Pathogenesis and Autointerference in a Virus Disease of Crabs

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The course of an infection apparently caused by a filterable virus of the blood of the shore crab, Carcinus maenas, has been experimentally studied in this host both at Roscoff, France, where it was originally found, and at Woods Hole, Mass., where the local species was also found susceptible. Although a portion of the infected animals die with the symptoms of inadequate blood clotting, recovery of this function occurred promptly in about two-thirds of the animals. Half of the animals that recovered this function did so within 4 to 6 days. Recovery was not accompanied by disappearance of the virus from the whole blood. In tests done as late as 40 days after recovery, virus was still present. Autointerference was demonstrated after acute infection. It was found in the serum of animals with manifest disease on all days tested and in whole blood of animals taken more than 2 days after the clotting defect appeared. It was not demonstrable within the whole blood within the first 2 days of disease. The role of this in the recovery phenomenon is discussed.

The recent discovery of several transmissible virus diseases, specifically of crabs (1, 4, 14), as well as of mollusks (5, 8, 9, 13), means that there is now an opportunity to study the course of virus infection in an individual invertebrate over a period of time. Thus one may now ask for invertebrates as well as vertebrates: Does recovery occur, and, if so, what is the mechanism? What is the relationship of persistence of the virus to pathological changes in the host? What factors influence recovery from disease? These questions have been difficult to study in insect virus disease because few individual animals may be repeatedly bled without killing them. In contrast, most of the relatively large crustacea may be repeatedly bled for indefinite periods of time.

In a previous paper (1) in which the disease was first described, I noted (i) the absence of visible or cultivable bacteria in the blood despite the high titer, (ii) the ease with which the agent could be filtered, and (iii) the presence of apparent virus particles as seen by electron microscopy of sections of amoebocytes of infected animals and their absence in normal animals. On this basis we have assumed the filterable nature of the disease. However, more work identifying the agent with the disease is needed.

This paper is concerned with a virus disease of the shore crab, Carcinus maenas, which is manifested by the failure of the amoebocytes to form an adequate cellular clot. Evidence is presented that: (i) there is recovery from the disease (a renewal of the ability of the blood to clot), despite the continued presence of the agent; (ii) autointerference may be detected in the blood; (iii) such interference may play a role in recovery; and (iv) recovered animals are susceptible again to relatively large amounts of virus.

MATERIALS AND METHODS

The work described here was done during July and August 1969 and 1971 at the Station Biologique, Roscoff, France, and July and August 1970 at the Marine Biological Laboratory, Woods Hole, Mass. C. maenas were caught by the supply departments of the respective biological stations and kept and fed in holding tanks with running sea water for a few days before use in the laboratory. None of the animals used in this study were fed after being brought to the laboratory. It was difficult to keep them alive in small isolated containers with feeding because of overgrowth of bacteria in the absence of running water. In Roscoff, groups of three to five animals varying somewhat in size (estimated from 1.5 to a little over 3 inches [about 3.81 to 7.62 cm] across the carapace) and of both sexes were kept in glass dishes, often stacked one on top of the other. (A layer of salt water was kept in the bottom of each jar to maintain moisture). The temperature of the laboratory varied from 18 to 21 C. At Woods Hole, special precautions were taken to avoid any possibility of introducing the virus, which had been isolated in France and which was of unknown contagiousness and pathogenicity,
to other marine invertebrates. For this reason, each *Carcinus* was kept in a separate screw-cap jar, the sea water was changed once a day, and all discarded sea water was sterilized either by boiling or being placed within a large steam sterilizer. The crabs themselves were all killed within the sterilizer at the end of the experiment. All syringes and needles were boiled after use. Infection was assumed to be present in the crab if, after inoculation with suspected material, they developed the characteristic failure of blood clotting previously described.

The clotting test is concerned with the capacity of freshly drawn blood to form a cellular clot after extrusion from the needle onto a glass slide. Rapid cellular clot formation is dependent upon the presence in the syringe and needle of normal amoebocyte extract (2), which may be added directly from a stock of extract (usually 10 times the concentration of the blood). Alternatively, the syringe and needle may be "treated" by simply using it for repeated bleedings of a normal animal with intermittent rinsing in clean fresh sea water. To improve the diagnostic significance of a positive test, failure of the clotting was not recorded as such unless there was no formation of contracting strands of amoebocytes within the first 15 to 30 s as observed in a microscope at a magnification of 20 to 70 times.

The reproducibility of the test was unaffected by variations in size of the needle between no. 18 and 22 in size. All animals were checked for normal clot formation before use, and thus an occasional confusing overwhelming bacterial infection that also prevents clotting was excluded. Failure of clot formation because of the lack of amoebocytes as a result of inadequate penetration of the needle into the open vascular system was easily detected by microscope observation because scattered bacteria and debris are not normally present in blood. Only those tests with a microscopically clean preparation of blood with amoebocytes present were considered. A failure to clot was then considered as positive evidence of virus. All crucial experiments to determine the presence of virus were further checked both by looking for characteristic small adherent clumps of glassy cells in epipodites of the animal under study and by testing for transmissibility of the clot failure effect to other animals. Disagreements between the three types of results (clot failure, epipodite examination, and transmissibility) were rare.

Animals. It is not known whether *C. maenas*, the shore crab or "crabe enragé" of the coasts of France and England, is really the same species as that present on the east coast of the United States. The species studied at Roscoff had a characteristic angry behavior when stimulated, and this seemed less apparent in the American specimens. However, no difference in susceptibility to the virus was noted.

RESULTS

Resistance and recovery. After the first transfers of the virus in 1969 and subsequently, it was noticed that animals that were inoculated with known infectious material did not all develop disease. With the first passage only 8 of 14 developed signs of disease. This irregularity has continued to be both a fundamental problem as to the cause of the resistance and a practical problem of testing for the presence of the virus.

Recovery after loss of clotting ability was frequently noted in the first year and subsequently was observed at the same rate in crabs at Woods Hole (Fig. 1). About 50% of the recoveries occurred within 4 to 6 days. A few crabs recovered within a day of the first loss of clotting and thus might have been considered as not responding to the virus if they had not been studied on the appropriate day. This may explain some of the original "resistant" animals. Recovery was accompanied by a return of the amoebocyte count toward the normal level, but the capacity of cells to form a cellular clot was not directly dependent on the number present.

![Fig. 1. Composite figures from several different experiments done during the course of the summer. The actual number of crabs inoculated and coming down with failure of blood clotting is presented on the left. The top curve in each case represents the number of these that survived. The lower curve represents crabs that survived and recovered the capacity to form blood clots. Thus, between day 5 and 10, half of the susceptible animals had recovered. The curves do not include animals that did not develop signs of infection even though inoculated with infectious material.](http://iai.asm.org/)
Loss of capacity to form a clot was often accompanied by a general weakness of the animals and a very characteristic stiffness of all of the appendages. This stiffness occurred about 10% of the time in infected animals only and in a few animals was gradually lost. It seemed to be more of a spastic inhibition of movement rather than paralysis, because when the crab was stimulated by heat just before death by boiling very active movement of all limbs was readily apparent.

**Mortality.** The number of crabs that died after the development of clot failure is shown in Fig. 1. In experiments in Woods Hole, 6 of 33 died in 38 days. In Roscoff, 15 of 47 died in 27 days. Thus there was a mortality of 18 to 32% within about a month, about twice the mortality of uninoculated animals. However, the conclusions that can be drawn from these data are limited because of the short duration of these experiments and the fact that the animals were repeatedly bled, could not be adequately fed, and were kept under highly artificial conditions.

**Persistence of virus.** Failure of the blood to clot after injection with virus was a reliable indicator of virus effect. However, animals inoculated with virus had virus in their blood several days later without showing signs of disease, and some that recovered also carried virus. Table 1 shows that subinoculations of whole blood from three animals that had all recovered by day 15 nevertheless produced characteristic disease in the inoculated animals. Thus the virus remains present in the blood (and probably in other tissues) for some weeks after the original infection. Recovery is thus not associated with complete loss of virus.

**Titrations.** Because the virus produced somewhat transient symptoms in the crab and because the response was not uniform in all infected crabs, it was difficult to determine accurately the amount of virus present in a sample by a dilution end-point method. However, an attempt was made to determine whether there was any relationship between the amount of virus inoculated and the prepatent period (time between inoculation and demonstration of clot failure). Two different titrations on whole blood were done, and the results are presented diagrammatically in Fig. 2. It is clear that there was a direct relationship between virus dosage and incubation period.

**Autointerference.** Immunoglobulins have as yet not been described in invertebrates (3). Therefore, any clue as to the mechanism of recovery from a virus disease in invertebrates would be of interest.

In one of the later titrations of whole blood done in 1970, lower dilutions of virus failed to produce the expected signs of infection, although dilutions of 1:100 and 1:1,000 did. This effect is similar to the phenomenon of autointerference in vertebrate virology. To study this question systematically at Roscoff in 1971, it was decided to inoculate a series of samples of blood by using both undiluted materials and 1:100 dilutions and to subsequently determine the incubation period and percentage of animals infected. Those samples in which the more diluted material caused disease later than the undiluted were considered as normal (N). Those in which the more diluted gave earlier disease were considered as showing autointerference (A), and those in which there were no clear differences were considered as intermediate (I). There was no evidence of interference in seven tests of fresh whole blood taken on either day 1

<table>
<thead>
<tr>
<th>Crab</th>
<th>Proportion of inoculated crabs that developed the disease</th>
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<tr>
<td></td>
<td>6*</td>
</tr>
<tr>
<td>11</td>
<td>2/3</td>
</tr>
<tr>
<td>13</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>7/8</td>
</tr>
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</table>

*Day of infection.

**Fig. 2. Relationship of average prepatent period to dilution of virus inoculated. Note: These two titrations were done with freshly removed whole blood taken from crabs during their first days of patency.**
or 2 of patent disease, but all of six samples of whole blood taken later in the course of disease either failed to show the expected dilution effect or actually showed a reversal (Table 2). These results were independent of the treatment of the blood, including freezing and thawing. Furthermore, none of the samples of fresh sera removed by centrifuging the blood immediately after it was drawn showed the expected normal effect of dilution, and five of the six showed auto-interference. Autointerference then seems more manifest in serum than in whole blood but is more apparent in the late specimens of whole blood. The extent to which it may be responsible for the recovery phenomenon will be discussed later.

Resistance to infection by recovered animals. This question was tested twice. In the first experiment, three groups of animals were inoculated: (i) a small group of recovered animals, (ii) a group of uninfected animals that had been previously continuously bled as controls, and (iii) a group of freshly caught animals. All were tested by inoculating them with a 1:10 dilution of serum from an infected animal (Table 3). In another test in which whole blood was used as the challenge, disease was produced in four of six recovered animals and two of five multiply bled controls. Thus, no evidence of immunity to reinfection produced by large amounts of virus was forthcoming.

**DISCUSSION**

The mechanism by which an invertebrate recovers from a virus disease is unknown. Largely because of the lack of suitable experimental procedures in insects the question has not previously been susceptible to study. Although there are now at least eight presumed virus diseases of marine invertebrates (three in crabs and five in mollusks), only the present one has been studied in terms of the sequence of changes that occur with time. In this infection there is first a definite loss of cell cloting as tested under specific in vitro conditions and then a regaining of the normal ability. This recovery occurred at much the same rate both in animals infected in Woods Hole or Roscoff, even though the animals may represent different genetic stocks and were handled under somewhat different conditions. This phenomenon of recovery, however, has been studied primarily in the blood, and, with the exception of the effect as demonstrated in the epipodites, the effect of the virus on other tissues is unknown.

In two small experiments not detailed here, lowering the temperature of the environment below the usual 18 C at which the animals were maintained was accompanied by a later appearance of clotting defect. Thus the effect of temperature on the course of infection, with particular reference to interference and the regularity of takes, needs intensive study.

Whether the interference demonstrated in this study is due to the virus itself, to an interferon-like substance, or even to interference from another associated virus is undetermined. The original agent was subjected to end-point passage in an attempt to free it of other agents, but of necessity the agent has been continually passed in animals of unknown past history.

Does the interference phenomenon play a role in recovery? This thought is attractive because

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**Table 2. Autointerference as demonstrated by the effect of different dilutions**

<table>
<thead>
<tr>
<th>Days of</th>
<th>Dilution response to*</th>
<th>Serum</th>
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<tbody>
<tr>
<td></td>
<td>Whole blood injected immediately</td>
<td>Whole blood after 0.5 standing</td>
</tr>
<tr>
<td>1</td>
<td>N</td>
<td>N</td>
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<tr>
<td>1</td>
<td>N</td>
<td>N</td>
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<tr>
<td>1</td>
<td>N</td>
<td>N</td>
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<tr>
<td>2</td>
<td>N</td>
<td>A, A</td>
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<tr>
<td>3b</td>
<td>I</td>
<td></td>
</tr>
<tr>
<td>4b</td>
<td>I</td>
<td></td>
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<tr>
<td>5</td>
<td>I</td>
<td>I</td>
</tr>
<tr>
<td>8</td>
<td>I</td>
<td>I</td>
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</tbody>
</table>

* Symbols: N, normal dilution curve response, i.e., more dilute material yields fewer and/or more delayed onset in recipients. A, reverse dilution response curve, i.e., undiluted yielded lower percentage of takes and/or greater incubation period than the 1:100 dilution. This is considered as evidence of autointerference. 1, intermediate.

b These samples consisted of a mixture of blood from several infected crabs.
interference, as we have defined it here, appeared in sera of all infected animals and was demonstrated repeatedly in whole bloods taken more than 2 days after the clotting defect had appeared, but not in those taken at the height of infection. One might then argue that the substance appears first in the serum, gains in titer, and is able to protect uninfected cells against a further spread of virus, but does not eliminate the virus from the cells that are already infected. It would be most important in the future to determine whether there is a correlation between the presence of interference and the subsequent course of infection in that infected crab. The present 'experiments' were not planned in this way because almost all of the animals tested for interference were sacrificed at the time of the study.

Study of this virus disease of the common shore crab is of interest in comparative virology and medicine in general because of the following:

(i) Invertebrates apparently lack classical antibody, and therefore mechanisms of recovery can be studied without being confused by this effect. A variety of substances acting in vitro somewhat like antibody and tentatively called "antisomes" has been known to occur in crustacea since Cantacuzène's studies more than 50 years ago (6). Their role in virus infection has not been explored.

(ii) The similarity of the clotting function of amoebocytes in invertebrates to platelets in vertebrates has been emphasized by hematologists for many years (7, 10, 11). Thus, as the mechanisms of this infection become understood, useful questions concerning virus infection and platelets in human hematology may be raised.

(iii) The large range of temperatures to which Carcinus may be easily subjected without apparent serious effect (8 to 25 C in our experience) means that the effect of different temperatures on the progress of a virus infection within the host is now open to study.

LITERATURE CITED