Phagocytic Activities of Haemocytes Separated by Two Simple Methods from Larvae of Two Lepidopteran Species, 
*Agrotis segetum* and *Galleria mellonella*

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Differential cell fractionation (D.F.) and nylon wool column (N.W.) methods, both utilizing different calcium ion requirements among immunocytes for adhesion to non-self, were adopted to prepare immunocyte monolayers from *Agrotis segetum* and *Galleria mellonella* larvae. Monolayers prepared by the D.F. method were occupied predominantly by granulocytes, and those prepared by both D.F. and N.W. methods showed high purities of plasmatocytes (PL) and granular plasmatocytes (GPL). Granulocytes separated by the D.F. method in *vitro* showed high phagocytic activities against Indian ink particles and FITC-labeled silica beads, while PL and GPL prepared by both methods actively engulfed only FITC-labeled silica beads, similarly to granulocytes, but they could not phagocytose the Indian ink particles. Essential similar results were obtained with monolayers from the two lepidopteran insects.

**Key words:** phagocytosis, granulocytes, plasmatocytes, granular plasmatocytes, haemoctye monolayer, insect immunity, *Agrotis segetum*, *Galleria mellonella*

INTRODUCTION

Among insect haemocytes, granulocytes and plasmatocytes in particular participate in cellular defence reactions to non-self substances by phagocytosis, encapsulation or nodule formation, but the haemocytes involved in these defence reactions have been reported to differ among insect species (GÖTZ and BOMAN, 1985; GUPTA, 1991). Because cell morphology involved in immune reactions is diverse in nature, there may be a high risk of being misled by the difficulties in distinguishing them by shape, size and inclusions. Thus, it is highly desirable to carry out experiments with monolayers composed of a specific cell type to elucidate their immunocytic function in insects.

Granulocytes and plasmatocytes from whole insect haemolymph readily settle out and attach to a glass surface. By utilizing the specific property of immunocytes to attach to non-self, i.e., the requirement of calcium ion for plasmatocytes, and the requirement of prophenoloxidase cascade activation for granulocytes, WAGO and ICHIKAWA (1988) succeeded in establishing a method to separately prepare both cell monolayers in *Bombyx mori*. In *in vitro* culture system, only granulocytes have been shown to take up foreign substances like mammalian erythrocytes and latex particles (WAGO and ICHIKAWA, 1988; WAGO, 1991).

Recently, a simple method to separate plasmatocytes after removing granulocytes by adhesion of the latter cells to nylon wool in the presence of anti-coagulant buffer with EDTA-citrate was successfully adopted to *Galleria mellonella* (WIESNER and GÖTZ, 1993).

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The plasmatocytes prepared by this method were found to actively engage in phagocytosis of hydrophobic silica beads (WIESNER and GÖTZ, 1993), and fluorescein-isothiocyanate (FITC)-labeled yeast cells (ROHLOFF et al., 1994).

In the present study, we compared the phagocytic activities of immunocyte monolayers prepared according to the two methods mentioned above from _Agrotis segetum_ and _G. mellonella_ larvae.

**MATERIALS AND METHODS**

**Insects.** Larvae of _A. segetum_ were reared on an artificial diet devised by KΟJIMA and NAKAYAMA (1979), at 25°C under a 16 h light and 8 h dark photo-regime. Larvae of _G. mellonella_ were reared on honey bee nest debris at 25°C under darkness. Two or 3 day-old final (sixth) instar larvae of these two species were used for the experiments.

**Buffers and reagents.** Insect tissue culture medium (GRACE’s medium) was purchased from Gibco Laboratories. Anti-coagulant buffer was prepared according to LEONARD et al. (1985): 93 mM NaCl, 100 mM glucose, 30 mM trisodium citrate, 26 mM citric acid and 10 mM Na₂-EDTA, pH 4.6. Insect physiological saline (IPS) was prepared according to WAGO and ICHIKAWA (1988): 150 mM NaCl and 5 mM KCl, pH 6.9. FITC-labeled silica beads (Silasorb 300 amine; diameter, 5 μm) were a kind gift from Dr. Vladimir MATHA (Galena Co., Ceske Budejovice, Czech Republic). Nylon wool fiber and trypan blue were purchased from Funakoshi and Katayama Chemical Co. Ltd., respectively.

**Preparation of immunocyte monolayers.** Before bleeding, the larvae were chilled at 4°C for 30 min and surface sterilized with 70% ethanol.

a) Differential cell fractionation (D.F.) method. This was done according to WAGO and ICHIKAWA (1988) with some modification. The ventral abdomen of the larvae was immersed in 5 ml of IPS with 30 mM Na₂-EDTA and a few crystals of phenylthiourea (PTU), the pH of which was adjusted to 6.9 by addition of 0.1 M Tris-HCl, pH 6.9, in a 4.2 cm Petri dish containing three round cover glass slips (d. 18 mm) at the bottom. Haemolymph from two _Agrotis_ or six _Galleria_ larvae was bled into the buffer by using a scissor to cut the prolegs which were directly immersed in the buffer. In the presence of EDTA, granulocytes (GR) adhered to glass surface, but plasmatocytes (PL) and granular plasmatocytes (GPL) remained floating in the suspension. After the specified time intervals at a constant temperature, the suspension was transferred to a centrifuge tube after gentle pipetting, and centrifuged at 40 × g for 5 min at 4°C. The haemocytes thus collected were resuspended in 1 ml of GRACE’s medium and transferred to a Petri dish containing 4 ml of GRACE’s medium with three round cover slips, on which PL and GPL settled and formed a monolayer. The slips with a GR monolayer were washed twice with 3 ml of GRACE’s medium in another Petri dish, and then maintained in GRACE’s medium for further experiments.

b) Nylon wool column (N.W.) method. Plasmatocyte monolayers were prepared by the method described by WIESNER and GÖTZ (1993). A 5 ml disposable syringe was filled with 80 mg of loosely packed sterilized nylon wool. Beforehand, the column was rinsed three times with 3 ml of anti-coagulant buffer, and then 3 ml of the buffer was added on the top of the wool layer. Haemolymph bled from two _Agrotis_ larvae or six _Galleria_ larvae was immediately mixed with a few crystals of PTU, added onto the buffer layer of the syringe column, and allowed to settle for 15 min, during which most of the GR adhered to the nylon wool. After the column was opened, non-adherent cells were collected in a centrifuge
tube. The eluates were collected together in the tube after two rinses with 3.5 ml of anticoagulant buffer. The haemocytes were centrifuged at 40 × g for 5 min at 4°C, and then resuspended in 1 ml of GRACE's medium. The suspension was added to a Petri dish containing 4 ml of GRACE's medium and three round cover glass slips, and incubated at a constant temperature to allow formation of a PL + GPL monolayer on the slips.

Phagocytic activity. The assay was conducted essentially according to the method described by ROHLOFF et al. (1994). Ten microliters of GRACE's medium suspension containing

Fig. 1. Photographs of haemocyte monolayers prepared from A. segetum (left figures) and G. mellonella (right figures) larvae. Granulocyte (GR) monolayers were prepared by differential cell fractionation (D.F.) method, and plasmocytes (PL) with granular plasmocytes (GPL) monolayers by the D.F. or nylon wool column (N.W.) method. GR monolayers: a, d; the PL+GPL monolayers by the D.F. method: b, c; the PL+GPL monolayers by the N.W. method: e, f. Bar is 50 μm.
FITC-labeled silica beads or Indian ink particles at a concentration of $2 \times 10^5$/ml was gently layered onto each cover slips immersed in GRACE's medium in a Petri dish, and the cell monolayers were incubated at a constant temperature. After the specified time intervals, the slips were transferred to other Petri dishes, and treated with 100 µl 0.2% of trypan blue solution in GRACE's medium to check cell viability (dye exclusion method). The slips were incubated for 5 min at room temperature, and then washed twice with GRACE's medium. After fixing with 4% formaldehyde, the number of cells phagocytosing FITC-labeled silica beads or Indian ink particles, was counted under a light microscope in three different fields (0.1 mm²), and phagocytic activity was evaluated on the basis of the averaged values.

RESULTS

Preparation of haemocyte monolayers

As shown in Fig. 1, GR monolayers obtained by the differential cell fractionation (D.F.) method exhibited a high purity, consisting of nearly 90% granulocytes (GR), and both PL + GPL monolayers prepared by D.F. and nylon wool column (N.W.) methods were predominantly comprised of plasmatocytes (PL) and granular plasmatocytes (GPL), which were similar to plasmatocytes in morphology but contained many granular particles in the cytoplasm, as first demonstrated in Spodoptera litura (Kurihara et al., 1992). PL and GPL adhered to cover slips by extending lamellipodia and filopodia.

As shown in Fig. 2, the attachment of GR and that of PL and GPL to cover slips was enhanced by higher temperatures when tested at 25, 28 and 31°C. As the levels of cell attachment were essentially the same when tested at 28 and 31°C, we adopted 28°C as the

![Graph](image1)

**Fig. 2.** Attachment of haemocytes prepared from *A. segetum* larvae to cover glass slips forming cellular monolayers at different temperatures. GR and PL + GPL were obtained by the D.F. or N.W. method (see Fig. 1), and incubated in GRACE's medium for 2 h at the temperatures given in the abscissa to allow for adhesion to the slips. Values are means with SEM (n = 4–15). Means with different letters are significantly different ($p < 0.05$, ANOVA test).

![Graph](image2)

**Fig. 3.** Attachment of haemocytes prepared from *A. segetum* larvae to cover slips to form monolayers after different periods of incubation. GR and PL + GPL obtained by the D.F. or N.W. method (see Fig. 1) were incubated in GRACE's medium at 28°C for the periods given in the abscissa to allow for adhesion to the slips. Values are means with SEM (n = 5–15). Means with different letters are significantly different ($p < 0.05$, ANOVA test).
incubation temperature, so as to maintain the monolayers in better condition for the phagocytosis experiments.

As shown in Fig. 3, the number of cells attached to cover slips increased with longer periods of incubation at 28°C when checked 15, 30 and 60 min later. Though no PL and GPL prepared by the N.W. method adhered after 15 min-incubation, full adherence occurred after 30 min- and 60 min-incubation, allowing us to carry out the phagocytosis experiments. Thus, we adopted an incubation time of 60 min to prepare monolayers of haemocytes in the following experiments.

**Phagocytic activities of haemocytes**

Phagocytic activities of haemocytes against FITC-labeled silica beads were compared

**Fig. 4.** Phagocytosis by haemocytes prepared from *A. segetum* larvae against FITC-labeled silica beads at different temperatures. Haemocytes obtained by the D.F. or N.W. method (see Fig. 1) were allowed to adhere to cover slips in Grace's medium for 1 h at 28°C, and the monolayers in Grace's medium were incubated with silica beads for 2 h at the temperatures given in the abscissa. Values are means with SEM (n = 5). Means with different letters are significantly different (p < 0.05, ANOVA test).

**Fig. 5.** Comparison of phagocytic activities of haemocytes prepared from *A. segetum* (A) and *G. mellonella* (G). GR and PL + GPL obtained by the D.F. or N.W. method (see Fig. 1) were allowed to adhere to cover slips at 28°C for 1 h and the monolayers in Grace's medium were incubated with FITC-labeled silica beads (I, II, III) or Indian ink particles (IV, V, VI) at 28°C for 2 h. Values are means with SEM (n = 5). Means with different letters are significantly different (p<0.05, ANOVA test).
at incubation temperatures of 25, 28 and 31°C for 2 h on monolayers from *A. segetum* prepared by the two methods described above. As shown in Fig. 4, phagocytic activities were low in GR, as well as in PL + GPL monolayers, and essentially not seen in the latter monolayer obtained by the N.W. method, when incubated at 25°C. The most active phagocytosis induced in all the haemocytes occurred when cells were incubated at 28°C, while activity of PL + GPL was more markedly suppressed at 31°C incubation compared to GR.

![Images of haemocyte monolayers](image)

**Fig. 6.** Photographs of haemocyte monolayers prepared from *A. segetum* and *G. mellonella* larvae, after incubation in Grace’s medium with different foreign substances. GR and PL + GPL obtained by the D.F. or N.W. method were allowed to adhere to cover slips at 28°C for 1 h and the monolayers in Grace’s medium were incubated with FITC-labeled silica beads (Beads) or Indian ink particles (Ink) at 28°C for 2 h. Types of haemocytes incubated are shown at the top of the four sets of photographs, and the foreign substances tested are given in the right side of the three sets of photographs. The upper six photographs are from *A. segetum*; the lower six photographs are from *G. mellonella*. FITC-labeled silica beads engulfed in haemocytes (B) remain yellow, while beads not taken up by them (B) are densely stained blue by trypan blue, when checked under a light microscope. Dead haemocytes are stained blue, but living cells are not. B, but no B, release fluorescence under fluorescent microscope (data not shown). Arrows shown the cells phagocytosing ink or bead particles. Bar is 50 μm.
Accordingly, we adopted an incubation temperature of 28°C in the subsequent experiments.

Phagocytic activities of haemocytes separated by the two methods from *A. segetum* and *G. mellonella* larvae were examined using silica beads and Indian ink particles, which were overlaid on the haemocyte monolayers. As shown in Figs. 5 and 6, GR of both species actively phagocytosed both silica beads and Indian ink particles, while PL and GPL separated by both methods engulfed only silica beads similar to GR, but not the Indian ink particles.

**DISCUSSION**

In the differential fractionation method established by WAGO and Ichikawa (1988), plasmatocytes (PL) are first attached to a Petri dish in the presence of an inhibitor to block activation of the phenoloxidase cascade (the essential process for adhesion of granulocytes (GR) to non-self). Non-adherent haemocytes are collected by centrifugation, and then GR are allowed to adhere to the glass surface. These cells are detached by pipetting at a low temperature, collected by centrifugation, and, after discarding the supernatant, the cells are again attached to the slide glass. The monolayers are used for the study of immune reaction. In this study, we improved their monolayer preparation method by allowing GR to adhere to cover slips in the presence of EDTA during the first step, and then allowing PL and granular plasmatocytes (GPL) collected by centrifugation of the non-adherent haemocytes to adhere to different slips. These cover slips with monolayers are directly used for the assay of phagocytic activity. Thus, the procedure for preparing monolayers consisting of particular types of immunocytes becomes much simpler and time-saving.

In contrast, the nylon wool column method is also simpler for preparation of PL + GPL monolayers, but not for GR monolayers, because the GR are removed first by adhering to the nylon wool in the presence of EDTA, which blocks the adhesion of PL and GPL to the wool. Our results proved that PL + GPL monolayers prepared by both methods actually consisted of these haemocytes at a high purity of over 90%, and essentially exhibited similar phagocytic activities. Thus, we recommend preparation of the PL + GPL monolayer by the nylon wool method, and the GR monolayer by the improved differential fractionation method. By combining these methods, the two monolayers are available within 90 min.

We could demonstrate that GR showed high phagocytic activities against Indian ink particles (diameter, ca. 0.1 μm) and FITC-labeled silica beads (d. 5 μm), while PL and GPL obtained by both methods were not able to take up any Indian ink particles. However, they actively engulfed the silica beads to the same degree as GR in both *A. segetum* and *G. mellonella* preparations. When Indian ink particles and FITC-labeled silica beads were injected into last instar larvae of *A. segetum* and *G. mellonella*, it was found that GR phagocytosed both particles, while PL and GPL engulfed only silica beads (Yokoo and Tojo, unpublished). In *B. mori*, GR attach to positively-charged Sephadex or polystyrene particles, but adherence to the negatively-charged these particles in the activated conditions of the phenoloxidase cascade was negligible (Wago, 1992). This fact suggests that surface charge is important to trigger adhesion of the immunocytes to non-self. Work is now in progress to elucidate whether PL and GPL can not phagocytose Indian ink particles, because of their small size, surface structure or physicochemical characteristics.

In monolayers prepared from *B. mori* by the differential cell fractionation method, only
GR but no PL engulf sheep erythrocytes (d. 5.7 μm), and latex particles (d. 5.7 μm) (WAGO and ICHIKAWA, 1988; WAGO, 1991). In G. mellonella quite contrary to the case in B. mori, PL has been shown to be the main phagocytic cell type (RATCLIFFE et al., 1984), and only PL but no GR separated by Percoll density gradient centrifugation is reported to ingest bacteria (ANGGRAENI and RATCLIFFE, 1991). PL purified by the nylon wool method from G. mellonella engulf hydrophobic but not hydrophobic silica beads (d. 5 μm) (WIESNER and GÖTZ, 1993), and also ingest FITC-labeled yeast (ROHLLOFF et al., 1994). Thus, there has been a great discrepancy in the phagocyte types between the two species of Lepidoptera, where extensive studies have been conducted on cellular immune reactions (GUPTA, 1991). Investigations should be carried out using the in vitro monoculture described in this report to elucidate the similarities and differences in the haemocyte types involved in phagocytic cellular defence.

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