmed by Crossley (1975) and Nappi (1975). According to Gupta (1985), the GRs are normally involved in the encapsulation of foreign material; thus, he labeled them immunocytes. Later, Gupta (1970) and Gupta and Han (1988) studied the German cockroach, *Blattella germanica*, and explained the contribution of the GRs, which had a higher number of microtubules and nuclear pores during encapsulation. Furthermore, Han and Gupta (1989) studied encapsulation by GRs after surgical suture of the nerve cord and plain gut of *Blattella germanica* and observed 20 subsequent layers of these haemocytes involved in encapsulation by electron microscope.

Pathological effect of the chemicals

The effects of application of the chemicals including insecticides on the morphological and histological characteristics of haemocytes in a number of insects were studied with histological methods. However, data on this aspect of insect haemotology are still inadequate to arrive at generalization regarding the toxicity of any one chemical or different chemicals on the haemocytes of a particular group of insects.

The effect of chemicals on haemocytes was investigated for the first time by Mcindoo (1917), who studied the exposure of *Apis mellifera* Eschscholtz, 1822 to nicotine vapours and observed overvacuolisation of its haemocytes. However, Tareeva and Nonjukov (1931) found abnormally large haemocytes in the grasshopper *Calliptamus italicus* L., 1758 due to the toxic effect of sodium arsenate. Shull et al. (1932) studied the effect of some toxic gases and vapours (fumigation) of ammonia, carbon disulphide, carbon tetrachloride, chloroform, 1,2, dichloroethane, hydrochloric acid, acetic acid, amyl acetate, trichloroethane, tetrachloroethylene, diethyl ether, hydrogen cyanide, benzene, nitrobenzene, chlorobenzene, paradichlorobenzene, cyclohexane, bromocyclohexane, methyle cyclohexane, xylene, cymine, toluidine, dimethylaniline, diethyl amine, methylsalicylate, pyridine, nicotine, nephthaline, tetrahydronaphthalene, limonine and D-camphor. They reported granulation of the cytoplasm and low encapsulation, and self and nonself recognition and variation in the THC and DHC of *Adesmia cancellata* Klug, 1830 haemocytes. The response of haemocytes to deoxyphylotoxin was also studied in *Leptocorisa varicornis* Fabricius, 1775 and *Nepa cinereia* L., 1758, and the applicability of a useful technique for separating different categories of haemocytes was investigated in *A. cancellata*. However, the selection of *N. cineria*, *L. varicornis*, *Dermestes vulpinus* Sturm, 1826 and *A. cancellata* was based on the absence of knowledge regarding the haemocytes of these species. Pilot (1935) also did not observe any changes in the haemocytes of locusts when these were exposed to sodium arsenite or sodium fluoride (0.1% and 0.2%). However, he reported the disintegration and destruction of haemocytes and an increased number of mitotically dividing haemocytes.

Lepesme (1937) reported frequent mitosis, vacuolisation and the complete breakdown of *Schistocerca gregaria* haemocytes from contact application of sodium arsenite. However, Woke (1940) did not find any change in the haemocytes of the southern army worm, *Prodenia eridania*, due to the oral consumption of phenothiazine. Yeager and Munson (1942) observed a clear response of the haemocytes of *P. eridania* following exposure to nicotine bentonite, rotenone, nicotine, peat, pyrethrum, phenothiazine, barium fluorosilicate, sodium fluoroaluminate, sodium fluoride, mercuric chloride, calcium arsenate, calcium arsenite, arsenic trioxide and lead arsenate, and reported a low glycogen concentration in the haemocytes. Furthermore, Yeager et al. (1942) observed a toxic effect of sodium arsenite on *P. americana* haemocytes, reporting 87%

mortality of India-ink-injected roaches and 40% mortality in normal roaches, and concluded that India ink impeded the efficiency of haemocytes to combat sodium arsenite. Arvy et al. (1950) observed cytolysis and nuclear deformation following oral consumption of DDT by the beetle *Leptinotarsa decemlineata* Say, 1824. Arnold (1952b) also observed cytological changes in the haemocytes of the Mediterranean flour moth, *Ephestia kuhniella* Zeller, 1879, following fumigation by dichloroethyl ether, carbon tetrachloride and methyl bromide. Swelling of the chromatin caused enlargement of the nuclei of *Pediculus humanus* L., 1758 haemocytes following topical application of carbon tetrachloride, and prolonged exposure caused lysis of the nucleoplasm and cytoplasm (Hopp 1953). Bandhopadhyay (1970) reported increased vacuolisation in the nuclei of *P. americana* haemocytes. Roy and Bagchi (1973) investigated the effects of parathion, endrin and thiodan on *P. americana* haemocytes and noticed granulation of the cytoplasm as well as increased vacuole formation. Bharvaga and Pillai (1976) found cellular changes in haemocytes following a topical application of a chemosterilant, apholate, on *Dysdercus koenigii*. Zaidi and Khan (1977) studied the effect of a topical application of technical aldrin and dipterex on the haemocytes of *D. cingulatus* Fabr. and observed pathological effects on different haemocyte cell types. The ADs and GRs were found to be the most susceptible cells even to the weaker concentrations of these chemicals, whereas OEs were the most resistant cell type remaining unaffected even by the strongest concentrations of these compounds. The pathological effect on the haemocytes was demonstrated by the formation of cytoplasmic extensions, abnormal vacuolisation, dispersal of the cytoplasmic contents and swollen nuclei. Behura and Dash (1978) observed increased vacuolisation in the haemocytes of some aphids following the application of 0.01% solutions of dimethoate, fenitrothion, parathion, methyl demeton or thiometon. Furthermore, these haemocytes of the affected aphids showed ruptured cell membranes, broken nuclei and shrinkage of the cell membranes. Nishi (1982) injected different doses of β-ecdysone (0.5 μg, 1.0 μg, 2.0 μg, 4.0 μg and 6.0 μg larva^{-1}) into the larvae of 5th and 6th instars (24 hrs old) of *Spodoptera litura* Hubner, 1808, and found that in the subsequent stages the haemocytes were pathologically affected in a dose-dependent manner, showing dislocation of the nucleus, cytoplasmic and nuclear vacuolisation, rupturing of the cells and finally complete disintegration. Farks (1984) found that injecting cholesterol, hydrocortisone or 10% glucose stimulated mitotic division of the haemocytes of *G. mellonella* and *Tenebrio molitor*. Ahmad (1986) performed a detailed comparative study of the haemocytes of *Spilosoma obligua* and *Spodoptera litura* following the topical application of sub-lethal concentrations of DDT and Furadan (an organophosphate). Following the application of sub-lethal concentrations of DDT (0.2, 0.3, and 0.4%) on the 3rd instar larvae of *S. obligua* Curtis, 1825, the ADs were the most damaged cell type in subsequent stages of development and were adversely affected by even the weakest concentration, whereas the OEs were the most resistant cells and were not completely damaged even by the strongest concentration (Ahmad & Khan 1987). In the case of *S. litura* larvae (3rd instar), the sub-lethal concentrations of DDT were 0.05, 0.1, and 0.2%. Following the application of these concentrations of DDT, there was concentration-dependent damage in all of the cell types in the subsequent stages. However, the OEs were comparatively the least affected even at the strongest concentration. Following the application of sub-lethal concentrations of Furadan (0.0125, 0.025, and 0.05%) on 3rd instar larvae of *S. obligua*, all types of haemocytes, but particularly the ADs and PLs, were damaged in the subsequent stages even at the weakest concentration (Ahmad & Khan 1987). When sub-lethal concentrations of Furadan (0.1, 0.2, and 0.3%) were topically applied on the 3rd instar larvae of *S. litura*, the PRs in the subsequent stages of development were comparatively undamaged, whereas PLs, POs and GRs were more damaged. The CYs and SPs were more rapidly affected than were other types of cells. In general, the pathological effect on all types of haemocytes was concentration-based (Ahmad 1986). In addition, Ahmad (1986) demonstrated that after feeding different doses of β-ecdysone (0.5 μg, 1.0 g, 2.0 μg, 4.0 μg and 6.0 μg larva^{-1}) to 5th instar larvae of *S. litura*, all types of haemocytes except the OEs were damaged in the 6th instar larvae. However, the ADs, CYs and SPs were intensely damaged even by the intake of 0.5 μg β-ecdysone. In contrast, the ingestion of these doses of β-ecdysone by the 6th instar larvae caused insignificant damage to the PRs even at the strongest dose. Cells other than OEs were most affected (Ahmad 1986). Ahmad and Khan (1988) demonstrated the toxic effect of sub-lethal concentrations of Triol (an analogue of ecdysone) and Makisterone A by injecting these insecticides into the haemocoel of 5th instar hoppers of *H. nigroreplectus*. They observed selective damage of the haemocytes 72 hrs after injection, but Triol was more effective than Makisterone A. Khan et al. (1990) summarised the toxic effects of Triol and Makisterone A on *D. cingulatus* and *Diacrisia obligua*

Walker, 1855 and described pathological changes in the haemocyte number and the DHC in two successive generations of these species following injection of sub-lethal doses of these compounds.

Discussion

Circulating haemocytes of different phylogenetic orders have been extensively studied although lacunae, discrepancies and pitfalls are destined to remain in this field. To achieve uniform terminology, the ultra-structural techniques of transmission electron microscopy (TEM) and scanning electron microscopy (SEM) should be applied to studies and, simultaneously, the physical condition of an insect should be closely monitored before preparing specimens for study because myriad factors are known to affect the shape and size of haemocytes. The hypothesis is that reaching an agreement on uniform terminology and the nature of the effects of biotic and abiotic factors would facilitate communication and information regarding the study of types of haemocytes. A common criterion to classify a haemocyte is based on structure fitting the function. Haemocytes in the open circulatory system play key roles in survival of insect species, and are of extreme economic and medical importance. However, there is no single method to determine the haemocyte cell types, so different methods must be used (Ribeiro & Brehlin 2006). Functional studies of phagocytosis and encapsulation depends on several factors before reaching conclusions, such as the size of a particular particle such as 1-μm thick India ink particles and 5-μm diameter silicon beads, for example in G. *mellonella* haemocytes, which easily engulf the silicon beads but fail to take in India ink particles (Tojo et al. 2000). Furthermore, phagocytosis depends on the formation of vesicles by the Golgi apparatus, and on lysosomes. The concept that haemocytes are interchangeable in their forms and structure in the different phases of the life cycle has been established by reports of the THC and DHC in different species. Ling and Yu (2006) studied phagocytosis and reported that biological particles are phagocytosed by granular haemocytes, whereas inert beads are phagocytosed only by plasmatocytes.

Monoclonal antibodies (MAb) should also be studied as tools for identifying the different categories of circulating haemocytes; such studies are scarce (Gardiner & Strand 2000). Use of the MAbs as specific markers of antigens shown to be specific for haemocytes must be based on studying the signaling pathways activated during differentiation (Lebestky et al. 2000). Another approach is lectin labeling, which has limitations similar to labeling by MAbs.

To conclude, the diverse nomenclature of haemocytes should be resolved by comparing the features of different cell types, and, clearly, there are far more similarities in the haemocyte profiles of different species than disparities; this should be resolved with consistent and uniform criteria for characterisation.

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