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The Cellular Immunity Responses In The Haemolymph Of Honey Bee Workers Infected By American Foulbrood Disease (AFB)

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Abstract: The cellular immunity responses resulted infected honey bees workers by American foulbrood (AFB), the most serious disease of bacterial origin, *Paenibacillus larvae* subsp. *larvae*, a Gram-positive and spore-forming bacterium, affected the larvae and pupae stages were studied at different developmental stages of bees. The physiological immunity could be prevail on the bacterial infection or their toxins by more than one method particularly at low infection levels. Self immunity reaction of immature and mature worker bees in infected honey bee colonies was distinguished by presence higher percentages of some blood cells and higher specialized of the morphological haemocyte changes. Granulocyte and Micronucleocyte cells plays an important role in the immunity system and consider one of the main sign of the disease recognizer.

Key words: Honey bee, haemocytes, blood cells, disease, American foulbrood, spores, Immunity,

INTRODUCTION

Honey bee colonies exposure to numerous parasites and pathogens affected on the economical honey bee industrial. One of the most important disease that attacks honey bee (*Apis spp.)* in the larval stage is American foulbrood. The American foulbrood is caused by serious bacterial disease *Panebacillus larvae* subsp. *larvae*[25]. Honey bee colonies susceptible to American foulbrood (AFB) or European foulbrood (EFB), resulted momentous damage[31]. Many factors affecting the distribution of the (AFB) disease as the ectoparazitic varroa mites which associated with honey bee pathogens and confirmed in some cases the vector of the disease[14]. Scavenge bees are more to be subjected to airborne bacterial spores in the infected bee colonies[17]. *Bacillus larvae* infection was quickly found in fat cells and all other larvae body tissues after percautaneous inoculation of 3-5 day old[13]. Honey bee larvae exhibited a high rate of mortality when infected with *P.larvae* at very young age (1-2 days old). *B. larvae* in dead larvae, increased in relation to the number of spores in the food[23]. The mortality decrease as the age of bees increased[22]. In spite of the disease affects only the larvae but in highly infectious it had sharply deadly to adult bees[6]. Social insects have evolved both communal and individual traits that reduce the impacts of their numerous parasites and pathogens[7]. If the bacteria succeed in reaching the haemocel through mechanically injured or enzymatically damaged anatomically protective barriers, they generate the internal immune responses in body cavity of the insect[10]. The immunity responses has have the potential to reduce the individual mortality and pathogens spread among colony members[7]The immunity proteins produced that kill bacteria caused by hymenoptaecin with apidaecins form a group of inducible responses proteins that protect honey bee well from saprophytic bacteria[9,15]. In the other side *B. larvae* produce substance(s) with proteolytic activity that interfere with the antibacterial immune mechanisms of the insect and destroyed some molecules of the insect humoral immune system[16,21]. The haemocytes are the major cellular components in the immunity process and involved in the cell mediated immunity reactions[24]. Haemocyte – mediated defense reactions of bees consist of phagocytosis, encapsulation, cytotoxicity and secretion of materials to damage foreign organisms or increases a percentage[10]. The aim of this investigation is to study the cellular immunity responses of the haemolymph in infected immature and mature stages of honey bee workers by American foulbrood disease.

MATERIALS AND METHODS

I: Diagnosis of American foulbrood disease: During summer season, 2006 out of 75 honey bee colonies of Carniolian hybrid, ten colonies were found infected with foulbrood disease. These colonies were located in an apiary at Giza region of Egypt. The American foulbrood disease was diagnosed according to symptoms described by Alippi,[2,3] and Prince and Dancer,[32]. Infected bee larvae were examined for the causative bacterium using a simple staining technique involves transferring the samples onto a microscopic slide using nigrosine stain the highly effective and used all over the world.

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II: Collection of haemolymph samples and preparation of blood films: The haemolymph samples were collected from the 2nd days old larvae, prepupae and adult nurse worker bees from healthy and infected bee colonies by (AFB) disease according to method of Gilliam and Shimanuki,[8]. The tested blood films were prepared and stained with Gimsa's stain based on the proceeding of Nappi and Streams,[19] and Gray[12].

III: Determination of bacterial density in the blood films: The bacterial density of American foulbrood (AFB) were detected in the blood films as follows;

- Films exposed low infection level density with the bacterial infection.
- Films exposed high infection level density with the bacterial infection.

The mean haemocytes count was calculated as mean 20 randomly spots/slide. The mean surface area of the plasmatocyte cells (μm²/cell) was calculated according to the formula of Maurizio,[18] as follow; the mean surface area = II X ab/2 (II=3.14, a = maximum length and b= maximum width). Analysis of variance (ANOVA) was performed for the obtained data according to the method of Waller and Duncan,[33].

RESULTS AND DISCUSSIONS

The haemolymph of healthy honey bee workers at different developmental stages showed the following various haemocytes as follows; Proleucocyte, Eosinophil, Basophil, Neutrophil, Oenocytoid, Pycnonucleocyte, Adipoleucocyte, Spherulocyte, Granulocyte Macronucleocyte, Micronucleocyte, Plasmatocyte and Spindle shaped cells[35] as shown in Fig.(1).

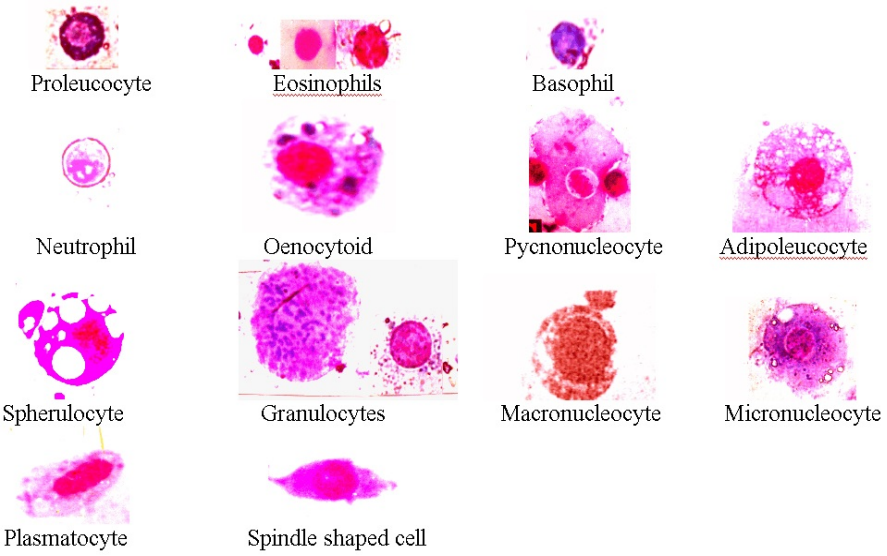


Fig. 1: Different haemocytes type in the haemolymph of healthy honey bee workers at different developmental stages. (X- 400)

Table 1: Mean number and percentage of the different haemocytes in the haemolymph of infected honey bee worker larvae by *Panebacillus larvae* subsp. *larvae*.

	Blood cells											
	Non-phagocyte cells							Phagocyte cells				
Item	Pro.	Eos.	Bas.	Net.	Oen.	Pyc.	Sph.	Grn.	Mac.	Mic.	Pla.	Spl.
Healthy larvae	26.12	9.25b	19.87	13.5	4b	1.12	0.0b	9b	7	5.5	10.75	9.5
	22.59%	8%	17.18%	11.67%	3.46%	0.98%	0.0%	7.79%	6.06%	4.76%	9.29%	8.22%
Low infection	26	20.66b	33.66	11	10a	0.16	0.0b	17.66b	11.83	8.5	6.83	4.66
	17.22%	13.69%	22.3%	7.29%	6.62%	0.11%	0.0%	11.69%	7.84%	5.64%	4.52%	3.08%
High infection	27.85	43.85a	78.42	12	1.28b	2.28	1.0a	202.8a	12.71	5.71	5.28	6.14
	6.99%	11%	19.67%	3.00%	0.32%	0.57%	0.25 %	50.72%	3.19%	1.43%	1.32%	1.54%
$LSD_{0.05}$	F=0.025	19.93	F=2.88	F=0.048	4.42	F= 1.481	0.346	99.07	F=0.68	F=0.48	F=0.191	F=0.95

Pro.:Proleucocyte. Eos.: Eosinophil. Bas.: Basophil. Net.: Neutrophil
Oen.: Oenocytoid. Pyc. : Pycnonucleocyte. Sph: Spherulocyte Grn.: Granulocyte.
Mac.:Macronucleocyte. Mic.:Micronucleocyte. Pla.:Plasmatocyte. Spl. : Spindle shaped cell.

The haemolymph of larval honey bee workers infected by American foulbrood (AFB) at the 2nd days old contained *Panebacillus larvae* subsp. *larvae* in chain shape (Vegetative stage) as shown in Figs. (2and8). Data presented in Table (1) showed clearly significant differences between mean number and percentage of some haemocytes between the haemolymph of healthy and infected worker bee larvae particularly with Granulocytes. It is interested to not that most of blood cells as Eosinophil, Basophil, Oenocytoid, Macronucleocyte and Micronucleocyte were increased

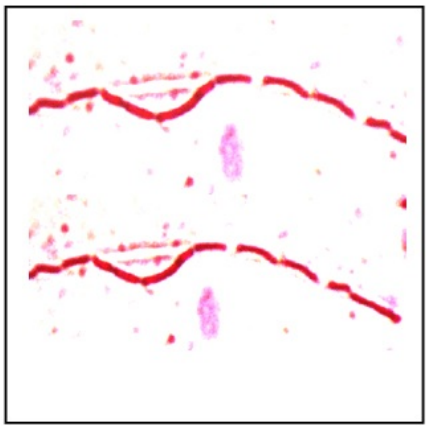


Fig. 2: Vegetative stage o*f* *Panebacillus larvae* presented in the hemolymph of infected bee worker larvae in chain shaped. (X-400)

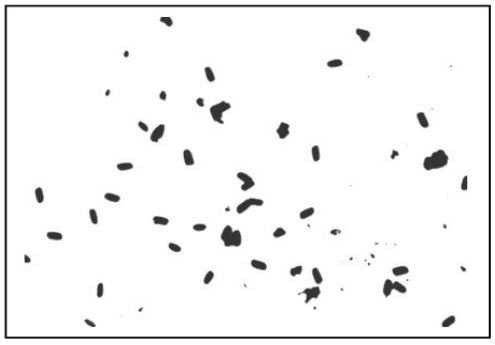
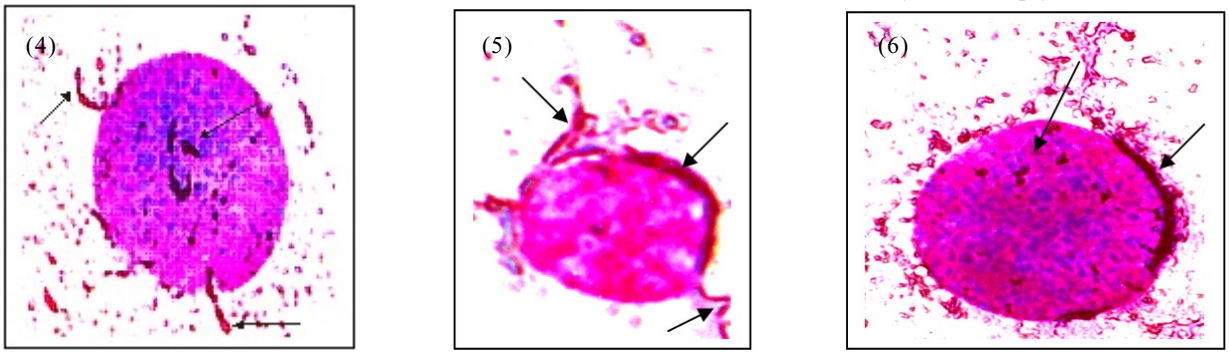


Fig. 3: *P. larvae* spores present in the haemolymph of worker bees at the prepupal stage. (X- 400)

with lower infection level and decreased with diseased level increase, that may be due to the immunity process in the haemolymph of infected bee stage, while other haemocytes as Proleucocytes, Neutrophils, Plasmatocytes and Spindle shaped cells were gradually decreased with the disease level increase. Granulocyte cells sharply increased with the disease infection level increase. They reached to 50.72% with highest infection level (Table1). The Granulocyte, Eosinophil, Proleucocyte and Plasmatocyte cells may be attack the bacterial infection by more than one way as adhesion and ingestion methods as shown in Figs.(4,5,6,7,8and9). Macronucleocyte and Micronucleocyte cells showed several morphological changes towards bacillus infection as cytoplasmic protuberances, vacuoles and gap swallowing in their internal structure (Fig.10).

The mean dimension of the Granulocyte and Macronucleocyte cells of infected worker bee larvae showed increases with lower infection level and decreases with the diseased level increase. The mean surface area (μm^2) of the Plasmatocyte cells were decreased with the disease level density increase (Table 2). From the obtained results, it could be concluded that the bacterial spores of *Panebacillus larvae* infected bee larvae of honey bee workers at the 2nd days old were tendency to the chain shape. The immunity mechanism had more than one side of the haemocytes responsiveness to the disease infection, variety from increasing their numbers, adhesion and ingestion methods. The mean dimension of the Granulocyte and Macronucleocyte cells of infected worker bee larvae showed increases with the lower density of the disease levels and decreases with the diseased level increase, while Plasmatocyte cells were gradually decreased with the disease level increase.

The haemolymph of infected prepupal stage of worker honey bees by (AFB) disease were contained bacillus larvae in vegetative and spore forms (Figs.2and3) that attributed to the bacterial developmental stages. Sharply decreased in the haemocytes percentage with all infection levels were recorded. The Basophile cells reached the maximum level percentage among other haemocytes present (90.88 and 75% with lower and higher infection levels, respectively). In heavily infection levels with (AFB) disease, the haemocytes sharply



Figs. (4,5 and 6): Bacterial ingestion stages of the Granuocyte cells in the haemolymph of infected bee larvae. (X-400)

Table 2: The haemocytes measurement of infected honey bee worker larvae by American foulbrood disease (±S.E.).

Blood cells	Granulocyte diameter (µ)	Macronucleocyte diameter		Plasmatocyte dimension		
		Cell (µ)	Nucleus (µ)	Length (µ)	Width (µ)	Mean surface area (µm2)
Healthy Larvae	16.788	5.666	3.277	14.326	9.166	206.173
	3.237	0.4106	0.2071	0.8169	0.5688	
Low infection level	19.134	6.0526	3.294	13.833	8.5476	185.594
	3.157	0.2826	0.1507	0.9214	1.229	
High infection level	17.735	5.7727	3.136	13.238	6.8452	142.268
	3.237	0.3714	0.1873	1.0804	0.7526	

Table 3: Different haemocytes count in the haemolymph of infected bee workers by *Panebacillus larvae spores* at the prepupal stage.

Item	Blood cells Non-phagocyte cells						Phagocyte cells				
	Pro.	Eos.	Bas.	Net.	Oen.	Pyc.	Grn.	Mac.	Mic.	Pla.	Sp.
Healthy prepupae	7.4	21.4	13.4	1.8	6.2	0.0	3.66	0.4	0.4	1.6	1.2
	12.88%	37.24%	23%	3.13%	10.8%	0.0%	6.37%	0.7%	0.7%	2.78%	2.08%
Low infection level	5	3	379	3	3	0.0	21	0.0	1	0.0	1
	1.2%	0.72%	91.11%	0.72%	0.72%	0.0%	5.05%	0.0%	0.24%	0.0%	0.24%
High Infection level	0.0	3	30	0.0	0.0	0.0	3	0.0	0.0	4	0.0
	0.0%	7.5%	75%	0.0%	0.0%	0.0%	7.5%	0.0%	0.0%	10%	0.0%

Pro.: Proleucocyte Eos.Eosinophil. Bas.: Basophil. Net.: Neutrophil.
Oen.: Oenocytoid. Pyc.: Pycnonucleocyte. Grn.: Granulocyte. Mac.:Macronucleocyte.
Mic.: Micronucleocyte. Pla.: Plasmatocyte. Spl.:Spindle shaped cell.

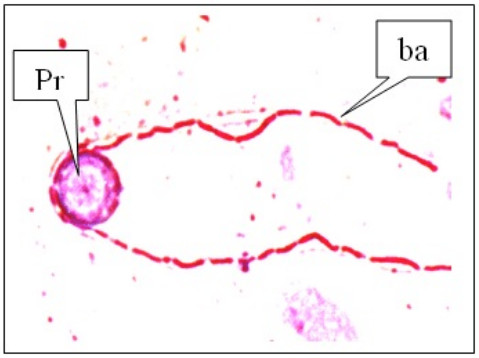


Fig. 7: Adhesion of the proleucocyte cell (Pr) to bacillus contamination (ba). (X- 400)

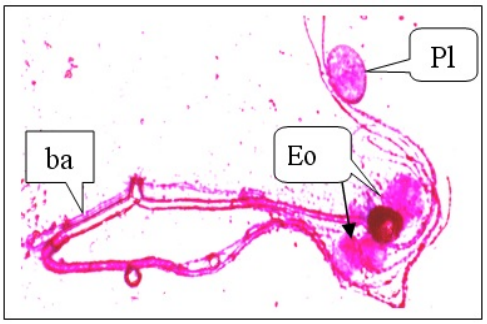


Fig. 8: Coherenced of the plasmatocyte (Pl) and Eosinophil cells (Eo) with the *bacillus larvae* (ba) at high infection level. (X-400)

decreased, dwarfed, surrounded by high density level of the bacterial infection (Fig.11) and died at the end of bee life as shown in Table (3) andFig. (12), that may be due to the fall down the immunity system.

From these results, it could be suggested that the *Panebacillus larvae* developed to the spore phase in infected honey bee workers at the prepupal stage. The immunity system was completely breakdown when the infection disease reached high level.

Self immunity reaction was distinguished in most blood cells of adult nurse worker bees in infected honey bee colonies, whereas they forced to increase their percentages (Table 4) and had more than one side of the defense mechanism responses towards the bacterial invasion. Oenocytoids, Micronucleocytes, Spherulocyte, Granulocyte and Pycnonucleocytes modified their shape as presence different vacuoles volume, increase size, cytoplasmic protuberances and gap swallowing in their boodies as shown in Figs. (13,14 and15). It could bee concluded that adult nurse worker bees were more exposure to the contamination with the disease spores and their blood cells showed demonstrated certain specialized immunity features in tested infected bee colonies.

From the obtained results it could be summarized that the *Panebacillus larvae larvae* in infected worker bee larvae take chain shape in their vegetative stage, while in the prepupal stage it development to spore conformation. There were significant differences in the mean number

Table 4: Blood picture in the haemolymph of adult nurse bee workers in healthy and infected honey bee colonies by (AFB) disease.

	Blood cells												
	Non-phagocyte cells								Phagocyte cells				
Item	Pro.	Eos.	Bas.	Net.	Oen.	Pyc.	Adp.	Sph.	Grn.	Mac.	Mic.	Pla.	Spl.
Nurse worker bees (A)	0.66	254.3	1.33	18b	13	0.66	9	1.0a	1.66	3.66	23	2	0.66
	0.20%	77.32%	0.40%	5.47%	3.95%	0.20%	2.75%	0.30%	0.50%	1.10%	7.0%	0.61%	0.20%
Nurse worker bees (B)	7	8.25	5.5	5	27	1.5	3	19b	16. 5	3.5	21. 5	48	2
	4.17%	4.92%	3.28%	2.99%	16.09%	0.89%	1.79%	11. 33%	9.83%	2.09%	12.82%	28.61%	1.19%
$LSD_{0.05}$	F=4.75	F=2.01	F=2.01	F=1.35	F=0.95	F=3.57	F=0.5	1.60	F=2.2	F=4.85	F=1.16	F=3.06	F=3.06

Pro.:Proleucocyte. Eos.: Eosinophil. Bas. : Basophil. Net.: Neutrophil.
Oen.: Oenocytoid. Pyc.: Pycnonucleocyte. Adp.: dipohaemocyte. Sph.: Spherulocyte.
Grn.: Granulocyte. Mac.: Macronucleocyte. Mic..: Micronucleocyte. Pla.: Plasmatocyte.
Spl.:Spindle shaped cell. (A) : Healthy colonies. (B) : Infected bee colonies by (AFB) disease.

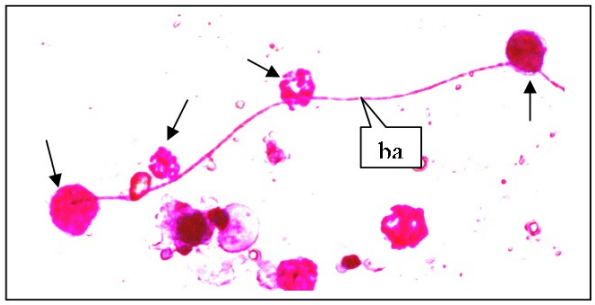


Fig. 9: Chain of the *Panebacillus larvae* (ba) attaked by some blood cells(arrows). (X- 400)

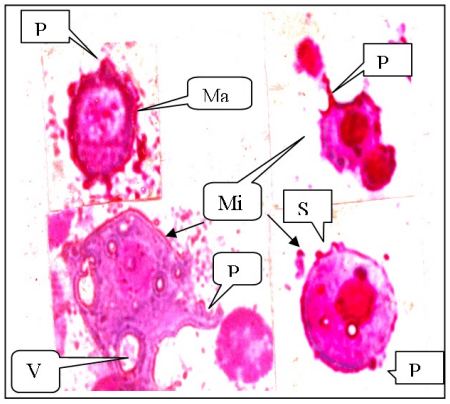


Fig. 10: Cytoplasmic protuberances (P), Gap swallowing (S)and vacuoles (V) presented in the Macronucleocyte (Ma) and Micronucleocyte (Mi) cells in infcted bee worker larvae by *bacillus larvae*. (X-400)

and haemocytes percentage in infected bee workers at different developmental stages. The haemocyte percentage increase with the lower infection level and decrease with the bacterial level increase. Granulocyte cells play an important role in the immunity process, they consider one of the main peculiarity of the disease recognizer, whereas, they increase with the lower disease infection level and decrease with the disease level

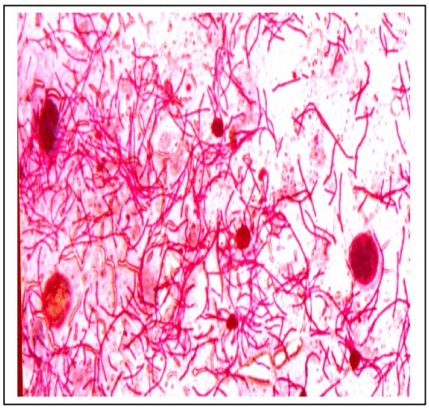


Fig. 11: Blood cells surrounded by sharply densities of the bacterial infection of *bacillus larvae*. (X- 400).

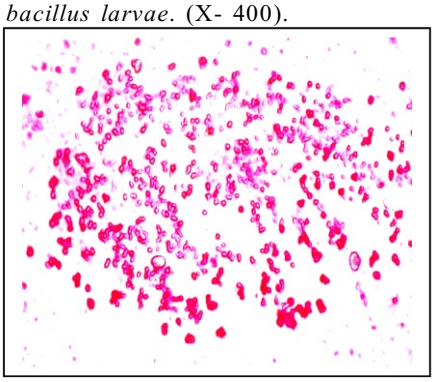


Fig. 12: Dwarf haemocytes in the haemolymph of the prepupal stage in the advanced cases of the infection level by *Panebacillus larvae*. (X - 400)

increase. The Basophile cells percentage sharply increased in the haemolymph of infected older bee larvae at the prepupal stage. When the immunity process downfall, the total number and haemocytes percentage were sharply decreased, dwarfed and died with end of bee life. There were one or more side of the physiological immunity process in the cytoplasm of the haemocytes of adult nurse bees in infected bee colonies, represented cytoplasmic

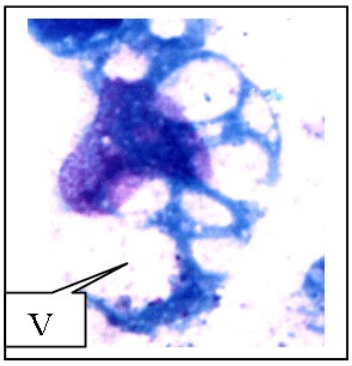


Fig. 13: Spherulocyte cell in the haemolymph of adult nurse worker bees in infected honey bee colonies by (AFB) disease showed large vacuole (V). (X-400).

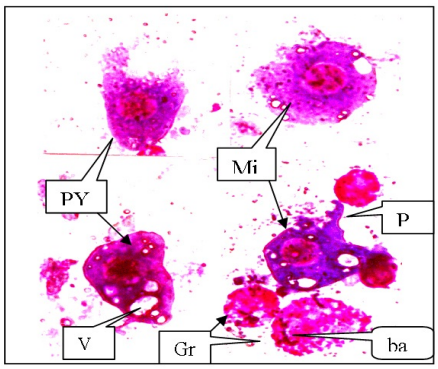


Fig. 14: Self immunity reaction of adult nurse worker bees showed different interaction defenses; Pycnonucleocytes (Py) had small to large vacuoles(V). Micronucleocytes (Mi) showed emergence an cytoplasmic protuberances (P). Granulocytes (Gr) had engation method to bacterial infection (ba). (X- 400).

protuberances, gap swallowing and different vacuoles dimension.

It could be summarized that haemocytes of immature and mature honey bee workers were sensitive to infection with American foulbrood (AFB) disease. Certain of adult nurse bees in infected honey bee colonies by (AFB) disease were distinguished by force self immunity responses, whereas they distinct by the highly haemocytes percentage among others present in normally one. They had more than one side of the defense mechanism reaction. The immunity system in the haemolymph of different infected stages of worker honey bees may be prevail on the bacterial activity or their toxins by more than one method particularly at low infection levels.

The obtained data are agreement with the finding of Riessberger *et al*.,[22], they decided that resistance nurse aged and winter bees to (AFB) disease may be contributed to substances produced by adult bees inhibits the growth of *P. larvae* spores. Evans and Pettis,[7] found

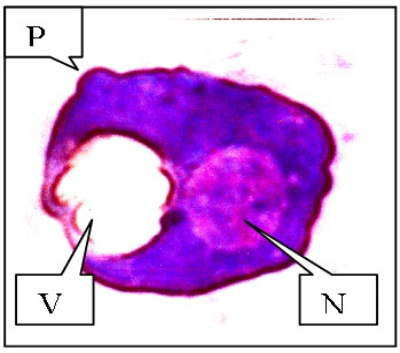


Fig. 15: Large vacuole (V) and cytoplasmic protuberances (P) in big Oenocytoid cell presented in the haemolymph of nurse worker bees in infected bee colonies by (AFB) disease. (X- 400) (N: Nucleus).

considerable variation across honey bee colonies infected by *P. larvae* in an immune trait important for survival and point toward a significant trade – off between this trait and colony productivity. The colony level disease rates were negatively correlated with the immune responsiveness of colony members. Palmer and Oldroyd,[29]and Spivak and Reuter,[28] reported that multiple mating by social insects particularly honey bee queens is adaptive because it increase intra colony genetic diversity and thereby reduces the likelihood that parasites or pathogens will catastrophically infect a colony, using (AFB) disease as model of pathogen. Yue Wen *et al.*,[34] found that adult Asian honey bee, *Apis cerana* hygienic behavior effectively decreased the level of spore contamination by American foulbrood disease. Glinski and Grzagorczyk,[11] found greatly increased in the haemocytes count in the haemolymph of infected bee workers by bacterial spores. Gregorc and Bowen,[13] found sign of lytic function in the haemocoel and degradation of the haemocytes resulted *P. Larvae* infection. Papadopoulou-karabela *et al*.,[30] found not differences in the Granulocytes percentage and mitoically dividing haemocytes between healthy and diseased bees by some bacterial spores. Zakaria,[35] mention that blood cells in the haemolymph of resistant honey bee workers to varroa mite infestation had some immunity responsivenes. The viability of Granulocytes and Oencytoids in the haemolymph of healthy honey bees workers ranged between 81.1% on day 5 and 90.2% on day 9[27]. Clearly significant differences in the blood volume and the haemocytes count percentage were recorded in the haemolymph of honey bee workers following infection by some bacterium spores[4,5]. Sorescu and Dragomir,[26] described the ways the proportion of the haemocyte populations changed with the main bacterial infection to honey bees; *Paenibacillusb larvae*, *Escherichia coli, P. alvei, B. laterosporus* and *B. circulants and* mycotic diseases (American and European foulbrood and Ascophaerosis).

Haemocytes count in healthy individuals of *A.mellifera.* and *A. cerana indica* were significantly higher than those found in the infested one with *Tropilaelaps clareae* mite[1]. Infected adult worker bee with Chronic Paralysis Virus (CPV) showed marker decreased in the mean number and blood cells percentage[20].

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