Progression, lethality and remission of hemic neoplasia in the bay mussel *Mytilus edulis*

R. A. Elston, M. L. Kent, A. S. Drum

Center for Marine Disease Control, Battelle/Marine Research Laboratory, 439 West Sequim Bay Road, Sequim, Washington 98382, USA

ABSTRACT: The course of the blood cell proliferative disorder (hemocytic neoplasia, HCN), was studied over 128 d in 40 bay mussels *Mytilus edulis*. Individuals obtained from a high prevalence cultivated population were maintained in the laboratory and sampled sequentially on 7 occasions for hemocytological analysis of HCN. Mussels were examined histologically at death or at the termination of the experiment. Ten (25%) of the individuals were disease-free throughout the experiment. Twenty (50%) developed the progressive disease. Of these, 12 had died and 6 of the remaining 8 were at an advanced stage at the end of the experiment. Eight (20%) mussels appeared to be in a state of remission at the end of the experiment and displayed an active host response to the abnormal blood cells. The remission phenomenon was characterized by the entrapment of abnormal cells in an extracellular matrix apparently secreted by normal, active hemocytes. It is concluded that (1) the disease is progressive and fatal but that some individuals have the ability to develop a host response resulting in remission, at least temporarily; (2) a synchronous transition hypothesis more accurately represents progression of the disease in *M. edulis* than a clonal progression hypothesis.

INTRODUCTION

Hemic proliferative disorders have been reported from a variety of bivalve molluscs around the world (Farley 1969, Alderman et al. 1977, Brown et al. 1977, Twomey & Mulcahy 1983). This proliferative disorder was first described in the bay mussel *Mytilus edulis* in 1969 from a population in Yaquina Bay, Oregon, USA (Farley 1969) and was later studied in more detail in that location in order to characterize the pathological manifestations of the disease, describe the ultrastructure of the affected blood cells and determine the association of the condition with environmental contaminants (Mix et al. 1979, Mix 1983, Mix & Schaffer 1983). In these studies, the affected blood cells were considered to have morphological characteristics in common with vertebrate neoplastic cells. No infectious agents were detected in association with the disease and although there was some association between the concentration of organic contaminants in the tissues and the occurrence of the disease, it was not possible to demonstrate that environmental contaminants induced or promoted the condition.

The condition has been most intensively studied in the soft shell clam *Mya arenaria*, in which it is considered to be a neoplastic process (Cooper et al. 1982a) and is referred to as hemocytic neoplasia (HCN). In *M. arenaria*, the condition is reported to be progressive and fatal in most cases (Farley et al. 1986) but can be chronic or even remissive in a small number of cases (Cooper et al. 1982a). It is reported to be transmissible and is hypothesized to result from a retrovirus infection (Oprandy et al. 1981) which is inducible by bromodeoxyuridine (Oprandy & Chang 1983). Some of the evidence for the interpretation of the involvement of a retrovirus in the *M. arenaria* disease is based on negatively stained electron microscopic material and is therefore open to question. The disease is characterized by the proliferation of enlarged circulating hemocytes with a high nucleus to cytoplasm ratio. Mitotic figures are common in these cells and the cells appear to replace both normal hemocytes and other tissue in the terminal stage of the disease.

In order to provide a basis for further mechanistic studies regarding the pathogenesis and etiology of this condition, we conducted in vivo experiments to elucidate the course and outcome of the disease. Specifically, the research reported here was performed in order to determine if the condition is progressive and fatal for affected mussels. In the course of the
experimentation. Important observations were also made on remission of the disease in some of the experimental specimens. Also reported is information on the use of hemocytological techniques for the characterization of the disease.

MATERIALS AND METHODS

Experimental studies. Mussels were obtained from Puget Sound, in Washington State, USA, in November 1986 from a cultured population containing a high prevalence of the disease (at least 40% detectable histologically in certain point-in-time samples). Forty individuals (shell length 41 to 65 mm), randomly selected for this experiment, were held for 128 days in continuously flowing seawater (30% salinity) at ambient temperature (9 to 12°C). Each individual was bled through the posterior adductor muscle at Days 0, 16, 29, 58, 78, 100, and 128 with a 21-gauge needle. The blood was withdrawn into a 3 ml syringe with 0.5 ml of 0.45 μm filtered seawater to give an estimated dilution of between 1/10 and 1/25, depending on the visually estimated concentration of hemocytes. A permanent slide record was made by allowing these cells to attach to glass and staining them as described below. In mussels in which the shell was not too thick, the posterior adductor muscle was easily located by placing one valve of the mussel against a fiber-optic light source and viewing the conductance of light through the refringent muscle on the opposite valve.

Hemocytology. In order to make a permanent record of the relative proportion of normal to abnormal hemocytes, 2 methods of allowing the cells to settle and attach to poly-L-lysine coated glass microscope slides were tested. The first method was the use of modified histological cassettes ('Farley Chambers') as described previously (Farley et al. 1986, Smolowitz & Reinisch 1986). Alternatively, drops of hemocyte suspension were placed individually on microscope slides and incubated in a moist chamber. Both room temperature (19 to 21°C) and 4°C, and incubation times of 15, 30, 45 and 60 min, were tested in all combinations. In initial trials, following the settling and attachment period, the overlying fluid was examined microscopically for residual unattached cells using both concentrated and diluted volumes of normal and diseased hemolymph.

Slides were fixed in methanol and stained with Feulgen picric-methyl blue as described previously (Farley 1968). The permanent preparations were examined and 100 cells rated as either normal or abnormal, as defined below. This method provided a means of permanently recording the relative number of normal and abnormal cells from sequential samples. Slides were examined by 2 investigators in order to define the criteria for designation as normal or abnormal but the final evaluation of the approximately 280 time-series preparations was made by the same investigator.

Criteria for designation of cells in hemocytological preparations. Histological and hemocytological studies by earlier investigators (Cooper et al. 1982b, Farley et al. 1986) and ourselves of normal and terminally sick molluscs demonstrated clear morphological differences between normal cells and those which were abnormal or transformed. These observations formed an initial approach to assigning cells examined by the hemocytological methods to either the normal or abnormal category. Thus, abnormal cells were those which were neither clearly normal hemocytes or clearly abnormal transformed cells but which were suggestive of cells in the transition from normal to neoplastic forms. This is discussed in detail below. However, these observations suggested that all observed blood cells were either clearly normal or unequivocally transformed or abnormal. In contrast, in preliminary studies, we had observed many morphological types of cells from some mussels which were neither clearly normal hemocytes or clearly abnormal transformed cells but which were suggestive of cells in the transition from normal to neoplastic forms. This is discussed in detail below. However, these observations required that, in addition to classifying cells displaying a continuum of characteristics by the criteria defined above, we set a numerical requirement in order to characterize the progressive nature of the disease by hemocytological methods. We adopted a 20% increase in abnormal cells over the baseline percentage of cells judged to be abnormal for any given individual to be the arbitrary indicator of the presence of progressive disease. Thus an individual was not considered to have a progressive disease until the proportion of abnormal cells increased at least 20% over the lowest level of abnormal cells recorded for that individual. This approach is supported by the results reported below in which the disease clearly becomes progressive when the proportion of abnormal cells reaches higher levels. The possible identities of 'abnormal' cells in mussels which did not develop the progressive disease are discussed below.

Histology. Mussels which died during the experiment and all live mussels at termination of the experiment were processed by routine paraffin histology methods. Preparations were stained with hematoxylin and eosin (H&E) and examined for histological stages of the disease as previously described (MIX 1983) and for other significant histological features. At the termination of the experiment, histological examination was used to verify if the remaining live mussels judged not
to display the progressive disease by hemocytological methods were actually disease-free (as indicated by a less than 20% change in cell types as determined hemocytologically), in an early stage of the disease, or in a state of remission.

RESULTS

Hemocytological methods

Both the 'Farley Chambers' and individual drops of hemolymph provided acceptable fields of attached hemocytes from both normal and diseased mussels. The drop method was adopted, however, since it was possible to place drops from 3 individuals on one slide and, in addition, was regarded as less cumbersome and time consuming than the chamber method. We were not able to detect any significant numbers of cells in the overlying fluids following the attachment period and therefore concluded that the cells on the slide faithfully represented the cell population removed from the mussels. Both normal and abnormal cells began attachment immediately after droplets were placed on the slides and were well enough attached to remain so through fixation after 15 min at both 4°C and room temperature. However, 30 min at room temperature was selected as the optimal condition for cell attachment. At 30 min normal cells were more spread out than at 15 min or at 30 min at 4°C. At 1 h at room temperature, degenerative morphological changes in nuclear structure (clumping or condensation of chromatin) were observed in some individuals. However, in spite of efforts to strive for uniform methodology, there was some unexplained variation among individuals with respect to the degree to which cells spread out on glass and the extent of degenerative nuclear changes. It should also be noted here that we used the term hemocytology to describe the preparations of blood cells attached to glass. The term 'histocytology' as proposed by Farley et al. (1986) literally means 'study of tissue cells' and as such its meaning is not clear or appropriate.

Hemocytology

Figs. 1 to 3 show representative hemocytological preparations of mussel hemolymph. Fig. 1 shows a normal individual which was given a rating of 2/100 abnormal cells. Fig. 2 shows a preparation from an individual which had 45/100 abnormal cells, and was clearly in a progressive stage of the disease. Note, however, that the cells in Fig. 2 with large nuclei and nucleoli are relatively more spread out and well attached to the glass substrate than the cells in the terminal stage shown in Fig. 3. Fig. 3 shows a preparation which contained 98/100 abnormal cells and thus represented an individual in the terminal stage of the disease.

Course of the disease

Table 1 summarizes the status of individual mussels at the beginning and end of the experiment. By the end of the experiment, twenty (50%) of the mussels had

Figs. 1 and 2. *Mytilus edulis*. Hemocytological preparations of mussel blood cells. Fig. 1. Normal individual which was given a rating of 2/100 abnormal cells. The typical nuclei of spread hemocytes are shown between the arrows. Fig. 2. Mussel with 45/100 abnormal cells. These cells contain large nuclei (arrows) but are relatively well spread out on the glass substrate as compared to the advanced abnormal cells shown in Fig. 3. Feulgen picr-methyl blue; bars = 10 μm.
Fig. 3. *Mytilus edulis*. Hemocytological preparation of blood cells from a mussel with advanced progressive disease (98/100 abnormal cells). The prominent nucleoli are indicated at the arrows. Feulgen picro-methyl blue; bar = 10 μm

Table 1. *Mytilus edulis*. Summary of the status of mussels at the beginning and end of the experiment

<table>
<thead>
<tr>
<th>Status</th>
<th>Number of mussels</th>
<th>Remarks on condition at Day 128</th>
</tr>
</thead>
<tbody>
<tr>
<td>Died – following advanced HCN</td>
<td>–</td>
<td>4 in remission</td>
</tr>
<tr>
<td>Alive – progressive HCN</td>
<td>2</td>
<td>8</td>
</tr>
<tr>
<td>Remission</td>
<td>–</td>
<td>2</td>
</tr>
<tr>
<td>No disease detected</td>
<td>38</td>
<td>10</td>
</tr>
<tr>
<td>Other*</td>
<td>–</td>
<td>2</td>
</tr>
</tbody>
</table>

*See footnote to Table 1

Table 2. *Mytilus edulis*. Summary of the status of mussels at the beginning and end of the experiment by the number of individuals in each of 6 category levels of the proportion of abnormal cells

<table>
<thead>
<tr>
<th>% of abnormal cell categories</th>
<th>Number of mussels in each category at Day 0</th>
<th>Remarks on condition at Day 128</th>
</tr>
</thead>
<tbody>
<tr>
<td>0–10</td>
<td>37</td>
<td>12</td>
</tr>
<tr>
<td>11–20</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>21–40</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>41–60</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>61–80</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>81–100</td>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td>Died – HCN</td>
<td>–</td>
<td>12</td>
</tr>
<tr>
<td>Died – other*</td>
<td>–</td>
<td>2</td>
</tr>
</tbody>
</table>

*See footnote to Table 1

died in an advanced stage of the disease or were alive and had the progressive disease. Remission, as confirmed histologically, was observed in 8 (20 %), while 10 mussels (25 %) remained disease-free. Table 2 shows that 6 of the 8 live mussels with the progressive disease were in an advanced stage (81 to 100 % abnormal cells) while 2 had between 21 and 40 % abnormal cells. Mussels in remission, as confirmed histologically, had up to 27 % abnormal cells at the end of the experiment. Mussels judged to remain 'disease-free' were those which did not develop a 20 % increase in 'abnormal cells'. In addition, no histological evidence of the disease was observed in any of these mussels at the termination of the experiment.

Fig. 4 shows representative examples of the course of the disease as determined by the hemocytological method. Animals 12, 23 and 35 show the typical progressive disease. Animal 12 was alive at the end of the experiment in an advanced stage of the disease with no histological evidence of remission. Animals 23 and 35 died in an advanced stage of the disease before the end of the experiment. Animal 34 was an atypical case showing some evidence of remission (decline from 56 to 22 % abnormal cells) but which died following the Day 78 sampling. Although this mussel showed hemocytological evidence of remission, it was categorized as progressive because it died following the progression of the disease to a relatively advanced stage. Animals 8, 26, and 40 were classified as being in a stage of remission. This is suggested by the sequential hemocytological data for Animals 8 and 40 but not for Animal 26. However, remission was definitively demonstrated by the distinctive host reactive response observed by the histological examination as discussed below.

It is important to note that by hemocytological methods, Animal 26 (in remission) is similar to Animals 17, 25 and 31 which had no evidence of the disease by either hemocytological or histological methods. The hemocytological profiles of Animal 26 (remission) and
Animals 25 and 31 (normal) are quite similar, while the profile of Animal 17 (normal) is quite similar to that of Animal 8 (remission). Thus, while the hemocytological method was useful for detecting advanced stages of the disease, histology is required to distinguish diseased from normal individuals when the disease is not in an advanced progressive stage.

Fig. 5 shows a histological section of a mussel in an advanced stage of the disease. The advanced disease essentially consisted of massive proliferation of the abnormal hemocytes. Typically, in the advanced stage of the disease these cells occupied all of the vascular spaces with relatively few normal hemocytes present. In individuals which were in a state of remission, the abnormal cells were often widely distributed in the vascular spaces and numerous normal granulocytic hemocytes were evident. These normal granulocytes appeared to secrete a fibrous extracellular matrix which entrapped the abnormal cells and, as confirmed by the hemocytological preparations, removed them from circulation in the vascular spaces. Focal areas in which normal hemocytes appeared to have sequestered abnormal cells were observed (Figs. 6 to 8).

Comparison of Fig. 5 with Figs. 6 to 8 shows that while the abnormal cells are widely distributed in individuals in remission they are not typically as densely packed as in the advanced disease and the presence of granulocytic hemocytes is very prominent. The concentration of these granulocytes around foci of abnormal cells suggests that they were forming a host response and defense against the abnormal cells, often resulting in a granuloma-like formation.

**DISCUSSION**

**Development of hemocytic neoplasia**

The hemocytological evaluation method used in this study provided a relative measure of the proportion of clearly abnormal cells. Farley et al. (1986) reported a similar approach using hemocytological material in studies of the condition in *Mya arenaria*. In that study, the authors evaluated 10 000 cells and therefore reported a sensitivity of 0.01% transformed cells, assuming that cells were either clearly transformed or normal. This point is further discussed below. Cooper et al. (1982b) and Cooper (1979) correlated a visual assignment (of phase-contrast-examined live cells) of 5 levels of neoplasia with hemocytology (identifying the number of abnormal cells per microscope field), concentration of abnormal cells and histological severity of the disease. The hemocytological approach provides a permanent record of the relative proportion of cells judged abnormal which can be obtained from repetitive bleedings.

The primary difference in interpretation of data in this study from the studies cited above is the recognition of morphologically transitional forms of cells. Many of these were assigned to the abnormal category...
Figs. 5 to 8. *Mytilus edulis*. Histological sections of diseased mussels. Fig. 5. Moderately advanced case of the disease with a mixture of normal and abnormal hemocytes. Bar = 50 µm. Fig. 6. Mussel in remission with a granuloma-like structure (arrows) containing a dense population of normal and abnormal hemocytes. Bar = 100 µm. Fig. 7. Higher magnification of granuloma-like structure in Fig. 6 (arrows). Note the fibrous matrix around the structure. Bar = 50 µm. Fig. 8. Granuloma-like structure containing normal and abnormal hemocytes with fibrous material (arrows). The fibrous material typically extends beyond the granuloma-like structure, throughout the vascular space in mussels in remission. H&E; bar = 50 µm.

in this study. However, their true identity may be either (1) transitional morphological types undergoing neoplastic transformation, or (2) normal undifferentiated stem cells (or a more differentiated but poorly understood population of normal cells). Their true identity cannot be discerned by morphological methods. Furthermore, the outcome of the disease in individuals which contain apparent transitional stage cells is not consistent since some mussels acquire the terminal progressive condition and others go into remission. For the purposes of this study, the imprecision inherent in assigning the morphologically transformed cells to one category or the other was compensated for by establishing the requirement that the proportion of cells considered abnormal increase by 20% or more.

A more important aspect of the difference in experimental observations and data interpretation between this study and the studies on *Mya arenaria* (Cooper et al. 1982b, Farley et al. 1986) is the conceptual recognition of 2 fundamentally different ways in which the disease may progress. In the *M. arenaria* studies, it is implicit in the methodology and data interpretation that a single morphologically fully transformed cell, out of a vastly larger population, clonally replicates and produces other fully transformed cells, leading to the advanced stage disease (the clonal hypothesis). In the present studies on *Mytilus edulis*, the observations suggest that a large proportion of hemocytes more or less synchronously go through a transformation process and that this process is manifested by the appearance of cells morphologically transitional between host stem cells and the easily recognized hemic neoplastic cell (the synchronous transition hypothesis). It is possible that the disease may progress by different mechanisms.
in different hosts. However, hemic neoplasia has been reported to be induced by a retrovirus (Oprandy & Chang 1983). While these results have been questioned by some investigators, the synchronous transition hypothesis of disease development is more consistent with an infectious etiology than the clonal hypothesis. In an infectious etiology, large numbers of cells are likely at risk of infection and subject to transformation rather than a single cell or small number of cells which subsequently undergo clonal proliferation. Finally, the observations in *M. edulis* of transitional forms support the concept of the hemocytic origin of the neoplastic cells, an hypothesis which has been questioned (Farley et al. 1986, Farley 1987) and remains to be definitively resolved. Thus one of the major questions for future research is to more accurately define the events which occur in the transformation of normal to abnormal cells and the resulting changes in cell behavior and morphology.

**Remission of the disease**

The discovery of mussels in a state of remission, with large numbers of sequestered abnormal cells, and which were clinically indistinguishable from those with no sign of the disease, demonstrates that hemocytological methods alone cannot always provide a definitive determination of the presence or absence of the disease. The hemocytological method is an extremely useful, non-destructive method for determining the sequential changes in stage of the disease where a host reactive response is not present. However, histological examination is essential to make a definitive determination if disease appears not to be present by hemocytological methods. Histology, while qualitative, is also more definitive with respect to the tissue distribution of the abnormal cells as well as the presence of a host reactive response. These findings appear to differ somewhat from those of Cooper et al. (1982b) in which the examination of wet preparations of blood cells was reported to be highly accurate in comparison with histological examination. However, examination of live cell preparations, like hemocytology, will not detect individuals in remission. In addition, examination of live cells is likely to be less sensitive in detecting the transitional form cells discussed above. Since Cooper et al. (1982b) did utilize histological methods at the termination of the experiment, and did not report a host reactive response, it may be concluded either that such a response does not occur in *Mya arenaria* as it does in *Mytilus edulis* or, alternatively, that the remission reported by those investigators was effected by a similar response but that all abnormal cells and evidence of the response had disappeared by the end of their 10 mo experimental period. Finally, it must be emphasized that the remission reported here may be temporary. The course of the disease over the longer term must be determined in additional studies.

**Progressive nature of hemic neoplasia in the mussel**

These studies clearly showed that the disease is progressive in some cases in *Mytilus edulis*. Interestingly, Cooper et al. (1982a) found an identical percentage of *Mya arenaria* (50%) showing a progressive disease process. In *M. arenaria*, however, only 10% were judged to be in remission compared with 20% in this study. In the previous studies, 40% of the *M. arenaria* were considered to be in a chronic stage of the disease while in the current studies, 25% of the population did not exhibit any signs of the disease when examined by histological methods. Cooper et al. (1982a) did demonstrate that higher levels of mortality existed in populations of clams with the disease than those without the disease. Our studies also demonstrated that, in cases in which the disease progressed to an advanced level, it resulted in the death of the mussels. The highest proportion of abnormal cells reached for which an individual subsequently demonstrated remission was 44%; in all other cases of remission, the proportion of abnormal cells as determined hemocytologically was never higher than 26%.

The specimens for this study were taken from a previously unstudied population of mussels with a high prevalence of the disease. While the prevalence of the disease is higher than previously reported for *Mytilus edulis*, it is comparable to prevalences reported for *Mya arenaria* (Farley et al. 1986). The prevalence and seasonality of the disease in the population of *M. edulis* will be the subject of future reports. It should be noted, however, that other studies found an increasing prevalence of this disease during fall and winter (e.g. Mix 1983). Thus, the initiation of this experiment in November may have been fortuitous in providing an opportunity to observe the progressive nature of the disease. Effects of temperature on disease induction merit further study.

These studies showed for the first time that the phenomenon of remission of these disorders in bivalves is, at least in some cases, the result of a host reactive response in which normal hemocytes, presumably granulocytes, secrete a fibrous extracellular matrix which sequesters the abnormal cells. It is possible that in some of the mussels which were normal histologically at the end of these experiments, remission was complete and all abnormal cells and evidence of a host reactive response had been removed. For example, Animal 17 (Fig. 4) had 24% abnormal cells in the Day
78 sample but had no histological evidence of the disease at Day 128. Thus the question of the ultimate outcome of the disease in mussels which enter a state of remission is an important question for future studies. In mussels in which the condition is progressive, the disease appears to satisfy the criteria for being considered a neoplasia. The characteristics which differentiate those individuals which can mount an effective host reactive response to the abnormal cells and those which succumb to it may have biological significance beyond the mussel. Thus, based on characteristics of the individual’s defense system, some animals are able, at least temporarily, to reverse the course of the disease while in others it progresses to death.

Acknowledgements. This work was supported by the National Cancer Institute and the US Army Medical Research and Development Command under Grant Number SRC(8), 5 RO1 CA 44269-02.

LITERATURE CITED


Responsible Subject Editor: Dr A. K. Sparks, accepted for printing on March 16, 1988