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Transmissible Disease, Probably Viral in Origin, Affecting the Amebocytes of the European Shore Crab, *Carcinus maenas*

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A transmissible infection of the shore crab ("crabe enragée"), *Carcinus maenas*, was discovered in one of about 700 of this species collected by the Station Biologique, Roscoff, France. The infection has been transferred by subinoculation through four serial passages in crabs collected at Roscoff and through six more passages in *C. maenas* collected at Woods Hole, Mass. The agent causes abnormal cellular clotting, a drop in the peripheral amebocyte count, clumping of amebocytes in the peripheral tissue and blood, and abnormal behavior of the amebocytes of the infected animal on glass. Mortality over a period of some weeks is negligible. Electron microscopy shows characteristic virus particles, about 55 to 125 nm in size, in the infected amebocytes. The agent is present in large amounts in the whole blood and serum, is filterable, heat labile, and has been preserved in the frozen state. Final identification will depend upon purification, growth in tissue culture, and further electron microscopy.

Despite the huge array of marine invertebrates, virus disease is essentially unknown among them. In fact, there is very limited knowledge of disease processes in most invertebrate phyla (F. B. Bang, Fed. Proc. **26**: 1664, 1967). This report concerns a filterable and infectious process which causes abnormal clotting of amebocytes of the shore crab, *Carcinus maenas*. It was encountered during a summer's study at the Station Biologique de Roscoff, Brittany (France). Its relationship to an agent which induced a slowly developing paralysis in the Mediterranean swimming crab, *Macropipus depurator*, described in a note by Vago (9), is unknown.

In searching for a ciliate previously found as a parasite in the blood of the shore crab at Roscoff (F. B. Bang, Fed. Proc. **26**:1680, 1967; reference 7), blood samples of hundreds of locally harvested crabs were individually examined microscopically. Using a single syringe and needle repeatedly, a blood sample from each crab was put on an uncovered glass slide and scanned for the ciliate. Each blood sample quickly formed a tight clot on the glass. However, the 679th crab failed to clot. Repeated tests of the blood from this animal showed that the amebocytes remained separate, settled individually on the slide, and slowly spread on the glass surface. Blood from this crab was transferred to three other crabs, two of which then developed the same clotting defect. The effect was transferred through four passages at Roscoff where some of its properties were tested experimentally. The agent was frozen and has since been carried through six additional passages at the Marine Biological Laboratory at Woods Hole. Some of the effects of the agent on host crab amebocytes and some characteristics of the agent will be described.

MATERIALS AND METHODS

A series of recently collected shore crabs from the Supply Department at the Station Biologique were examined for the presence of abnormal clotting, and then groups of three to five were placed in separate round glass aquaria about 35.6 cm (14 inches) in diameter and 15.3 cm (6 inches) in depth in small amounts of seawater. Each animal was identified and inoculated by injection of 0.05 ml of test material into one of the leg joints. Each was subsequently bled daily or every two days to determine whether abnormal clotting had developed. The failure of the blood to clot on glass at room temperature (20 C) was considered evidence of infection. A number of other changes which accompanied this failure to form a clot will be described in the text. Each grouping of crabs was kept continuously in the same glass jar, and members of that group were bled with a freshly sterilized syringe

and needle which had been treated with amebocyte extract (*see below*). Since the experiments were of brief duration, the animals were not fed. The jars were washed in running seawater each day. There was no evidence of cross contamination from jar to jar. A series of uninoculated or seawater-inoculated crabs were maintained as controls for each experiment; these remained negative (continued to form good clots) throughout the study period.

Amebocytes were counted by placing 0.05 to 0.1 ml of a 0.01 M solution of *N*-ethyl-malemide (NEM) in a clean syringe, then withdrawing 0.1 to 0.15 ml (making a 1:2 or 1:4 dilution) of blood into the syringe, mixing by inverting the syringe several times in the presence of a bubble, and then discarding the first part of the mixture through the needle, placing a drop of the mixture in a Malagasey hematocyte chamber and counting the cells. Four outer squares were counted and figures were corrected for dilution and expressed as cells per centimeter, square.

In tests for clotting, 0.05 ml of blood was withdrawn from the base of a leg joint, retained in the syringe for about 30 sec, and then expelled on a glass slide. On normal animals the same needle and syringe were used in sequence, flushing seawater through the apparatus between individual bleedings. On first using a newly sterilized needle and syringe, however, a drop or two of normal amebocyte extract was drawn into the apparatus, retained for a minute, and then expelled before bleeding the first crab. This was necessary because normal crab blood does not form the characteristic clot unless the apparatus has first been in contact with a factor which has been released from crab amebocytes, i.e., amebocyte extract. This factor is evidently heat labile and is destroyed when needle and syringe are boiled.

Boiled apparatus and freshly prepared extract were always used when it was necessary to avoid transmitting the agent from one crab to another. In each of the experiments, uninfected controls and infected crabs were bled at the same time, and, except for one crab which was kept in close contact with infected animals, none of the controls developed clotting failure.

When clots formed, an irregular loose aggregation of amebocytes formed in the drop before the cells touched the slide and gradually contracted into a central mass or clot.

For purposes of this discussion, a crab was considered infected if its blood developed a failure to clot. In the titrations and studies for the presence of the agent in tissues, etc., a result was not considered positive for infection (failure of the blood to clot) unless the test was repeated or unless several animals in the test developed the defect. Negative (failure of infection) tests were more difficult to evaluate since the incubation period was variable and many of the tests could not be continued long enough to allow for a long incubation period.

RESULTS

Of the three crabs originally inoculated with blood from the spontaneously infected crab, two developed infection, one at 2 days and one at 13 days. The third animal was discarded 20 days after inoculation after a series of negative tests.

TABLE 1. *Titration of whole blood and serum from an infected crab*[a]

Dilution	Whole blood[b]		Supernatant	
	Fraction positive	Incubation of positive animals (days)	Fraction positive	Incubation of positive animals (days)
Undiluted	3/5	3.8	5/5	5.4
10^{-1}	2/5	5.0	5/5	5.8
10^{-2}	4/5	10.0	2/5	7.0
10^{-3}	1/5	10.0	4/4	8.0
10^{-4}	1/5	19.0	2/5	10.0

[a] Infected crabs studied for 19 days; end point determined by failure of blood from injected crabs to clot.

[b] Each group of animals kept together. Freshly sterilized syringes were used to test each group. All crabs were tested every other day for the first ten days and thereafter at 2- and 3-day intervals. Blood for infection was taken from experimentally infected crab on the second day of infection.

A series of ten different experiments was done at Roscoff with fresh blood or serum taken from the original infected crab, representing three successful passages and ten successful transfers of the agent. The agent has been through six additional passages at Woods Hole.

Disease. Incubation period varied from 2 to 19 days even when all the crabs were inoculated with about 0.05 ml of undiluted blood taken from the original, spontaneously infected crab. The earliest indication of clotting failure in this first group occurred 2 days after inoculation but often was not fully manifest until 4 to 7 days. One abnormal case in which clot failure appeared at 21 days may have been a cross-infection within a group of crabs kept together in one container. In a number of subsequent experiments, however, the incubation varied from 1 to 19 days. As may be seen in Table 1, in which 0.2 ml of different dilutions of whole blood or freshly separated serum in boiled seawater was injected into five crabs each, the incubation period was directly related to the dilution of the inoculum and varied from 5 to 10 days, except for one animal which was positive at 19 days. Table 1 also shows that the infection was characteristically irregular Of 73 crabs inoculated with known positive material, 74% developed clot failure within the 10-day period of observation. None of 73 controls failed to clot during the 10-day period of study. None of 250 uninoculated crabs examined after the original finding showed spontaneous clot

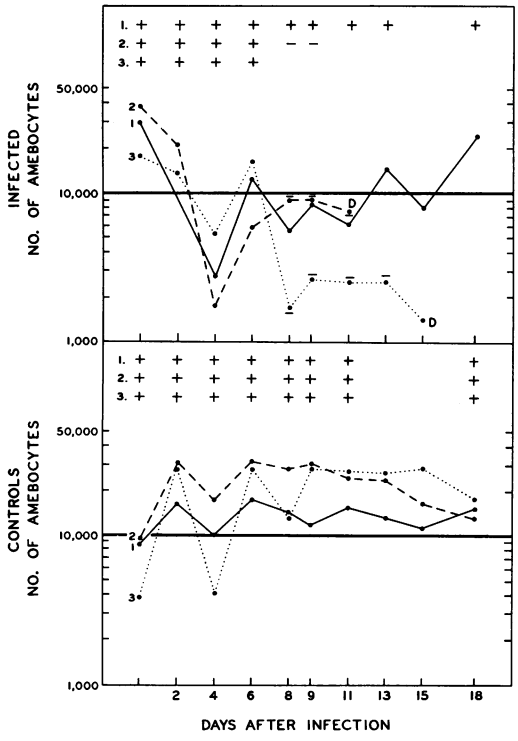

FIG. 1. *Comparison of amebocyte counts in normal and infected crabs over an 18-day period. Counts are presented on a logarithmic scale, and the heavy line in each case represents a rough estimate of the lower limit of the normal count. The plus and minus signs represent a test for clotting. As may be seen for the upper group, the amebocyte count in all three infected individuals dropped well below normal before the failure to clot developed.*

failure. In this experiment the end point of the titration was not reached.

Mortality and possible recovery. Although Fig. 1 shows that two of three infected crabs died a few days after abnormal blood clotting was detected, it is not definite that this mortality was due to the agent since the unfed animals were kept closely confined and were frequently bled. A number of crabs apparently recovered from the failure of clot formation, at least temporarily. No difference in susceptibility seemed related to the size or sex of the individuals.

Effect of the disease on the amebocytes. In addition to the direct observation that cellular clots did not form in the acutely infected animal, the agent apparently had a direct effect on the amebocytes themselves. There was a decrease in the number of circulating amebocytes as early as 2 days before the abnormality in clotting was manifest (Fig. 1). In one case the blood count had returned toward normal before the abnormality in clotting was detected even though abnormal clumps of cells could be seen in the peripheral blood (*see below*). However, in most of the individual counts done throughout the study lowering of the amebocyte count and abnormal clotting occurred simultaneously. The day to day analysis suggests that the low count in itself is not the cause of abnormal clotting.

The tissues of the infected animals were directly examined by breaking off an epipodite at its base, placing it on a slip-covered slide, and looking at it under a compound microscope. Epipodites are additional lobes on the protopodites (jaws or modified forelimbs) of some crustacea. In this way the free cells could be seen in the tissue through the transparent integument. There was a sharp difference between amebocytes of normal and infected animals. In normal crabs they were discoid, granular, unattached to the surrounding tissue, and moved freely within the channels whenever pressure was placed on the overlying cover slip. Infected animals showed amebocyte clumps which were usually bound together so that individual cells could not be distinguished, and the whole clump was often so adherent to adjoining tissue that it moved back and forth only slightly with cover slip pressure. Table 2 summarizes the number of animals examined for this change. Small packets of these adherent cells were also found frequently in the blood of infected animals taken for clot examination.

The fourth abnormality was the reaction of the cells after they were expelled from the syringe and placed on glass. Examined with phase microscopy at 300×, cells from normal animals had long filaments, each with a central rib, which often connected one cell with another. Cells from infected animals had fewer filaments but had short, paddle-shaped extrustions which had no central ribs and were often bent and deformed. They seemed to have fewer granules and often appeared glassy. This cellular abnormality is presumably the basis for the failure of clot formation. Invertebrate fibrinogen was apparently available within the abnormal cells, for a phase-negative precipitate formed around the abnormal cells as rapidly as around normal amebocytes.

Presence of agent in blood. Blood was tested for presence of the agent on days 2, 3, and 4 after inoculation by subinoculating three crabs from each animal each day with 0.05 ml of freshly drawn whole blood. Table 3 shows that the agent was present in the blood as early as 2 days after infection and that it persisted in all of the four originally inoculated animals at all times, except for crab no. 3 which was negative on the third day of infection. It was also continuously present in

TABLE 2. *Appearance of abnormal amebocyte clumps in epipodites*

Crab	Day of infection									
	1	2	3	4	5	6	7	14	15	18
Infected	0/2	2/3	2/2		1/1		1/1	1/1	1/1	1/1
Control	0/2	0/3	0/2		0/1		0/1		0/1	

TABLE 3. *Tests for presence of agent in whole blood of crabs days 2, 3, and 4 after inoculation and course of infection in the same crabs on day 5 to 8*

Crab[a]	Organism	Day				Determination	Day			
		0	2	3	4		5	6	7	8
1	Culture[b]	0	50	0	0	Amebocyte clot[c]	−	−		−
	Virus		+	+	+	Amebocyte count (no./mm³)			1,925	
2	Culture	0	50	0	50	Amebocyte clot	−	−		−
	Virus		+	+	+	Amebocyte count			2,950	
3	Culture	50	3	0	0	Amebocyte clot	+	−		+
	Virus		+	−	+	Amebocyte count			10,650	
4	Culture	50	0	0	0	Amebocyte clot	+	+		+
	Virus		+	+	+	Amebocyte count			17,550	
5	Culture	0	0	0	0	Amebocyte clot	+	+		+
	Virus		−	−	−	Amebocyte count			33,200	
6	Culture	0	S[d]	0	0	Amebocyte clot	+	+		+
	Virus		−	−	−	Amebocyte count			18,400	
7	Culture	20	0	0	0	Amebocyte clot	+	+		+
	Virus		−	−	−	Amebocyte count			20,050	
8	Culture	0	S	0	0	Amebocyte clot	+	+		+
	Virus		−	−	−	Amebocyte count			14,250	

[a] Crabs 1 to 4 were inoculated. Crabs 5 to 8 were uninoculated controls.

[b] Culture: numbers represent number of colonies per 0.02 cc of Zobell's seawater-agar. Virus: virus present (+) or absent (−) in the three subinoculations from each crab.

[c] Amebocyte clotting and failure to clot are indicated by plus or minus signs.

[d] S: bacterial spreader.

one inoculated animal (no. 4) which showed neither a drop in the amebocyte count nor abnormal clotting. All uninoculated crabs were tested for the agent in the same way and were negative throughout. There was no correlation between the occasional presence of bacteria and the infectiveness of the blood. Titrations of the amount of virus in the whole blood or serum at different times have not yet been done. However, in one titration done at Woods Hole (blood taken on day 4 after injection), the end point was reached at $10^{-9.2}$ with the incubation period varying from 4 to 18 days.

Nature of the agent. The marked effect of the agent on amebocytes, the complete absence of visible bacteria, either within infected cells or free in the plasma, the presence of the agent in concentrations as high at 10^9 all suggest that the agent is a filterable virus.

Three experiments were done to test its capacity to pass a filter. To test the efficacy of the filter, blood from acutely infected crabs was diluted 1:5 in seawater containing bacteria, a Swinney filter was attached to a syringe containing the material, and the filtrate was pushed through under moderate manual pressure. In two tests a filter which withheld particles above 450 nm was used and in one test a filter which withheld particles

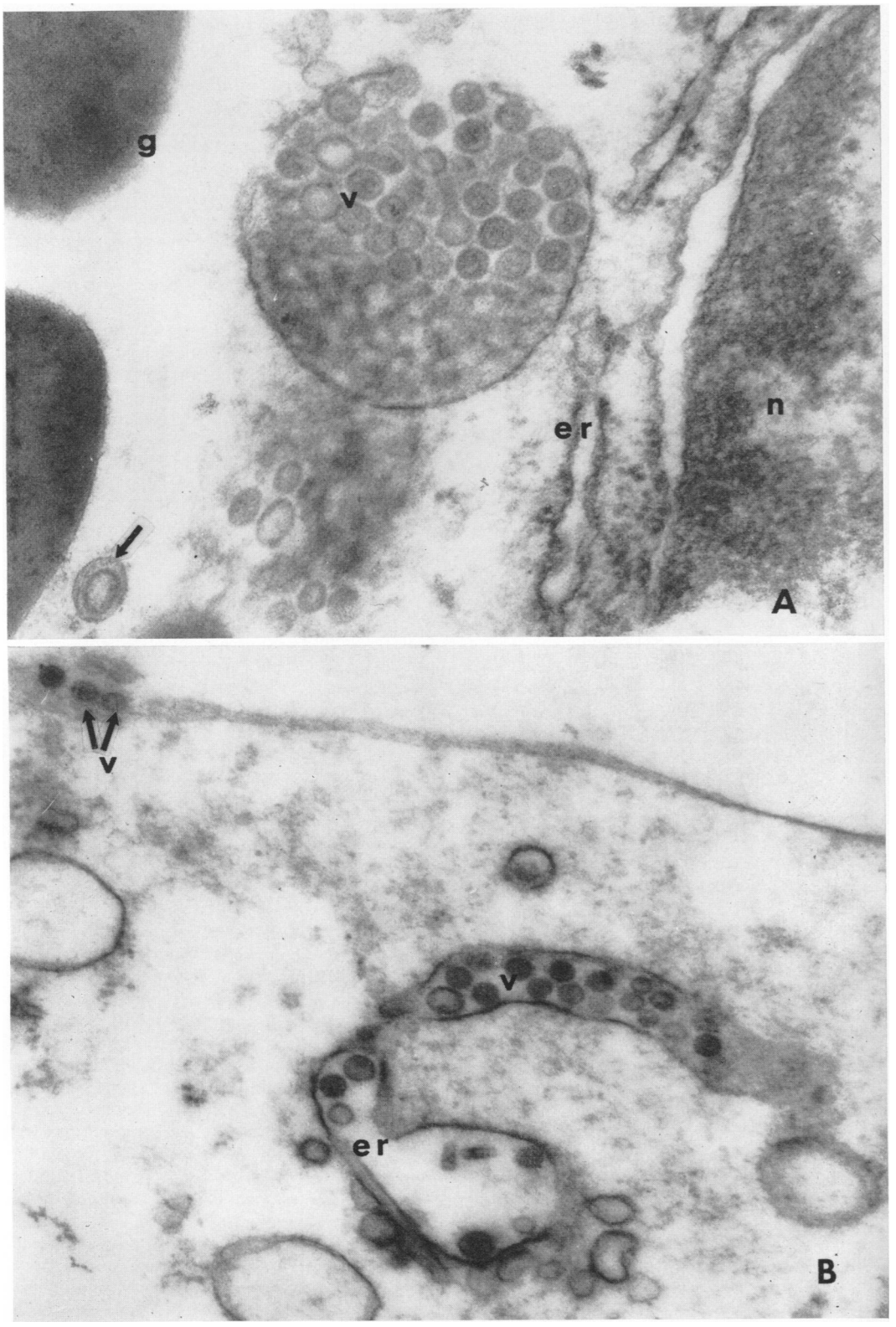

FIG. 2. *Electron micrograph of portions of amebocytes of a crab inoculated 5 days previously and showing failure of clotting two days previously. Virus particles (V), cell granules (g), nucleus (n), and endoplasmic reticulum (er) are apparent. (A) A packet of particles, some of which show an apparent club-shaped extension. One particle (arrow) shows a characteristic double wall, seen also in separate particles in the cytoplasm. × 96,000. (B) A group of single membrane particles within a packet of endoplasmic reticulum. The relationship of the individual rod-shaped particles to the round ones is unknown. × 75,800.*

over 220 nm. In all three the filtrate was free of bacteria as tested on seawater-agar, and in all three crabs were infected by the filtrate. Crabs inoculated with the 450-nm filtrate developed disease in four of five and three of five individuals, whereas those inoculated with the 220-nm filtrate developed it in five of five inoculated animals. The incubation period with the filtrate did not differ significantly from that following inoculation of unfiltered material.

Heat stability. Two samples of serum from an acutely infected crab which had been inoculated with the 450-nm filtrate were heated to 70 and 100 C for five min. This serum produced no infection in six crabs each. The same preparation after slow freezing and maintenance at −25 C produced infection in three of six crabs in 2 to 4 days. In addition, material kept at 4 C in the laboratory refrigerator remained infectious for 4 to 7 days. One ampoule of material kept at −70 C for 10 months was reinoculated into four *C. maenas* at Woods Hole and infected all four.

Electron microscopy. The agent has many properties of a filterable virus, but final identification must await more complete characterization. Electron microscopy of amebocytes of a crab which had been inoculated 5 days previously and shown infection for 2 days shows characteristic virus particles (Fig. 2) which have not been found in normal cells. About one half of the cells in this preparation showed particles of this nature. Sections of blood from two normal animals and three crabs at later stages of infection did not show such particles. These measure 55 to 75 nm in internal diameter and 95 to 125 nm outside.

DISCUSSION

Among invertebrates in general only virus diseases of insects have been extensively studied (8), and a great deal is known about the infecting agents. But small size of the hosts and their relative susceptibility to trauma inhibit their usefulness as animals in which infection and recovery can be studied in individual animals. Since shore crabs can be easily maintained in the laboratory and repeatedly bled over a period of months, it is possible to follow the course of viral infection and host response in some detail. Further, transmissible infections of marine invertebrates take place in animals which have an osmolarity close to that of the surrounding seawater, so that in nature other mechanisms for reaction to viral infections may be involved.

A number of bacterial and protozoan diseases have been reported in marine arthropods, particularly in crustacea. Many of these involve major abnormalities within the clotting system. In a bacterial disease of *Limulus*, degranulated amebocytes stick to the walls of the open vascular system and there is a concomitant intravascular gelation (5). In a protozoan disease of *Carcinus*, first described by Poisson (7), the invading ciliate eats the host's amebocytes. However, no other diseases in which the amebocytes themselves seem to be infected have been reported. Since crab amebocytes are phagocytes in addition to their role in clotting, it will be of interest to study phagocytic function in infected animals and to determine whether infection with the Roscoff agent increases the susceptibility of the host to bacterial infection.

Farley (4) has described a pathological process in oysters and clams which resembles leukemia in that excessive numbers of amebocytes appear in the blood and throughout the tissues, a phenomenon which may be a disease of the amebocytes of molluscs; transmissibility of the process was not reported.

In the present infection the mechanism by which clotting of the peripheral blood is inhibited is not known nor is its importance to the host. Since crustacean amebocytes are concerned with phagocytosis and clot formation, it might be expected that frequent bleeding of infected crabs would rapidly exsanguinate the animals through the puncture wounds. Since this does not happen, wound closure in normal and infected animals should be comparatively studied. The formation of a gel around individual amebocytes from infected animals suggests that gelation could contribute to the closure of small wounds. In the Hermit crab *Pagurus*, study of the formation of cellular clots at the tips of cut antennae has shown that the process of clot retraction is apparently associated with the formation of large arrays of microtubules in the multiple processes which project from the clotted amebocytes (1). Possibly the Roscoff agent either inhibits or prevents microtubule formation in *Carcinus*.

Although disease processes analogous to the one here reported are not known among the invertebrates, there is a strikingly similar process in man. If one considers the closely similar function of mammalian platelets and crustacean amebocytes in the formation of cellular clots (3, 6), then human thrombocytic purpura comes immediately to mind: the number of circulating platelets in the peripheral blood is reduced much as the number of amebocytes is reduced in the crab disease; peripheral thrombi with platelets form in the capillaries of man in a way comparable to the clumps of abnormal amebocytes found in peripheral tissues of the crab; and the abnormal behavior of human platelets is not necessarily associated with deficiency of fibrinogen, just as in the crab there is apparent continued normal fibrin

function despite the abnormal amebocyte behavior.

However, the pathogenesis of platelet thrombi in man is still poorly understood, and therefore the comparison cannot be pushed too far.

ACKNOWLEDGMENTS

I am indebted to Naomi Taylor for preparation of the amebocytes for sectioning and electron microscopy.

ADDENDUM IN PROOF

Since the preparation of this paper for publication, an article entitled "An Electron Microscope Study of the Distribution of Tomato Spotted Wilt Virus in Systemically Infected *Datura stramonium* Leaves" (R.I.B. Francki and C. J. Grivell, Virology **42:** 969–978, 1970) has been published which shows virus particles in the plant tissue which appear very similar to those present in the European shore crab, *Carcinus maenas*. It is of some interest that the virus of tomato spotted wilt is also transmitted by Thrips and thus grows in arthropods.

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