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ON THE EXISTENCE OF IMMUNITY PRINCIPLES IN INSECTS.[1]

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During the course of my work on various diseases of insects, I have often been confronted by results which seemed to point towards the existence of immunity principles. I failed to become convinced, however, till I instituted a series of experiments meant to prove or disprove my views. Other workers, also, on investigating caterpillar and grasshopper diseases, have been unable to explain some of their results without assuming the possibility of physiological immunity, but direct proof for their contentions has been lacking.

In physiological work of this sort it is very difficult to obtain quantitative data for the reason that the amount of blood obtainable from a particular insect amounts to only one-tenth to one-fifth of a cubic centimeter. In one series of experiments I managed to obtain quantitative results. The other data are qualitative but, I hope, no less important.

The Question of Phagocytosis.

Since all entomological text-books emphasize the importance of the blood cells in ridding the insect body from the invasion of foreign substances this question was first investigated. In practically all the literature on the subject, insect blood cells are compared with the mammalian white blood corpuscles. An exceedingly aggressive nature is attributed to them and their movements are described as actively amæboid, engulfing foreign substances with great avidity. I was greatly astonished to find that this view was incorrect and that the blood cells are visibly rather passive. Of course, we know that the so-called amæbocytes play an important rôle during metamorphosis, and I do not wish to create the

[1] Contribution from the U. S. Bureau of Entomology in coöperation with the Bussey Institution of Harvard University. (Bussey Institution, No. 143.)

impression that I depreciate the importance of these cells. However, even during metamorphosis their action is not confined to an aggressiveness manifested by movement but rather, I think, to an increase in the secretion of proteolytic and perhaps other enzymes. These break down the larval tissues and prepare the various proteins and other substances for assimilation by the imaginal disks that form the adult tissues.

One gains the impression from text-books that the insect blood cells, called amæbocytes, during metamorphosis, bodily attack those larval tissues destined to destruction; that they swallow masses of such tissues, digest them and then wander over to the imaginal disks where they surrender the digested matter. I will attempt to show that insect blood cells are nor quite as aggressive, as we have been persuaded to suppose, and that one can stimulate the formation of certain substances acting extracellularly. It may be true that these substances are formed by the cells but, on the other hand, it is also possible that they are formed by the blood plasma or serum. After they are formed, however, they act independently of any cellular organization.

During 1916 and 1917 while studying certain bacteria pathogenic to caterpillars, and others pathogenic to grasshoppers, I had occasion to inoculate many insects with different cultures. In some of my experiments several of the insects lived in spite of the fact that enormous numbers of microörganisms, supposedly pathogenic, were introduced. For example: Ten mature female grasshoppers (*Melanoplus femur-rubrum*) were each injected with $^1/_{10}$ c.c. of a 24 hour bouillon culture of *Bacillus poncei* Glaser. *B. poncei* is a highly motile organism which I obtained from the Honduran government in 1915. The bacterium is ordinarily pathogenic to *M. femur-rubrum*. After intervals of $\frac{1}{2}$, 1, 2, and 24 hours the animals were killed and a large metathoracic leg removed from each by breaking the joint between the trochanter and femur. The blood that oozed from each animal was caught on a separate sterile cover-slip. Some of these preparations were fixed by passing through a Bunsen flame, others were immersed in 70 per cent. alcohol, while still others were fixed with Schaudin's corrosive sublimate solution. After fixation, the preparations were dried and stained with methylene blue. Excessive staining can be remedied, of course, by treatment with alcohol. After mounting

the preparations were studied and I was astonished to find that they were surprisingly free from *Bacillus poncei*. Six of the smears showed no microörganisms whatever, the remaining ones showed a few bacilli scattered about here and there outside of the blood cells. On examining each smear carefully by studying ten fields with the oil immersion lens, I found only one blood corpuscle with *B. poncei* embedded in its cytoplasm. If the grasshoppers had been permitted to live, I feel sure that only the four revealing any bacteria in the blood would have finally died of the disease. The remaining six would have lived till they succumbed to natural causes. Two animals were examined after ½ hour; two after 1 hour; two after 2 hours and four after 24 hours. The bacteria were found in one case examined after ½ hour, in two examined after 2 hours and in one examined after 24 hours. This experiment was repeated with similar results.

In many of the inoculation experiments with *B. poncei* from one-fourth to one-half of the animals did not die and I then assumed that the blood acted antagonistically towards the introduced bacteria. The blood tests cited above seemed to be evidence in favor of this view. The blood of a certain number of the inoculated animals managed to rid itself of *B. poncei* and, moreover, this riddance was not accomplished by hungry amæbocytes as the textbooks would have us believe. If the grasshopper blood cells had phagocytised large numbers of the bacteria I surely would have noticed this in the stained smears. In some cases the blood, however, acted antagonistically towards the bacteria and I will later show more clearly that the antagonistic substances are extracellular and therefore in the blood plasma or serum.

I thought that the tissue culture method might offer some interesting possibilities in studying, *in vitro*, the behavior of insect blood cells towards bacteria. The method for preparing such cultures is very simple and does not differ materially from the well known methods used by Harrison, Carrel, etc. for the cultivation of embryonic mammalian tissue. I shall not describe a method familiar to all biologists.[1]

The results of the following four experiments may be considered characteristic for a large series performed with both grasshopper

[1] Those interested in the cultivation of insect blood cells may be referred to R. W. Glaser: "The Growth of Insect Blood Cells in Vitro." PSYCHE, Vol. XXIV, No. 1, 1917.

and army worm blood. The blood of *Melanoplus atlanis* was used for these four experiments and *Coccobacillus acridiorum* d'Herelle, pathogenic to grasshoppers, was the organism used for the artificial contamination of two of the four tissue culture slides. Two slides were considered as checks. They were prepared by mixing a drop ($^1/_{10}$ of a c.c.) of the grasshopper blood with a drop of sterile, neutral, nutrient bouillon. The first day all of the blood cells appeared perfectly normal. On the third day some showed signs of disintegration, whereas others remained normal. On the sixth day the cells destined to disintegrate were completely disorganized. The others remained normal and showed cell division with the formation of syncytial, tissue-like masses. After two weeks the cells still appeared normal and the syncytia had increased considerably in size. The observations were not continued after two weeks. Throughout the entire period the slides had remained perfectly sterile showing that all technical precautions, observed during their preparation, had been adequate. At no time, not even during the first day before the formation of fibrin, did I observe any independent movement on the part of the blood cells. They remained passive and the only visible independent activity observed consisted in cell division on and after the sixth day.

The two experimental slides were prepared by mixing a drop of the grasshopper blood with a platinum loop-full of a 24 hour culture of the *Coccobacillus acridiorum* d'Herelle, a highly motile organism. The slides were examined as soon as prepared. The blood cells appeared to be perfectly normal and remained entirely passive. The preparations were swarming with the motile bacteria and in ten to twenty minutes many of the bacteria made their way into the cytoplasm of the blood cells. The latter did not engulf the bacteria which seemed to bore their way into the cytoplasm.[1] On the third day the bacteria were no longer motile. They seemed to be multiplying, but appeared in bunches simulating agglutination masses. Some of the blood cells had disintegrated; others appeared perfectly normal and bacteria were no longer visible within the cytoplasm. On the sixth day the bacteria seemed to be in about the same condition; multiplying, bunched and

[1] This may have been due to surface tension. It may be called phagocytosis if the word is used in a broad sense.

motionless. The blood cells showed cell division and the formation of the tissue-like, syncytial masses. Bacteria were not found within the cytoplasm of any of the cells. In two weeks the two culture slides presented much the same condition with the exception of the blood cell syncytia which were much larger. The observations were discontinued after two weeks.

The foregoing experiments were repeated with army worm and gipsy moth caterpillar blood. The results were in perfect harmony with the grasshopper blood observations.

From the tissue culture work, we are forced to conclude that in a mixture of insect blood cells and bacteria, the blood cells are not the visible aggressors. However, the blood seems to be able to overcome bacterial invasion to a certain extent. Substances are elaborated which antagonize the bacteria. On the culture slides, the quantity of the blood is not sufficient, and metabolism is lowered, so that antagonistic substances are not formed so rapidly nor so abundantly as is the case within the body of the insect. For this reason, although the bacteria were rendered ineffective on the culture slides and permitted the blood cells to grow, they were not killed. All of these questions will be more clearly elaborated in the next section.

Extracellular Antagonistic Substances.

In a large series of experiments with grasshoppers (*Melanoplus femur-rubrum*) and *Bacillus poncei* four animals remained alive after two weeks. These animals, like the remainder, which died, had been injected with $^1/_{10}$ of a cubic centimeter of a 24 hour bouillon culture of *B. poncei*. I suspected that these four animals were immune and thought it might be possible to demonstrate the existence of some immunity principle, such as an agglutinin. Of course, on account of the small amount of blood obtainable from a grasshopper, it is extremely difficult to perform a Widal test with all the high dilutions, but I am confident that my experiments are significant in spite of this shortcoming.

Four depression slides were prepared from the four supposedly immune animals. A leg from each grasshopper was broken and a drop of blood from each was caught on a separate sterile cover-slip. To each cover-slip I added one platinum loop-full of a 24 hour bouillon culture of *B. poncei*. The culture was first examined

microscopically and the organisms were found to be highly motile. The cover-slips were kept under observation and at first the bacteria swarmed about everywhere at an exceedingly lively rate. The motility seemed to diminish in a few minutes and in 20 minutes to ½ hour the bacteria had agglutinated in large masses and seemed to be dead to all appearances. The four tests were identical and I never saw a better reaction with *Bacillus typhosus* and typhoid serum.

Four depression check slides accompanied the four used in the experiment. These were prepared by adding *B. poncei* to normal *Melanoplus femur-rubrum* blood. The bacteria remained motile till the next day.

The eight slides were prepared under sterile conditions and the edges of the coverslips sealed with sterile vasalene, so that I was able to keep them for six days. At the end of that time when I examined the preparations the agglutination masses presented the same appearance in all four experimental slides. The blood corpuscles, however, had divided and formed syncytia. I inoculated culture tubes from these four slides, but obtained no growth, proving that all the bacteria had been killed. The four check slides proved to be interesting in a different way. On them the bacteria were not motile, but long chains were visible showing life. In some places the bacteria were bunched, but no true agglutination masses were found. On check slide 4 the blood showed signs of growth through the formation of syncytia. The blood corpuscles seemed not to have grown on the other three preparations. Culture tubes were inoculated from these check slides and pure cultures of *B. poncei* were obtained from all.

I thought it would be interesting to obtain some quantitative data in regard to the bactericidal action of immune insect blood. Sixteen *M. femur-rubrum* grasshoppers were injected each with $^1/_{10}$ c.c. of a 24 hour bouillon culture of *B. poncei*. In ten days all but three had died, and since no deaths were recorded for four days I assumed that the three living animals had acquired immunity against the bacteria. Small samples of blood removed from each showed no bacteria microscopically. Under sterile conditions the following experiments were performed in small test tubes. Adequate checks accompanied the series.

Experiments.

(1) 2 drops[1] ♀ *femur-rubrum* immune blood + 1 c.c. bouillon + 1 loop of *B. poncei.*
(2) 2 drops ♀ *femur-rubrum* immune blood + 1 c.c. bouillon + 1 loop of *B. poncei.*
(3) 2 drops ♂ *femur-rubrum* immune blood + 1 c.c. bouillon + 1 loop of *B. poncei.*

Checks.

(1) 2 drops bouillon + 1 c.c. bouillon + 1 loop of *B. poncei.*
(2) 2 drops ♀ *femur-rubrum* normal blood + 1 c.c. bouillon + 1 loop of *B. poncei.*
(3) 2 drops ♂ *femur-rubrum* normal blood + 1 c.c. bouillon + 1 loop of *B. poncei.*

The six test tubes were incubated for 24 hours at 35° C. At the end of that period neutral potato agar plates were poured from the six cultures using the customary one, two, and three dilution method. Each experiment and each check was represented by three plates making eighteen plates altogether. They were incubated for three days at 35° C. after which a count was made of the developed colonies. The colonies on the six first dilution plates (experiments and checks) were too numerous and confluent for counting. Those on the six second dilution plates were also extremely numerous. At a glance one could tell that the colonies on the check plates were more numerous than on the experimental plates. By taking counts of sections of all the plates I arrived at the conclusion that the ratio of check to experimental colonies was about 10: 1.

With the six third dilution plates I was able to make actual colony counts for the three experiments and three checks.

NUMBER OF COLONIES ON THIRD DILUTION PLATES.

	Checks.	*Experiments.*
(1)	7	0
(2)	4	0
(3)	6	0

As can be seen from the foregoing table Checks 1, 2, and 3 gave 7, 4, and 6 colonies respectively, whereas nothing at all was obtained on the three third dilution experimental plates.

These experiments taken in conjunction with the other results I have presented show that it is possible to bring about the formation of bacteriacidal properties in some insects. From my tissue culture work discussed under phagocytosis, I have shown that normal insect blood is somewhat antagonistic towards bacteria,

[1] Two drops equals 1/6 of a cubic centimeter. This is the maximum amount of blood which one can obtain from one animal at a time.

but this antagonism is much more evident in animals that do not die after a bacterial infection, i.e., animals which are immune.

If it were only possible to inject an insect more than once without producing fatal results, I am sure one could obtain still more interesting results. I have often made two trials but grasshoppers and caterpillars, at least, do not seem able to overcome the effects of a second injection.

SUMMARY.

1. Entomological text-books emphasize the importance of phagocytosis in ridding the insect body of foreign matter, but in reality insect blood cells are visibly rather passive.

2. Grasshopper and caterpillar blood cells do not seem to phagocytise bacteria in an amæboid fashion.

3. When bacteria are found within the blood cells, they may have gained entrance through their own aggression or physical factors may have been involved.

4. The blood of normal insects, however, is somewhat antagonistic towards bacteria.

5. This antagonism acts extracellularly.

6. Actively immunized grasshopper blood shows a high degree of antagonism towards the bacteria used in producing this immunity.

7. An agglutinin was found in immune grasshopper blood.

8. Some quantitative data on the bacteriacidal action of immune grasshopper blood were obtained.

LIPEURUS DOVEI *NOM. NOV.*

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It was recently brought to my attention by Dr. A. Hassall through Dr. L. O. Howard that in naming *Lipeurus lineatus*[1] I have used a preoccupied name.[2] Therefore, as a substitute for *L. lineatus* I propose, as above recorded, the name *L. dovei*, in honor of Mr. W. E. Dove of the Bureau of Entomology who has been instrumental in collecting several new and interesting species of Mallophaga.

[1] PSYCHE, Vol. 24, No. 4, p. 114, 1917.
[2] Zeitsch. f. Ges. Naturw., Vol. 28, p. 384, 1866.