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Original article

Flow cytometric analysis of lectin-stained haemocytes of the honeybee (*Apis mellifera*)

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Abstract – The study evaluated the technical feasibility of distinguishing the different honeybee blood cells by flow cytometric analysis with and without staining by 3 lectins: soybean agglutinin (SBA), concanavalin A (ConA) and wheat germ agglutinin (WGA). Flow cytometric analysis of unstained cells provided a forward scatter-side scatter dotplot with no distinct haemocyte population. Examination of blood samples stained with FITC-labelled ConA or WGA revealed a notable population of fluorescently marked cells on FL1-histograms. Microscopic analysis, run parallel, demonstrated low fluorescence of the granular cells, strong fluorescence of the plasmatocytes, with P1's stained all over their surfaces and P2's with a rather dotted appearence. Prohaemocytes were not stained at all. SBA-FITC did not stain honeybee haemocytes as demonstrated by both the flow cytometric and the microscopic examinations

honeybee / haemocytes / flow cytometry / lectin staining

1. INTRODUCTION

Flow cytometry is a technology in which a variety of measurements are made on cells, cell organelles, and other objects suspended in a liquid and flowing one at a time, at rates of several thousand per second, through a sensing region of a flow chamber. The methods of measurement include absorption and scattering of the light beam by the particle, fluorescence of attached fluorescent dyes, and the shape of the detected signal (Dean, 2000). Flow cytometry is applicable in a broad range of disciplines. Many applications can be found in human and veterinary medicine, with the immunophenotyping of leukocytes using monoclonal antibodies grouped in so-called Clusters of Differentiation (for example CD4, CD8, etc.) being one of the most important (Erber, 1990). In the past decade flow cytometry also has been introduced in the typology of invertebrate blood cells from oysters (Ford et al., 1994), snails (Amen et al., 1992), crustaceans (Sequeira et al., 1995) and also from insects (Willott et al., 1994).

The current haemocyte typology of the worker honeybee (*Apis mellifera* L.) is mainly based on the studies of D. Vansteenkiste at the University of Ghent (Van Steenkiste, 1988). He described 5 main haemocyte subsets based on light and electron microscopic examinations and studies of their function. In short, he distinguished (1) plasmatocytes (PL), which represent more than 90% of the haemocytes in circulation and which can be subdivided in 4 subtypes named

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P1 to P4. P1's are small (diameter = 5–12 μm), round cells with a dense, central nucleus; P2 is a transitional stage between P1 and P3; P3's are large oval, discoid cells (length = 8–20 μm, width = 8–12 μm); and P4's are fusiform cells (length = 10–30 μm, width = 6–10 μm). Further he described (2) prohaemocytes (Pr): small (diameter = 6–10 μm), round cells that attach rapidly and flatten thereafter, making them larger on haemolymph smears (length = 10–20 μm, width = 4–10 μm); (3) granular cells (GC): rather large (length = 10–25 μm, width = 8–20 μm) cells with various inclusions in their cytoplasm; (4) oenocytoids (OC): very similar to GC's and (5) coagulocytes (CC): very unstable cells that mostly burst during sampling, leaving only a free nucleus. Unlike most previous papers, Van Steenkiste (1988) followed the standard reference work on insect haemocyte typing of Gupta (1979), permitting the interpretion of honeybee blood cell research in a broader context of insect haematology. Nevertheless, honeybee haemocyte typing remains complex. For instance, the differentiation between plasmatocytes and prohaemocytes, and between oenocytoids and granular cells is only possible by interference contrast or phase contrast microscopic examination. Studies on the proportional occurrence of the different subsets due to age, disease or physiological condition are hampered by the current typology as they demand the patient and conscientious differentiation of many hundreds of blood cells to allow statistical analysis of the data. It is likely that some of the disadvantages of the traditional microscopic approach of honeybee haematology might be addressed by introducing flow cytometry.

This study evaluates the technical feasibility of distinguishing different honeybee blood cells by flow cytometric analysis, first by examining unstained cells, then by examining cells that were labelled by fluorescein isothiocianate (FITC)-conjugated markers. As insect haemocyte surfaces were shown to harbour several carbohydrate moieties (Hypsa and Grubhoffer, 1997; McKenzie and Preston, 1992; Nappi and Christensen, 1986; Richards et al., 1989), lectins were retained as marker molecules. The lectins were chosen based on their reactivity in a simple spotblot test. To interpret the flow cytometric analysis correctly, an interference contrast microscopic examination of the lectin stained haemocytes was run parallel.

2. MATERIALS AND METHODS

2.1. Honeybees and haemolymph sampling

For the spotblot analysis we used haemolymph from bees of mixed age collected at the hive entrance. All other experiments were performed with haemolymph from standardized adult worker bees collected as they emerged from a sealed brood comb incubated at 34 ^{o}C. If the bees could not be used immediately after emerging, they were kept for maximum 7 days in Liebefeld cages with tap water, sugar syrup and pollen paste ad libitum (Jacobs, 1979).

Haemolymph was sampled between tergite II and III using a stretched Pasteur pipette. Samples were fixed in phosphate buffered saline (pH 7.2) containing 1% formaldehyde for spotblot analysis, in Laemmli sample buffer (Laemmli, 1970) for Westernblot analysis or in 0.1 M cacodylate buffer (pH 7.4) containing 0.12 M sucrose, 0.05% $CaCl_2 \cdot H_2O$ and 4% paraformaldehyde (Raes et al., 1989) for microscopic or flow cytometric analysis.

2.2. Haemolymph spotblot and Western blot analysis

Spotblots were made by spotting 25 μL fixed haemolymph on polyvinylidine difluoride membrane strips and drying them in an oven at 100 ^{o}C. For Westernblot analysis 10 μL of the denaturated haemolymph samples was separated on a 12% acrylamide gel and blotted onto polyvinylidine difluoride membrane by a semi-dry method at 0.8 $mA \cdot cm^{-2}$ for 1 h. The protein bands were visualized by a reversible Ponceau-staining and the blot membrane was then cut in separate lanes.

Both spotblot and Westernblot membranes were blocked in 50 mM Tris-HCl buffer (pH 7.4), supplemented with 140 mM NaCl, 5 mM EDTA, 0.05% Triton X-100, 0.25% gelatin and 1% bovine serum albumin (= blocking buffer). Thereafter the membranes were subsequently incubated in biotin-labelled lectin (between 1/100 and 1/2000 in blocking buffer), streptavidin-peroxidase (1/2000 in blocking buffer) and developed in 3,3'-diaminobenzidine tetrahydrochloride.

Spotblots were stained with the following lectins: concanavalin A (ConA), *Glycine max* (soybean)

agglutinin (SBA), *Tetragonolobus purpureas* lectin (TPL) or *Triticum vulgaris* (wheat germ) agglutinin (WGA). The negative control was incubated in blocking buffer instead. Westerblots were stained with ConA, SBA, and WGA.

2.3. Microscopic examination of lectin-stained haemocytes

The haemocytes from fixed haemolymph samples were attached to albumin-coated microscope slides using a cytospin apparatus (centrifugation at 100 *g* for 20 min). The haemocytes were stained with FITC-labelled (excitation at 490 nm; fluorescence at 525 nm) ConA, SBA or WGA. Lectin dilutions (1/80 or 1/160) and wash steps were in cacodylate buffer (see above) supplemented with 1% bovine serum albumin (= cacodylate blocking buffer) and the air-dried preparations were mounted with glycerin. Microscopic examination was done with a Reichert-Jung Polyvar microscope in the interference-contrast or epifluorescence mode.

2.4. Flow cytometric analysis of lectin-stained haemocytes

Lectin staining of the haemocytes for flow cytometric examination occurred as follows: the cells (approximately 100 000 haemocytes per sample) were spun down (200 *g*, 10 min), resuspended and incubated (30 min) in FITC-labelled lectin (1/160 in cacodylate blocking buffer) and finally washed 3 times in cacodylate buffer. Unstained cells served as a negative control. Flow cytometric analysis was done on a FACS Vantage^{TM} (Becton Dickinson). Forward scatter (FSC), side scatter (SSC) and fluorescence (FL1 detector range: 530 ± 30 nm) were recorded. The fluorescence detector was adjusted at 560 V, with the amplitude gain in the "linear 8" mode. From each sample 5000 haemocytes were scanned.

3. RESULTS

Spotblot analysis revealed a brown spot when SBA, ConA or WGA was used (Fig. 1). No coloration was observed with the negative control or TPL. Therefore the latter was not retained for further analysis.

Westernblot analysis revealed only minor coloration with SBA (Fig. 2). ConA revealed a smear of positivity between 21.5 and 31 kDa and in the higher molecular mass region. When WGA was used several bands above the 45 kDa marker-protein were recognized (Fig. 2).

Figure 3 shows the results of the microscopic examinations of haemocytes stained with FITC-labelled lectins. Observations made by interference contrast microscopy were placed next to epifluorescent examinations of the same cells, which permitted the identification of any lectin-stained haemocytes. It was found that SBA did not stain any of the known haemocyte types. Only some unidentified structures – probably free nuclei of burst cells – were fluorescently stained. In contrast, ConA stained a broad range of honeybee haemocytes, including the plasmatocytes types 1 and 2 and the granular cells. P1's showed an overall surface staining with a fluorescent "rim" (produced by a vertical view of surface staining in different focus), whereas P2's showed rather dotted fluorescent marks. The GC's were less

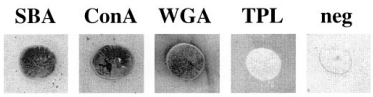


Figure 1. Spotblots of haemolymph stained with soybean agglutinin (SBA), concanavalin A (ConA) and wheat germ agglutinin (WGA) gave a positive reaction, whereas *Tetragonolobus purpureas* lectin (TPL) and the negative control remained unstained.

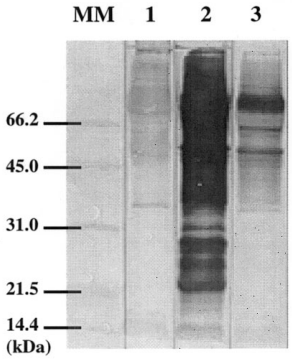


Figure 2. Westernblot analysis of haemolymph stained with soybean agglutinin (1) revealed only minor coloration. Concanavalin A (2) recognized a smear of glycoproteins and wheat germ agglutinin (3) stained several bands above the 45 kDa marker-protein.

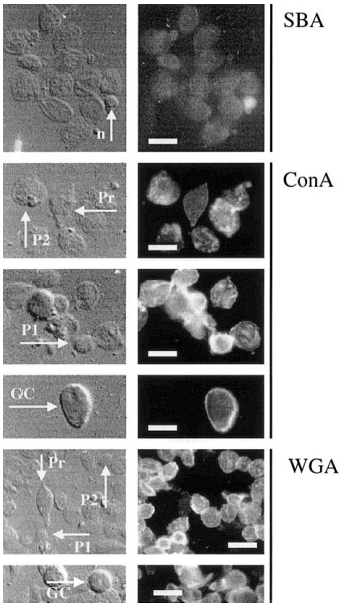


Figure 3. Microscopic examination of haemocytes stained with the FITC-labelled lectins soybean agglutinin (SBA), concanavalin A (ConA) and wheat germ agglutinin (WGA). The chosen microscopic fields were evaluated both by interference contrast (on the left) and epifluorescence (on the right) microscopy. SBA did not stain any of the known haemocyte types; only some unidentified structures (probably free nuclei = n) became fluorescent. The fluorescence after staining with ConA and WGA was very similar: the plasmatocytes type 1 (P1) showed an overall staining, whereas the type 2 (P2) showed a dotted fluorescence; the granular cells (GC) were less brightly stained and the prohaemocytes (Pr) were not stained at all. Bar = 10 μm (WGA) or 5 μm (SBA and ConA).

brightly stained by the FITC-labelled lectin than the two plasmatocyte types. Prohaemocytes were not stained at all. The same observations with respect to the P1's, P2's, GC's and Pr's were made using WGA.

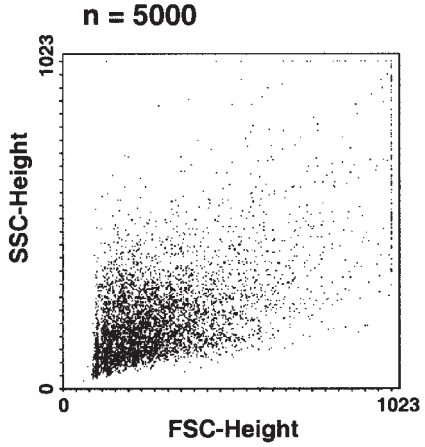
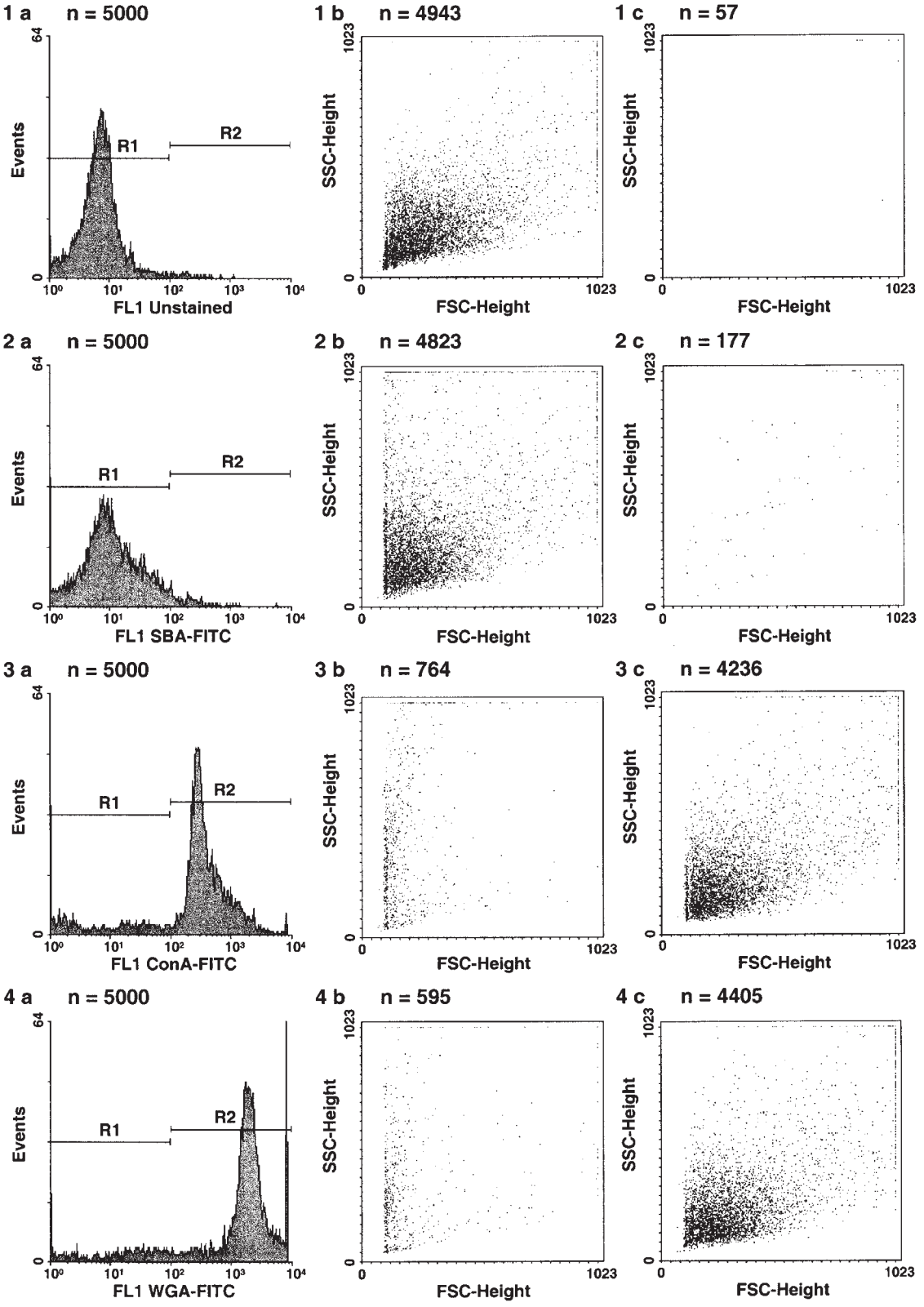


Figure 4. Flow cytometric analysis of unstained haemocytes. In the FSC-SSC dotplot no distinct haemocyte population could be observed.

Flow cytometric analysis of unstained haemocytes showed a FSC-SSC profile with most cells plotted in the lower left quarter (Fig. 4). This FSC-SSC profile did not distinguished a distinct haemocyte population. The FL1-histogram in Figure 5.1a showed the background fluorescence that was used to define the cut-off value (= 10^2) of positivity and to differentiate between the regions R1 (= no fluorescence) and R2 (fluorescence). Only 57 of the 5000 examined cells crossed this cut-off in the unstained sample. In the SBA-FITC treated sample there was only a negligible shift to the right on the FL1-histogram, with 177 cells found in the R2 region. In contrast, both ConA-FITC and WGA-FITC treatment resulted in a major peak of fluorescent cells, with population sizes of 4236 and 4405 cells respectively. To characterize the corresponding populations, FSC-SSC dotplots were made of the R1 and R2 gated cells. The R1 gated cells (no fluorescence) appeared to be a distinct

Figure 5. Flow cytometric analysis of unstained haemocytes (1a-c) and of haemocytes stained with the FITC-labelled lectins soybean agglutinin (SBA; 2a-c), concanavalin A (ConA; 3a-c) and wheat germ agglutinin (WGA; 4a-c). First a histogram of the FL1-channel was given (marked as "a" in each series), which permitted to define the regions R1 (no fluorescence) and R2 (fluorescence). In the last two dotplots of each series only the R1 (in "b") or the R2 gated cells (in "c") were plotted in an FSC-SSC frame. ConA-FITC and WGA-FITC resulted in a major fluorescence peak. In both cases it was shown that the fluorescent (R2 gated) population was plotted all over the FSC-SSC dotplot with strong accumulation in the lower left quarter, whereas the unlabelled cells (R1 gated) appeared to be a distinct population with low FSC-values. SBA-FITC treatment caused only a negligible shift to the right on the FL1-histogram.


1 a
n = 5000
Events
R1
R2
FL1 Unstained
1 b
n = 4943
SSC-Height
FSC-Height
1 c
n = 57
SSC-Height
FSC-Height
2 a
n = 5000
Events
R1
R2
FL1 SBA-FITC
2 b
n = 4823
SSC-Height
FSC-Height
2 c
n = 177
SSC-Height
FSC-Height
3 a
n = 5000
Events
R1
R2
FL1 ConA-FITC
3 b
n = 764
SSC-Height
FSC-Height
3 c
n = 4236
SSC-Height
FSC-Height
4 a
n = 5000
Events
R1
R2
FL1 WGA-FITC
4 b
n = 595
SSC-Height
FSC-Height
4 c
n = 4405
SSC-Height
FSC-Height

population of small-sized cells (low FSC-value) with variable granularity (broad range of SSC-values). The R2 gated cells (fluorescence) were distributed all over the FSC-SSC dotplot with strong accumulation in the lower left quarter. This was observed both in the ConA-FITC and the WGA-FITC treated samples. The only difference between the latter two treatments was that FL1-fluorescence was even more pronounced in the WGA-FITC sample.

4. DISCUSSION

Flow cytometry of total blood samples from man and many other higher vertebrates (most of the studied species are farm animals) show distinct populations of erythrocytes, lymphocytes, eosinophils, neutrophils and platelets on a simple FSC-SSC dotplot, even without any previous staining (Sintnicolaas et al., 1991). As it was recently demonstrated that dotplotted forward-scatter versus side-scatter parameters of the crustacean species *Procambarus zonangulus* (crayfish) also resulted in distinct populations of granular, semigranular and hyaline haemocyte types (Cardenas et al., 2000), it was reasonable to assume that a similar result would be obtained with the unstained honeybee blood samples. However, this was not the case: the FSC-SSC dotplot did not distinguish a distinct haemocyte population. This finding can be explained partially by the fact that the differences in size between the haemocyte types of the honeybee are relatively small compared to the range in size within a single haemocyte subset (Van Steenkiste, 1988). This means that the potential of flow cytometry in honeybee haematology highly depends on the availability of fluorescent-labelled specific markers.

The spotblot proved to be a simple and easy method for rapid screening of marker molecules for their affinity. In its present form with spotting of whole haemolymph samples, the technique can not differentiate between affinity for cellular or non-cellular structures. Carbohydrate moieties present on molecules that are found freely in the plasma also can be stained. Further, we chose to first screen haemolymph from worker honeybees of mixed ages to maximize the chance of a positive spotblot if certain carbohydrate determinants would appear only in a narrow age-group. Positive spotblots were confirmed in all cases by later methodologies except for SBA. SBA-sensitive carbohydrate moieties do not yet occur on the haemocytes of the very young bees that were sampled for Westernblots, microscopy and flow cytometry.

Examination of both ConA and WGA stained blood samples by epifluorescence microscopy and flow cytometry revealed a notable population of recognized cells. The microscopic examinations demonstrated that identical cells were stained by the two lectins, and that the observed fluorescence had the same pattern: low fluorescence of the granular cells, strong fluorescence of the plasmatocytes, with P1's stained all over their surfaces and P2's with a rather dotted appearence. These two lectins are known to recognize different carbohydrate determinants, ConA reactive against α-D-mannose and α-D-glucose, and WGA reactive against $(\text{N-acetyl glucosamine})_2$ and N-acetyl neuraminic acid. Thus, the similarity in fluorescence patterns might indicate that the carbohydrate moieties that are recognized by these two lectins are shared by the same glycoproteins or occur on different glycoproteins that are closely associated. The fact that on the Westernblot analysis some bands are cross-reactive for the two lectins supports the former.

Our microscopic findings differ from those on other insect species. In *Blaberus craniifer* and *Extatosoma tiaratum* ConA recognition was found in all haemocyte types whereas WGA recognition was only found in plasmatocytes and the so-called "spreading granular cells" from *Extatosoma* (Richards et al., 1989). In the larvae of *Calliphora vomitoria* haemocytes were not recognized by ConA, whereas 40–50% of the cells were stained by SBA and 100% by WGA (McKenzie and Preston, 1992). The same authors demonstrated that SBA staining was associated with phagocytic cell types. We did not find any indication that this occurred in the honeybee; it would imply that the majority of the PL's and GC's were phagocytic at the moment of sampling, which seems unlikely. In *Triatoma*

infestans WGA bound on internal granules, whereas ConA stained the whole cells (Hypsa and Grubhoffer, 1997). These authors found only weak reactivity in prohaemocytes, as our results also indicated, which they thought was due to the low state of differentiation of this cell type. Indeed, the only clear similarity in lectin recognition among different insect species is the lack of carbohydrate determinants on prohaemocytes. So far, we have no indication that different insect species follow a similar pattern of glycosylation of their haemocyte surface molecules during maturation and differentiation. Therefore, the use of lectins for typing insect haemocytes across the species level remains highly questionable. It should be noted that insect blood cell classification is very difficult. There is probably more variation in the morphology of the haemocytes of a single insect Order than in the leukocytes from all vertebrate groups put together (Ratcliffe and Gotz, 1990). Nevertheless, haemocyte staining with FITC-labelled lectins might provide a valuable aid for the differentiation of the haemocyte subsets within a single insect species. In honeybee haematology lectin-dependent fluorescence might be helpful for the difficult differentiation of PL's, GC's and Pr's.

The present flow cytometric analysis of lectin stained samples demonstrated that the use of FITC-labelled markers allows a rapid and easy count of a fluorescent population of haemocytes of the honeybee. However, the epifluorescence microscopy that was run parallel could not serve to verify the flow cytometric counts within the defined regions R1 and R2. Yet, in general terms the flow cytometric data reflected the microscopic examinations: no recognition by SBA, and strong fluorescence of a large cell population by ConA and WGA. The present flow cytometric results failed to distuinguish between the marked PL's and GC's as they were all grouped in a single fluorescence peak. We believe that the search for marker molecules of the honeybee haemocytes should be extended to a broader range of lectins or to monoclonal/polyclonal antibodies with a high specificity for a single haemocyte subset. This will be a crucial step for flow cytometric typing of the cells present in a haemolymph sample of the honeybee.

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Résumé – Analyse par cytométrie de flux des hémocytes de l'Abeille domestique (*Apis mellifera*) après coloration par des lectines. La cytométrie de flux est une technique qui permet de faire une variété de mesures sur les cellules, les organelles des cellules et autres objets en suspension dans un liquide et s'écoulant un par un à raison de plusieurs milliers par seconde, à travers une zone de détection d'une chambre de flux. Cet article a pour but d'évaluer s'il est techniquement possible de distinguer les différentes cellules sanguines de l'Abeille domestique (*Apis mellifera* L.) par l'analyse en cytométrie de flux, en examinant d'abord des cellules non colorées, puis des cellules marquées avec des marqueurs conjugués à l'isothiocianate de fluorescéine (FITC). Puisqu'il a été montré (Hypsa et Grubhoffer, 1997 ; McKenzie et Preston, 1992 ; Nappi et Christensen, 1986 ; Richards et al., 1989) que la surface des hémocytes d'insectes hébergeait plusieurs fragments d'hydrates de carbone, on a retenu les lectines comme molécules de marquage. Les lectines agglutine du soja (SBA), concavanaline A (ConA) et agglutine du germe de blé (WGA) ont été retenues sur la base de leur réactivité lors d'un test simple de spotblot. L'analyse en cytométrie de flux de cellules non colorées a fourni un nuage de points dispersés vers l'avant et latéralement sans populations distinctes d'hémocytes ; l'histogramme FL1 de la figure 5.1a montre la fluorescence de fond qui a été utilisée pour définir la valeur limite (= 10^2) de positivité et pour séparer les régions R1 (sans fluorescence) et R2 (avec fluorescence). Dans l'échantillon traité avec SBA-FITC il n'y a eu qu'un décalage vers la droite sur l'histogramme FL1, avec 177 cellules trouvées dans la région R2. Au contraire, les traitements par ConA-FITC et par WGA-FITC ont donné un pic important de cellules fluorescentes, avec des tailles de populations de 4236 et 4405 cellules respectivement. Pour caractériser les populations correspondantes, on a fait des nuages de points FSC-SSC des cellules sorties en R1 et R2. Les cellules sorties en R1 (sans fluorescence) sont apparues comme une population distincte de cellules de petite taille (valeur FSC faible) avec une granulation variable (large fourchette de valeurs SSC). Les cellules sorties en R2 (avec fluorescence) étaient réparties

sur tout le nuage de points FSC-SSC avec une forte accumulation dans le quart inférieur gauche. Cela a été observé aussi bien dans les échantillons traités par ConA-FITC que dans ceux traités par WGA-FITC. L'analyse microscopique faite parallèlement a montré une faible fluorescence des cellules granuleuses, une forte fluorescence des plasmatocytes, parmi lesquels les P1 étaient colorés sur toute leur surface et les P2 de façon discontinue. Pourtant, selon les résultats présents, la cytométrie de flux n'a pas permis de séparer les PL marqués des GC marqués car ils étaient tous groupés en un seul pic de fluorescence. Les prohémocytes n'étaient pas du tout colorés. Les analyses en cytométrie de flux et au microscope montrent que la SBA-FITC n'a pas coloré les hémocytes d'Abeille. L'étude présente montre que le potentiel de la cytométrie de flux pour l'hématologie de l'Abeille dépend de la disponibilité de marqueurs spécifiques fluorescents. Il est néanmoins nécessaire d'étendre la recherche de molécules de marquage à un plus grand éventail de lectines ou à des anticorps monoclonaux/polyclonaux ayant une forte spécificité pour un seul type d'hémocytes. C'est une étape cruciale pour parvenir à caractériser par cytométrie de flux les cellules présentes dans un échantillon d'hémolymphe d'Abeille.

***Apis mellifera* / hémocyte / cytométrie de flux / coloration aux lectines**

Zusammenfassung – Analyse von Lectin gefärbten Hämocyten der Honigbienen (*Apis mellifera* L.) im Durchfluss – Cytometer. Durchfluss – Cytometrie ist eine Technologie für verschiedene Messungen von Zellen, Zellorganellen und anderen Objekten, die in Flüssigkeit verteilt sind. Sie werden in Anzahlen von mehreren Tausend pro Sekunde einzeln durch eine empfindliche Region, die Fließkammer, geleitet. In dieser Arbeit wird untersucht, ob es technisch möglich ist, mit der Analyse im Fließcytometer die verschiedenen Blutzellen der Honigbienen zu unterscheiden, zunächst durch eine Prüfung der ungefärbten Zellen und danach durch Prüfung von Zellen, die mit Fluorescein Isothiocianat (FITC) konjugierten Markern gekennzeichnet waren. Da die Oberfläche von Insektenhämocyten mehrere Carbohydratanteile enthält (Hypsa und Grubhoffer, 1997; Mc Kenzie und Preston, 1992; Nappi und Christensen, 1986; Richards et al., 1989), wurden Lectine als Markermoleküle angelagert. Die Lectine Agglutinin der Sojabohne (SBA), Concanavalin A (ConA) und das Weizenkeim Agglutinin (WGA) wurden auf Grund ihrer Re-Aktivität in einem einfachen Spotblot Test angelagert. Die Analyse von ungefärbten Zellen mit dem Durchfluss – Cytometer ergab eine vorwärts-seitenverteilte Punktwolke (forward scatter-side scatter dotplot), aber keine unterscheidbaren Gruppen von Hämocyten. Das FL1-Histogramm in Abbildung 5, 1a weist die Hintergrundfluoreszenz auf, die benutzt wurde um den cut-off Wert (= 10^2) zu definieren und zwischen den Regionen R1 (keine Fluoreszenz) und R2 (Fluoreszenz) zu unterscheiden. In den mit SBA-FITC behandelten Proben ergab sich nur eine zu vernachlässigende Verschiebung zur rechten Seite auf dem FL1 – Histogramm, 177 Zellen wurden in der R2 Region gefunden. Im Gegensatz dazu ergaben sowohl die Behandlung mit ConA-FITC als auch mit WGA-FITC größere Peaks mit fluoreszenten Zellen, mit einer Anzahl von 4.236 bzw. 4.405 Zellen. Um die sich entsprechenden Gruppen zu charakteriseiren, wurden FSC-SSC Punktwolken der in R1 und R2 eingegrenzten Zellen gemacht. Die in R1 eingegrenzten Zellen (keine Fluoreszenz) schien eine distinkte Gruppe von kleinen Zellen (kleiner FSC Wert) mit verschiedenartiger Körnung (breite Verteilung der SSC – Werte) zu sein. Die vom R2 eingegrenzten Zellen (Fluoreszenz) waren über die gesamte FSC-SSC Punktwolke verteilt mit einer starken Verdichtung im linken unteren Teil. Das wurde bei beiden Proben, bei den ConA-FITC als auch bei den WGA-FITC Färbungen beobachtet.
Bei den zur gleichen Zeit durchgeführten mikroskopischen Analysen zeigte sich ein geringe Fluoreszenz bei den granulären Zellen, starke Fluoreszenz in den Plasmozyten, von denen Typ P1 auf der ganzen Oberfläche gefärbt war während die Oberflächen von P2 gefleckt erschienen.
Nichtsdestotrotz gelang es nicht, mit den jetzigen Ergebnissen zwischen den markierten Plasmozyten und den Granulocyten zu unterscheiden, da sie sich alle in einem einzigen Fluoreszenzpeak gruppierten. Prohämocyten wurden gar nicht gefärbt. SBA-FITC färbten keine Hämocyten der Honigbienen, wie es sowohl mit der Durchflusszählmethode als auch mit den mikroskopischen Untersuchungen gezeigt wurde. Diese Untersuchung zeigt, dass das Potential einer Bestimmung der Hämozyten der Honigbienen mit einem Durchfluss-Cytometer in hohem Maße von dem Vorhandensein spezifischer fluoreszenter Markierungen abhängt. Die Suche nach solchen Markierungssubstanzen muss auf eine größere Auswahl von Lektinen oder auf monoclonale/ polyclonale Antikörper mit hoher Spezifität für einzelne Hämocytenzellen ausgedehnt werden. Dies ist der entscheidende Schritt, um die Durchfluss Cytometrie zur Bestimmung der verschiedenen Hämocyten in einer Hämolymphprobe der Honigbienen anzuwenden.

***Apis mellifera* / Hämocyten / Durchfluss Cytometrie / Lectinfärbung**

REFERENCES

Amen R.I., Aten J.A., Baggen J.M., Meuleman E.A., de Lange-de Klerk E.S., Sminia T. (1992) *Trichobilharzia ocellata* in *Lymnaea stagnalis*: a flow cytometric approach to study its effects on hemocytes, J. Invertebr. Pathol. 59, 95–98.

Cardenas W., Jenkins J.A., Dankert J.R. (2000) A flow cytometric approach to the study of crustacean cellular immunity, J. Invertebr. Pathol. 76, 112–119.

Dean P.N. (2000) Flow cytometry instrumentation, in: Robinson J.P. (Ed.), Current protocols in cytometry, John Wiley & Sons, Inc., New York, pp. 111–118.

Erber W.N. (1990) Human leucocyte differentiation antigens: review of the CD nomenclature, Pathology 22, 61–69.

Ford S.E., Ashton-Alcox K.A., Kanaley S.A. (1994) Comparative cytometric and microscopic analyses of oyster hemocytes, J. Invertebr. Pathol. 64, 114–122.

Gupta A.P. (1979) Hemocyte types: their structures, synonymies, interrelationships and taxonomic significance, in: Gupta A.P. (Ed.), Insect hemocytes, Cambridge Unversity Press, Cambridge, pp. 85–129.

Hypsa V., Grubhoffer L. (1997) Two hemocyte populations in *Triatoma infestans*: ultrastructural and lectin-binding characterization, Folia Parasitol. 44, 62–70.

Jacobs F.J. (1979) Eine standardisier methode zur *Nosema*-forschung: eine notwendigkeit im labor und am bienenstand, Apidologie 10, 75–76.

Laemmli U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature 227, 680–685.

McKenzie A.N., Preston T.M. (1992) Functional studies on *Calliphora vomitoria* haemocyte subpopulations defined by lectin staining and density centrifugation, Dev. Comp. Immunol. 16, 19–30.

Nappi A.J., Christensen B.M. (1986) Hemocyte cell surface changes in *Aedes aegypti* in response to microfilariae of *Dirofilaria immitis*, J. Parasitol. 72, 875–879.

Raes H., Bohyn W., De Rycke P.H., Jacobs F. (1989) Membrane-bound iron-rich granules in fat cells and midgut cells of the adult honeybee (*Apis mellifera* L.), Apidologie 20, 327–337.

Ratcliffe N.A., Gotz P. (1990) Functional studies on insect haemocytes, including non-self recognition, Res. Immunol. 141, 919–923.

Richards E.H., Ratcliffe N.A., Renwrantz L. (1989) The binding of lectins to carbohydrate moieties on haemocytes of the insects, *Blaberus craniifer* (Dictyoptera) and *Extatosoma tiaratum* (Phasmida), Cell Tissue Res. 257, 445–454.

Sequeira T., Vilanova M., Lobo-Da-Cunha A., Baldaia L., Arala-Chaves M. (1995) Flow cytometric analysis of molt-related changes in hemocyte type in male and female *Penaeus japonicus*, Biol. Bull. 189, 376–380.

Sintnicolaas K., de Vries W., van der Linden R., Gratama J.W., Bolhuis R.L. (1991) Simultaneous flow cytometric detection of antibodies against platelets, granulocytes and lymphocytes, J. Immunol. Methods 142, 215–222.

Van Steenkiste D. (1988) De hemocyten van de honingbij (*Apis mellifera* L.): typologie, bloedbeeld en cellulaire verdedigingsreacties, Ph.D.-thesis, University of Ghent.

Willott E., Trenczek T., Thrower L.W., Kanost M.R. (1994) Immunochemical identification of insect hemocyte populations: monoclonal antibodies distinguish four major hemocyte types in *Manduca Sexta*, Eur. J. Cell Biol. 65, 417–423.