Alternative Model of Cellular Immune Reactions in Insect

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ABSTRACT

The cellular immune reactions involve mainly the insect blood cells or hemocytes and consist of adhesive reactions of hemocytes against microbes or parasites. Among these various hemocytes, the granulocytes and plasmatocytes are thought to be the most important hemocytes involved in insect cellular defense. According to the number and size of the foreign invaders in the insect hemocoel, three major cellular defense reactions can be classified: Phagocytosis, encapsulation and nodule formation. Many of the initial interactions leading to coagulation and phagocytosis reactions in insects are sugar-lectin binding reactions, involving homeostasis- and defense-related receptors, such as scavenger receptors and mucin-like immune receptors. However, the mechanism that cause the formation of endocytotic vesicles are not known. The major aims of this study are 1) To test the induction of cell-spreading and macropinocytosis, 2) To investigate the role of lectins in coagulation reaction. The observations suggest that in the presence of lectins hemocytes can either form coagulation products, such as globules, or perform cellular functions, such as adhesion and macropinocytosis. Since coagulation and cell activities are quite disparate processes, the balance between the two types of reactions is quite relevant for the overall outcome. In cell-free hemolymph (plasma) only coagulation reactions occur in the presence of externally added GalNAc-specific lectins.

Keywords : Cellular, immune, insect, lectin

INTRODUCTION

Naturally, insects are continuously exposed to potentially pathogenic microorganisms, but only a few encounters result in infection. A complex defensive response is induced in insects after infection is stimulated by non-pathogenic and a few pathogenic bacteria (Kaaya 1993). Several evidences suggest that there are similarities between invertebrates and vertebrates defense system, for example, insects have an immunoglobulin-like protein in hemolymph (Sun *et al.* 1990), and common antibacterial proteins (Lee *et al.* 1989). Although insects lack immunoglobulins, they possess a complex and efficient system of biological defense against pathogens and parasites (Pathak1993). The defense mechanisms of insects principally involve both cellular and humoral responses.

The cellular immune reactions involve mainly the insect blood cells or hemocytes and consist of adhesive reactions of hemocytes against microbes or parasites. Insect cellular defense reactions have been studied for about 100 years. Changes in morphology, behavior and types of cell composition during an infection have been widely researched with microscopic techniques, lectin and monoclonal antibody markers (McKenzie 1992, Strand 1994, Theopold 1995, Theopold 1996). Hemocytes phagocytose bacteria, trap microbes in nodules and encapsulate large parasitoid eggs (Vinson 1990). Hemocytes are also involved in other immune responses such as the phenoloxidase cascade and hemolymph coagulation (Brehelin 1979, Gregoire 1974).

Hemocytes from different insect species vary in their morphological and functional characteristics. Among these various hemocytes, the granulocytes and plasmatocytes are thought to be the most important hemocytes involved in insect cellular defense reactions (Ratcliffe 1993). According to the number and size of the foreign invaders in the insect hemocoel, three major cellular defense reactions can be classified: Phagocytosis, encapsulation and nodule formation.

At least two cell surface recognition systems have been proposed to exist that account for internalization of particles. One ‘receptor’ is proposed to mediate binding of particles such as bacteria containing terminal glucose residues, while the other ‘receptor’(termed the non-specific receptor) mediates binding of particles including latex beads by more hydrophobic interaction (Cardelli 2001).

Since phagocytosis is a ubiquitous process within the animal kingdom, occurring in numbers of all invertebrate phyla and vertebrates, it may be regarded as the primary

cellular response again invading microorganisms. The main steps for phagocytosis are recognition, endocytosis, ingestion and killing (Gupta 1999, Götz 1985). These processes have been studied in different insect species. Generally, granulocytes and plasmatocytes are the major hemocytes involved in phagocytosis. The surface of the hemocytes contains binding sites that recognize cell wall components of foreign objects. Thus, when the foreign object encounters the hemocytes, the surface components of its cell wall will be recognised by the hemocytes. The bound objects are then surrounded by pseudopods and ingested by coated vesicles. Finally the engulfed particles are lysed by lysozyme and antibacterial peptides (Götz 1985).

Many of the initial interactions leading to coagulation and phagocytosis reactions in insects are sugar-lectin binding reactions, involving homeostasis- and defense-related receptors, such as scavenger receptors (Franc *et al.* 1999) and mucin-like immune receptors (Theopold *et al.* 1996). A range of sugar-lectin interactions have been documented to be involved in phagocytosis events (Wilson *et al.* 1999). Interactions involving mucin-like glycoproteins containing Gal and GalNAc are particularly interesting given the presence of these receptors in immune related tissues, such as epithelial (Pendland & Boucias 1996) and blood cells (Chen *et al.* 1993, Theopold & Schmidt 1997). The *Drosophila* immune receptor hemomucin is a major glycoprotein on larval hemocytes (Kramerov *et al*. 1997, Theopold *et al.* 1996) and hemocyte-like *mbn-2* cells (Theopold *et al.* 1996), which are used as a model system for the study of lectinophagocytosis. Addition of *Helix pomatia* lectin (HPL) to *mbn-2* cells causes immune induction (Theopold *et al*. 1996) and endocytotic vesicles (Fabbri *et al*. 2004. Although the process of lectin-mediated endocytosis which is frequently described as lectinophagocytosis is mainly restricted to the uptake of microbes (Ofek *et al*. 1995, Oka *et al*. 1998, Pendland & Boucias 1996, Rainho *et al*. 1999), Gal-specific glycoconjugants (Pendland & Boucias, 1996) and corresponding lectins (Abe *et al*. 1999) may play a central role in the initiation and functional completion of endocytotic processes in general. Therefore, the term of lectinophagocytosis is used in a broad context including endocytosis processes involving microbes, inert objects, and soluble substances, such as bacterial toxins.

Endocytosis is the process that occurs in all eukaryotic cells by which cells internalized portions of the plasma membrane, including associated proteins and a portion of the extracellular space. This is the route by which cells absorb nutrients, recycle membrane proteins and lipids, receive some chemical and molecular signals, and down-regulate membrane receptors in response to ligands and/or environmental changes (Cardelli 2001, Baggett & Wendland 2001).

The cellular capacity to internalise objects, involving attachment, engulfment and uptake by endocytotic processes, exists in virtually all organisms. Many endocytosis reactions are based on cell-cell interactions. However, the mechanisms that cause the formation of endocytotic vesicles are not known.

In this experiment therefore rise the question whether the induction of macropinocytosis is induced by monomeric lectins The major aims of this study are 1) To test the induction of cell-spreading and macropinocytosis, 2) To investigate the role of lectins in coagulation reaction.

MATERIALS AND METHODS

Insect culture and cell culture

Galleria mellonella larvae were reared on an artificial diet in the dark. *Drosophila melanogaster* Canton S flies were kept on cornmeal/yeast food at a photoperiod of 14 h light: 10 h dark at 25°C. Fourth instar of *Plutella xylostella* larvae used in this experiment were reared on fresh cabbage leaves at 23^{o}C with a photoperiod of 16 hrs light and 8 hrs darkness.

Mbn-2 cells are kept in Schneider medium at 25ºC and *Plutella* cells are kept in lepidopteran medium. Schneider cells are cells derived from embryonic tissues of *Drosophila melanogaster*. *Mbn*-2 cells are derived from a blood tumor mutant (Gateff *et al* 1980). These cells were grown in Schneider's Insect Medium supplemented with 10% FCS and grown in the dark at 25ºC. Schneider and *mbn*-2 cells were supplied by the Biochemistry Department at the University of Adelaide.

Isolation of hemolymph, hemocytes, fat body and gut

Final instar *G. mellonella* larvae were surface sterilised with 70% ethanol and a pair of prolegs was cut off with microscissors, the larvae were bled into a drop of insect ringer on a piece of Parafilm 'M' (American National Can, Chicago, IL, USA) on ice. To avoid melanogenesis, 10 larvae were bled into

200 ml of insect ringer and immediately centrifuged at 760g to precipitate the hemocytes. The recovered supernatant is the cell-free hemolymph.

To isolate fat body and gut, the sterilised caterpillar was dissected in PBS with fine forceps under a stereo-microscope. Fat body and gut were collected in PBS and kept in -80°C or used directly to extract soluble proteins. Fat body tissue was mixed and incubated with 2× volume of DDW and homogenized. Cell debris was pelleted by centrifugation at 13,000 rpm (full speed) for 10 minutes, and supernatant, containing most of the hemolymph-specific proteins was used immediately for experiments to avoid spontaneous aggregation.

Endocytosis assays

Cell lines from MBN2 and Plutella xylostella were placed into tissue Culture Plate, 24 well with 1 ml of medium until the cells attached into the surface. After attachment, the cell in each well were treated with different lectins in different concentration (0.5, 1.0 and 2 ul/1 ml medium) from 1 mg/ml lectin solution. The treated and untreated cells were kept and observed after 3 hr, 6 hr, 9 hr , 24 hr , 48 hr and 72 hr after treatment. The percentage of endocytosis and foem-like cell were recorded

Hemocyte staining with lectin

Hemocytes were bled from individuals of the last larval instar. *Drosophila* larvae were torn apart as little as possible but sufficiently to release a drop of hemolymph. For bleeding *Ephestia caterpillars*, a proleg was +cut off to release hemolymph. In both cases, hemolymph was collected into anticoagulant ringer (100 mM Tricine-HCl, pH 6.8, 100 mM NaCl, 40 mM KCl, 15 mM $MgCl_2$, 20 mM EDTA, saturated with phenylthiourea). After allowing the cells to attach for a few minutes, FITC-conjugated PNA (50 µg/ml in the same buffer as above including 0.05% Na-azide) was added.

Phagocytosis assay

Cells were seeded 30 min before addition of lectins. PNA and HPL were added to a concentration of 50µg/ml to SL2 and mbn-2 cells. 5 hours later, the cells were photographed using phase contrast microscopy.

RESULTS AND DISCUSSION

Lectin-mediated spreading and endocytosis

Lectins were tested in the two cell lines for induction of cell-spreading and macropinocytosis. The GalNAc- and Gal-specific tetrameric lectins from *Vicia villosa* (VVL) and *Arachis hypogaea* (peanut agglutinin, PNA) were found to induce spreading and macropinocytosis in *mbn-2* cells and *Plutella* cells (Fig. 1, 2). In the *mbn*-2 cell line, the presence of HPL and ConA had little effect on the induction of macropinocytosis (Fig. 4). In contrast, HPL induced macropinocytosis in *P. xylostella* cells together with VVL and PNA, although the induction process was delayed compared to *mbn2*-cells (Fig. 5). Inspite of ConA-binding glycoproteins present in lepidopterans (Fig. 1), ConA was not an effective inducer of macropinocytosis (Fig. 6A,B).

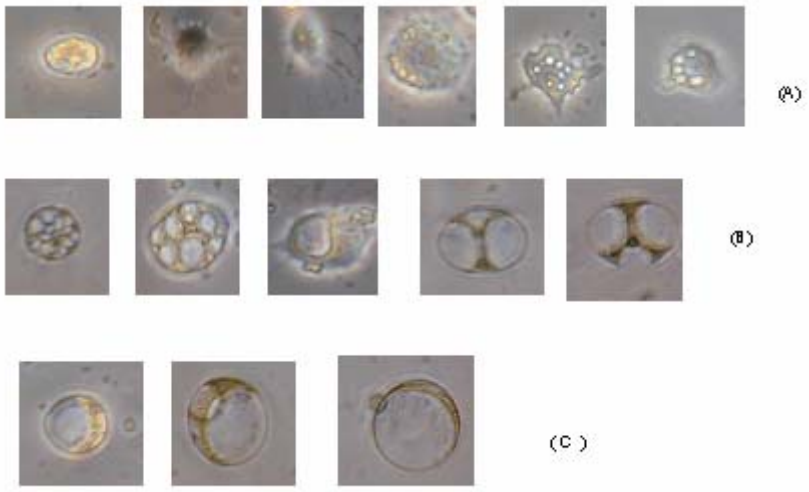


Figure 1. The process of macropinocytosis in a cell line of *P. xylostella*. Single cells were observed from attached and spred forms to initiation of multiple macropinocytosis events A), Sometimes endocytosis vesicles increased in size B), leading to foam cells C).

Since nothing is known about the properties of GlcNAc-containing proteins in lepidopteran cells, further experiments are required to examine the difference in binding and endocytosis induction. The fact that HPL binds to *mbn2*-cells, causing hemomucin clustering (Theopold and Schmidt, 1997) and immune signaling (Theopold *et al.*, 1996), but is unable to induce macropinocytosis, suggests that additional factors may be required to induce endocytosis. Alternatively, hexameric HPL may not interact with relevant components as do tetrameric VVL. To examine possible structural features of lectins, we used monomeric and oligomeric lectins.

Induction of cellular functions by oliogomeric lectins

In both cell lines, the Gal/GalNAc-specific monomeric lectin from *Psophocarpus tetragonolobus* (winged bean, WBL) had no significant effect on spreading and macropinocytosis in mbn2 and *Plutella* cells (Fig. 1, 2). To investigate whether VVL, PNA and WBL bind to the same sites on glycoproteins, we performed competition experiments, were we pre-treated the cells with WBL before adding VVL and PNA. In the presence of WBL the induction of macropinocytosis by VVL or PNA was reduced (Fig. 7). This suggests that WBL binds to glycodeterminants that are related to those used by VVL and PNA, but instead of inducing cell spreading and macropinocytosis, WBL is inactive under the conditions used in the experiment. The reasons for the lack of adhesive cross-linking activities are not known. Recent reports on the lectin conformation (Manoj *et al.*, 2001) and stability of the tetrameric complex (Srinivas et al., 1998) suggest that under certain conditions the lectin may be instable and act as a monomer. Since lectins are known to retain sugar-binding specificity as a monomer (Reddy *et al.*, 1999), it is possible that monomeric WBL can compete with tetrameric lectins for binding sites.

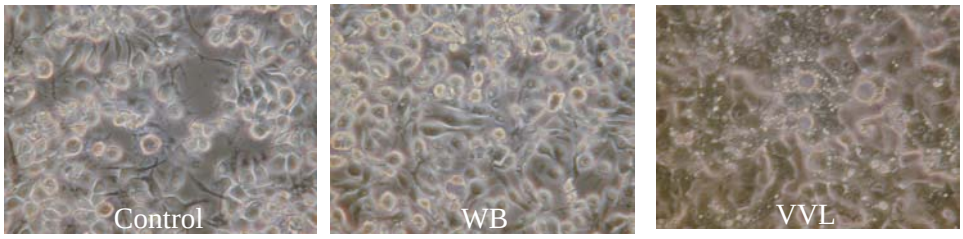


Figure 2. Attachment and spreading of *mbn-2* cells in the presence and absence of lectins. Lectins from *P. tetragonolobus* (winged bean, WB) were similar to control and did not show any significant attachment and endocytosis. In contrast, tetrameric lectins with GalNAc- and Gal/GalNAc-specific binding properties such as (VVL) and (PNA, not shown), induced attachment and macropinocytosis.

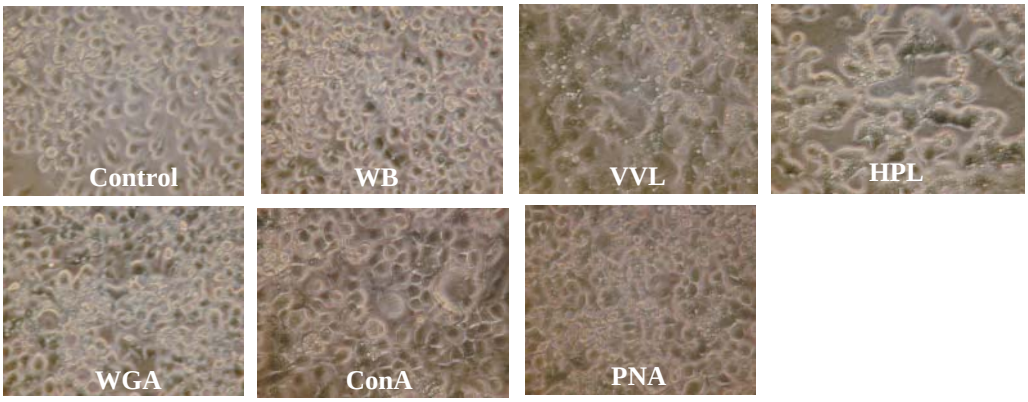


Figure 3. Attachment and spreading of *Plutella* cells in the presence and absence of lectins. Lectins from *P. tetragonolobus* (winged bean, WB) were similar to control and did not show any significant attachement and endocytosis. In contrast, tetrameric lectins with GalNAc- and Gal-specific binding properties such as (VVL) and (PNA), induced attachment and macropinocytosis.

To confirm that the monomeric lectin (WBL) competes for the same binding-sites in macropinocytosis, it was applied in conjunction with other tetrameric lectins and shown to reduce macropinocytosis (Fig. 7). Although this experiment was only performed twice, the observation that WBL may compete with GalNAc-specific VVL and Gal-specific PNA could be due to dual specificities of WBL (Manoj et al, 2001) or spatial hindrances of closely located lectin binding-sites on the mucin domain. Taken together, these observations suggest that binding of the carbohydrate-recognising domain (CRD) to a glycoprotein receptor is a precondition but not sufficient for macropinocytosis induction. Induction of macropinocytosis may require specific structural arrangements and cross-linking properties, which are only possible in oligomeric lectins.

The observation that HPL is able to aggregate hemomucin on the cell surface (Theopold and Schmidt, 1997) and induce an immune responses (Theopold et al., 1996), but is unable to induce macropinocytosis in *mbn2*-cells (Fig. 4), suggests that clustering of receptors is probably required but not sufficient for endocytosis.

Interestingly, HPL was able to induce macropinocytosis in *P. xylostella* cells, after a time delay, which suggests that additional components may be required for macropinocytosis and may be induced in lepidopteran, but not in Drosophila cells.

In the presence of lectins hemocytes can either form coagulation products, such as globules, or perform cellular functions, such as adhesion and macropinocytosis. Since coagulation and cell activities are quite disparate processes, the balance between the two types of reactions is quite relevant for the overall outcome.

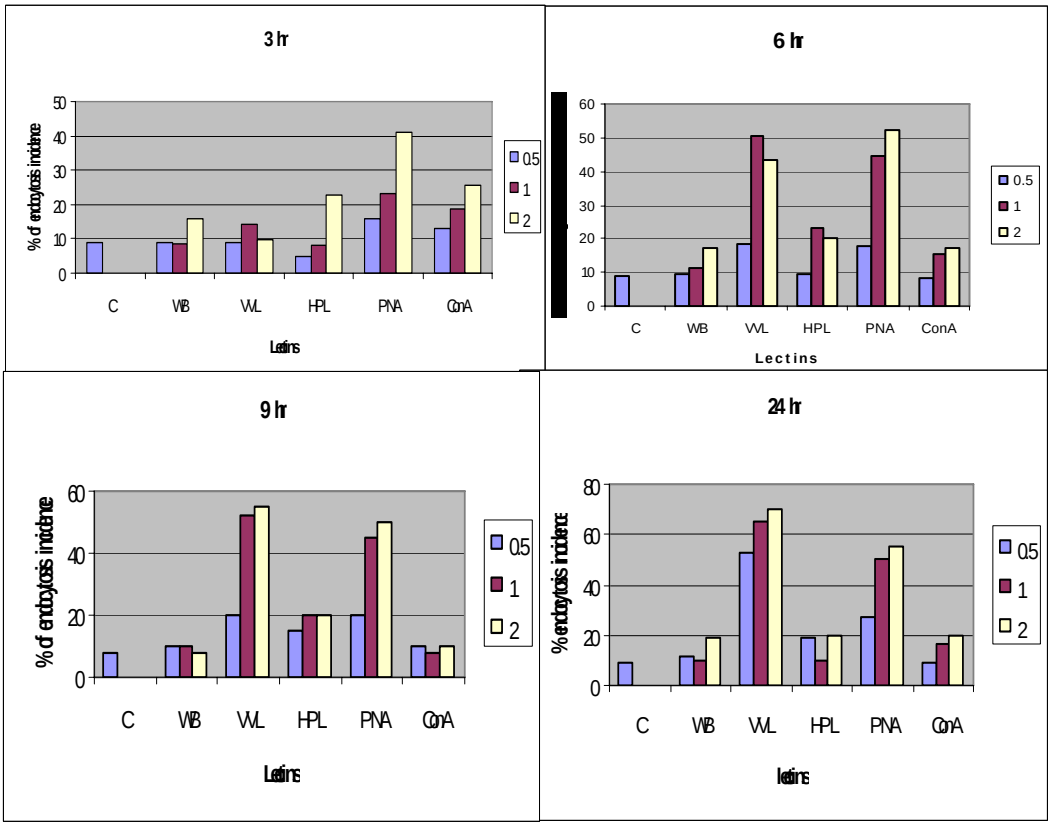


Figure 4. Macropinocytosis induced by oligomeric lectins. After addition of lectins at various concentrations, the relative number of cells with macropinocytosis was monitored over different time points. Lectin induction in mbn2-cells. Tetrameric lectins, such as VVL and PNA showed significant macropinocytosis, whereas WB and hexameric HPL were less effective.

In cell-free hemolymph (plasma) only coagulation reactions occur in the presence of externally added GalNAc-specific lectins. This suggests that GalNAc-lectin-mediated coagulation reactions may not normally occur in plasma, but are comfirmed to Gal-containing glycoproteins stored inside granules and Gal-specific lectins found in the plasma (Castro *et al*. 1987) . In the presence of hemocytes GalNAc-containing glycoproteins may attach to the cell surface to mediate cellular functions.

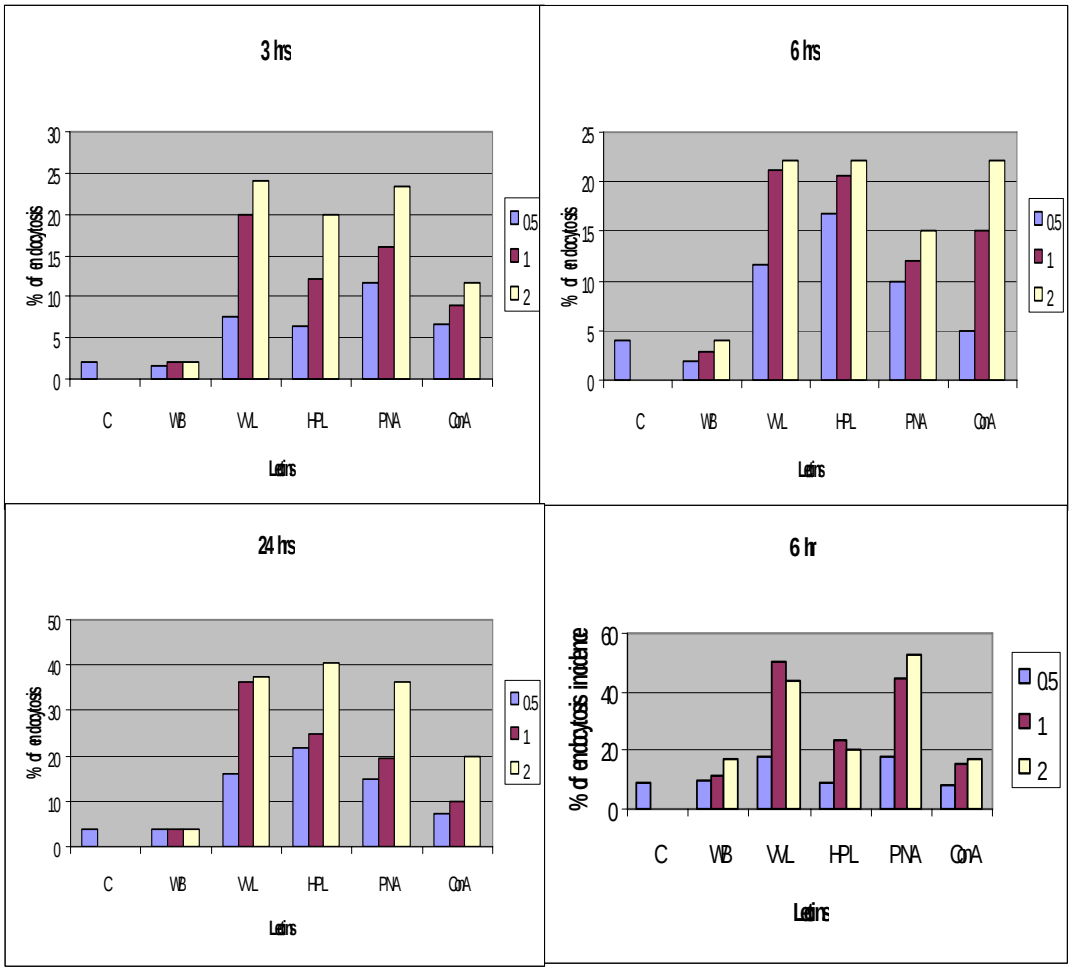
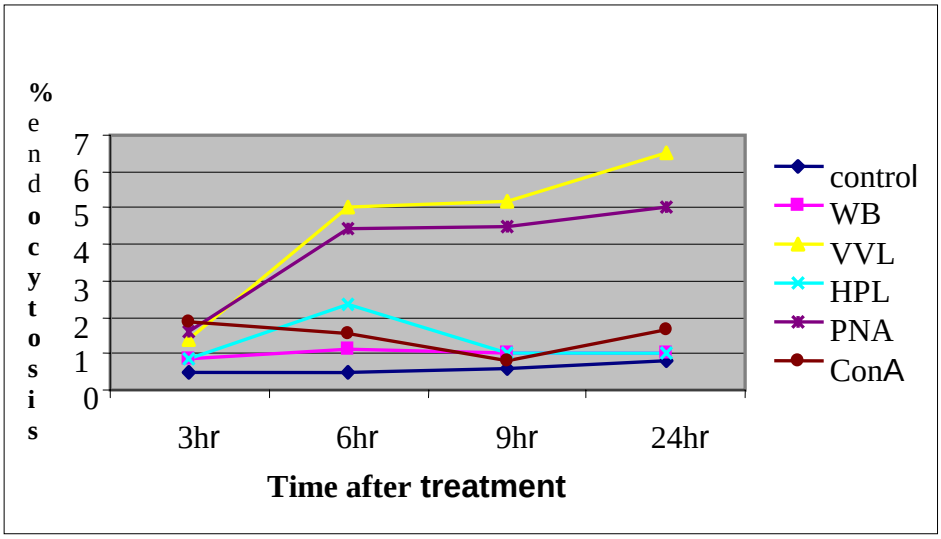


Figure 5. Lectin-mediated macropinocytosis in *P.xylostella* cells. After addition of lectins at various concentrations, the relative number of cells with macropinocytosis events was monitored over different time points. Compared to *mbn2*-cells, induction in *P. xylostella* cells is delayed. In these cells HPL is an effective inducer of macropinocytosis, whereas ConA is less effective.

Table 1. Properties of lectins conjugated that been tested in the experiments

No	Common name	Taxonomic Name	Mol Wt x 10^3	Sub Unit	Sugar specificity
1	Winged Bean	*Psophocarpus tetragonolobus*	35	1	Gal
2	Hairy vech	*Vicia vilosa*	139	4	GalNac
3	Roman Snail	*Helix pomatia*	79	6	GalNac
4	Peanut	*Aeachis hypogaea*	120	4	Gal, GalNac
5	Wheat germ	*Triticum vulgaris*	36	2	GlcNac,GalNac, NeuNac
6.	Con A	Concavalin A	1-2	4	Man, Glc



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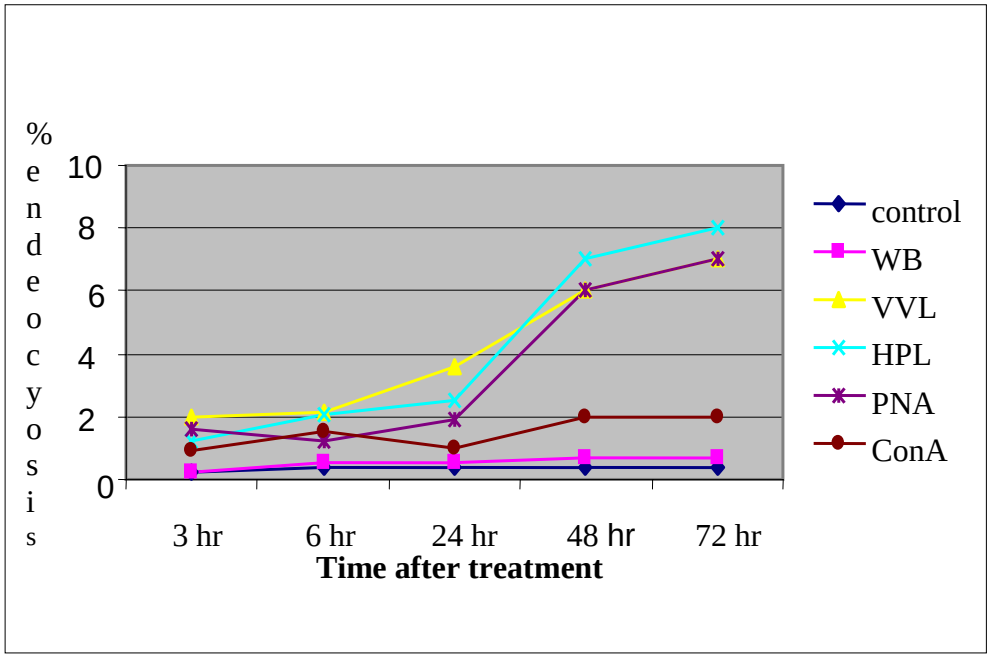

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Figure 6. Number of endocytosis events after treatment with various lectins. a) Diagram of macropinocytosis induction in *mbn2*-cells. B) Diagram of macropinocytosis induction in *P. xylostella* cells.

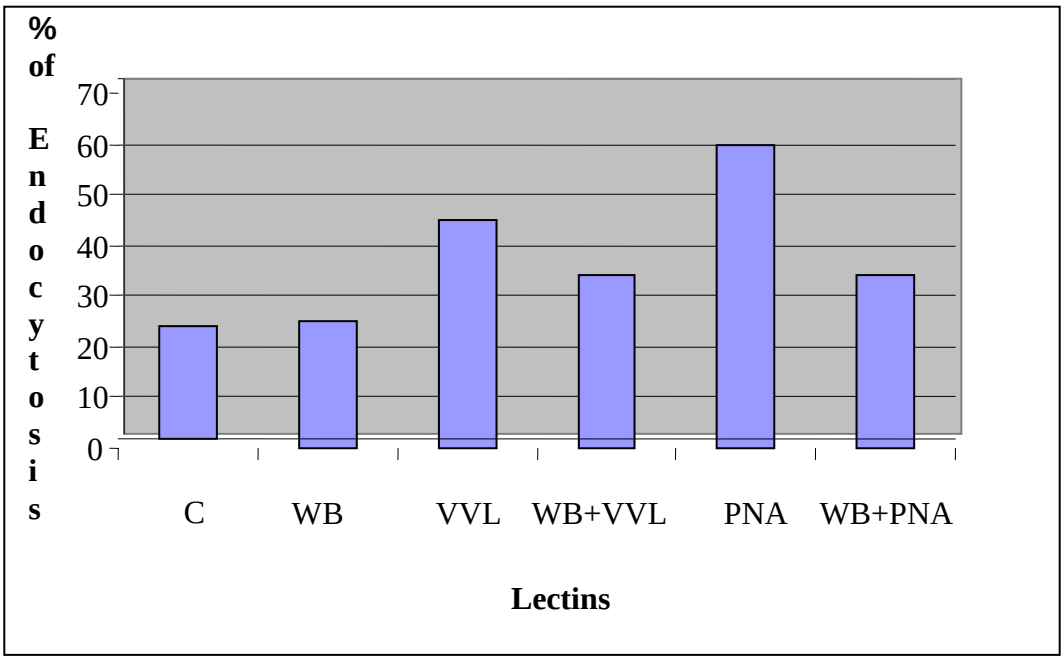

Figure 7. Macropinocytosis induction in *mbn2*-cells by oligomeric lectins is reduced in the presence of WBL. To test, whether WB was able to compete for binding sites of tetrameric lectins, the induction of macropinocytosis was monitored in the presence of both lectins. Each bar represents an average of two independent experiments.

CONCLUSION

Hemocytes can form coagulation products, such as globules, or perform cellular functions, such as adhesion and macropinocytosis in the presence of lectins. The balance between the two types of reactions is quite relevant for the overall outcome, since coagulation and cell activities are quite disparate processes. In cell-free hemolymph (plasma) only coagulation reactions occur in the presence of externally added GalNAc-specific lectins.

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