AN ABSTRACT OF THE THESIS OF

Marcia House for the degree of Doctor of Philosophy in Microbiology presented on September 18, 1997. Title: TRANSMISSION OF DISSEMINATED NEOPLASIA IN THE SOFT SHELL CLAM, *MYA ARENARIA*

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Disseminated neoplasia (DN) is a proliferative cell disorder that occurs in the circulatory system of bivalves. The condition is progressive and lethal. At least 15 species of bivalves over a wide range of geographic locations have been reported to contract DN. Prevalence levels of disseminated neoplasia can reach up to 90% in some populations.

In the laboratory, the condition can be transferred to healthy individuals by injection of hemolymph from animals of the same species with high intensity levels of DN. Studies were conducted to investigate transmission of disseminated neoplasia in the soft shell clam, *Mya arenaria*. It was determined that soft shell clams from two Oregon bays were susceptible to DN by injection, and that the lack of DN in these west coast populations of soft shell clams was not due to disease resistance in these animals. Additionally, it was demonstrated that onset, development of DN, and survival were directly correlated to the number of neoplastic cells injected into the animal. Experiments investigating water-borne transmission showed that the disease is infectious, and an exposure to DN cell in the hemolymph of highly affected clams was sufficient to cause disease. In a cohabitation study, transmission of DN from one DN positive animal to healthy animals was observed, with specific information collected on the length of exposure and DN intensity of the animals involved. Finally, transmission of disseminated neoplasia was not found to be successful using cell-free filtrates prepared from DN cells and DN positive soft shell clam tissue. A PCR enhanced reverse transcriptase assay was employed, and reverse transcriptase activity was detected in samples prepared from DN positive materials.

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Transmission of Disseminated Neoplasia in the Soft Shell Clam, *Mya arenaria*

By

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I understand that my thesis will become part of the permanent Collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

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Marcia House, Author

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CONTRIBUTION OF AUTHORS

The work presented in this thesis was done under the direction of Dr. Paul Reno in his laboratory at the Coastal Oregon Marine Experiment Station, Hatfield Marine Science Center, Department of Microbiology, Oregon State University, Newport, Oregon.

The work in Chapter 3, "Susceptibility of west coast soft shell clams to disseminated neoplasia and dose dependence of transplantation", was done in cooperation with Dr. Dale Leavitt at Woods Hole Oceanographic Institution (WHOI), Woods Hole, Massachusetts. Dr. Leavitt was essential in collection of the east coast soft shell clams, and in performing the studies at WHOI.

The work in Chapter 5, "Soft shell clams, *Mya arenaria*, with disseminated neoplasia (DN) demonstrate reverse transcriptase activity, but unaffected clams do not", was done in cooperation with Dr. Carol Kim in the Department of Microbiology at Oregon State University in Corvallis, Oregon. Dr. Kim performed PCR and Southern Blot analysis in the PCR enhanced reverse transcriptase assay.

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DEDICATION

This thesis is dedicated with love and admiration to my Mom and Dad
who have always encouraged and supported my explorations.
Thanks for taking me to the beach.

TRANSMISSION OF DISSEMINATED NEOPLASIA IN THE SOFT SHELL CLAM, *MYA ARENARIA*

CHAPTER 1

THESIS INTRODUCTION

Disseminated neoplasia (DN) is a proliferative disorder that occurs in the circulatory system of bivalves. DN can be chronic, with low numbers of DN cells in circulation, or develop into a more severe disease with high numbers of neoplastic cells leading to death of the animal. The condition was initially reported in the native oyster, *Ostreola conchaphila*, from Yaquina Bay, Oregon in 1969 (Jones and Sparks 1969). Since that time, DN has been identified in at least 15 species of bivalves including soft shell clams, *Mya arenaria*, (Yevtich and Barszcz 1976), mussels, *Mytilus edulis*, (Farley et al. 1969a) and European cockles, *Cerastoderma edule* (Twomey and Mulcahy 1984). The prevalence of DN varies among affected populations, reaching up to 90% (Reinisch et al. 1984). DN has not been detected in soft-shell clams from Oregon estuaries that have been examined, namely Coos Bay (Mix 1986), Yaquina Bay, Nestucca Bay, and Alsea Bay (House and Reno, unpublished observations). Complexities in the occurrence of DN suggest that several aspects of the animals' physiology as well as environmental conditions may be involved in determining the presence and severity of DN in a population.

Laboratory experiments have shown that DN can be transplanted to healthy animals by the injection of hemolymph taken from animals with high numbers of DN cells in circulation (Farley et al. 1986, Twomey and Mulcahy 1988, Elston et al. 1988b). Water-borne transmission of DN has been shown to occur by holding healthy clams in the effluent of a tank containing DN positive clams (Appeldoorn et al. 1984). Elston et al., (1988b) and Kent et al. (1991) found that untreated negative control mussels in transmission studies developed disseminated neoplasia, probably due to DN positive mussels in the laboratory water supply. Transmission of the disease with a cell-free homogenate prepared from DN cells from mussels, *M. edulis*, was successful within the same species (Elston et al. 1988b, Kent et al.

1991, Moore 1993). The etiological agent of DN has not been isolated, but some evidence indicates that a viral agent, specifically a retrovirus, may be involved (Oprandy et al. 1981, Oprandy and Chang 1983).

The goal of the studies presented in this thesis was to investigate transmission of disseminated neoplasia under laboratory conditions in order to gain a better understanding of how the disease is spread and maintained in natural populations. Chapter 2 is a review of literature which provides information on the biology of the soft shell clam, and the history and current knowledge of disseminated neoplasia. Chapter 3 describes studies designed to provide information about working with disseminated neoplasia using local soft shell clams. One study was performed to determine if west coast soft shell clams are susceptible to disseminated neoplasia, and a second study was designed to determine if the prevalence, onset and intensity of DN, as well as the survival of clams injected with neoplastic cells, are related to the dose. The studies in Chapter 4 were designed to gather specific information concerning water-borne transmission of DN. In the first study presented in the chapter, groups of clams were exposed to different doses of DN cells in order to determine if dose of DN cells affects onset, prevalence, and intensity of DN. The second study in Chapter 4 monitored DN in donor and recipient animals in shared tanks to determine the efficiency of transmission of disseminated neoplasia. Chapter 5 describes experiments using cell-free filtrates prepared from DN cells and tissues of DN positive animals in order to determine if a filterable agent causes disseminated neoplasia in soft shell clams. Additionally, a PCR enhanced reverse transcriptase assay was utilized to determine if the enzyme reverse transcriptase (RT), an enzyme essential to retroviral replication, was present in preparations of DN cells and DN positive tissues of soft shell clams.

CHAPTER 2

LITERATURE REVIEW

BIOLOGY AND ECOLOGY OF THE SOFT SHELL CLAM

The soft shell clam, *Mya arenaria*, is an estuarine bivalve mollusk found on both the Atlantic Ocean and Pacific Ocean coasts of the United States. This species was introduced to the west coast with the introduction of oysters for aquaculture endeavors at the turn of the century in the San Francisco Bay area. Reports from that era indicate that the spread of the clam up the west coast was assisted by ship's captains who planted the shellfish in areas that seemed likely to support a population (Stearns 1881). The clams survived and spawned, establishing populations in several Oregon and Washington estuaries (Carlton 1979). Factors that determine the distribution of soft shell clams in an estuary are temperature, availability of food, water flow, sediment quality, and salinity (Newell and Hidu 1986, Appeldoorn et al. 1984).

Initiation of broadcast spawning depends on the environmental conditions, and usually begins with the release of sperm into the water. The female soft shell clam can produce 120,000 (Brousseau 1978) to one million eggs (Newell and Hidu 1986) depending on the size of the animal. The eggs are released into the water column where they are fertilized. The fertilized egg develops into a veliger which feeds on algae, and remains planktonic for 2-3 weeks, the timing depending on local conditions. At the conclusion of the larval stage, the clam attaches to eelgrass or filamentous algae, or drops to the bottom and can attach to the substrate (Newell and Hidu 1986). It then metamorphoses into a bottom-dweller called spat, losing its velum and developing a muscular foot. The spat can float or crawl for 2-5 weeks, sometimes attaching to substrate with a byssal thread, a tough strand secreted by a gland in the foot. As the spat grows, it begins to burrow, but its location may still be governed by the movement of substrate in the estuary. As the size of the animal increases, it burrows deeper and becomes stable in one location.

Soft shell clams are filter-feeders; the gill is made up of sheets of filaments that are used to filter particles from the water that is pumped in through the incurrent siphon. The currents are generated by the lateral cilia, one of the three specialized

types of cilia on the gills. As water is drawn in by the beating cilia, suspended particles are captured by laterofrontal cilia, and pushed to the surface of the gill where the frontal cilia move the particle to the food groove, toward the mouth. The particles are sorted by the palp lamelli based on size and weight, with small, light particles retained for ingestion. If particles are too large, they are rejected for ingestion, and eliminated as pseudofeces. Langdon and Siegfried (1984) found that bivalves were able to efficiently filter and ingest particles ranging from 2-15 microns in diameter. The selected particles proceed to the stomach where they are mechanically ground and enzymatically digested by the action of the crystalline style. Soluble components continue on to the digestive diverticula, where intracellular digestion by digestive cells and hemocytes occurs. Nutrients are transported to tissues by the hemocytes and serum. The hemolymph moves through the open circulatory system, bathing the tissues and collecting in sinuses throughout the body of the animal.

Hemocytes function not only in transport of nutrients in the bivalve, but also in excretion, wound repair, shell repair, and internal defense (Cheng 1981). Several classification systems of bivalve hemocytes have been proposed, with the characterization of granulocytes and hyalinocytes being generally consistent among the various systems. Both the granulocytes and hyalinocytes are phagocytic, although the granuloctyes are more active in this respect than the latter. Particles can be phagocytized and digested intracellularly by the enzymes contained in the lysosomes of granulocytes. If a particle cannot be successfully phagocytized, then it can be walled off, or encapsulated. Leucocytosis, or the increase in number of hemocytes, has been viewed as a precursor of phagocytosis or encapsulation. It is not clear whether the overall number of hemocytes increases, or if the concentration of hemocytes increases only in a localized area. Finally, the release of lysosomal materials into the hemolymph has been noted as protective 'humoral' factors that have anti-microbial factors. No reports of vertebrate types of antibodies have been made. Cheng (1981) noted that evolutionarily, the initial functions of molluscan phagocytes were geared toward nutrient digestion and circulation, and that the immune function of the hemocyte is a function that became important later.

HISTORY AND EPIZOOTIOLOGY OF DISSEMINATED NEOPLASIA

In 1969, Jones and Sparks first described abnormal cells in the hemolymph of the native oyster, *Ostreola conchaphila* (formerly *Ostrea lurida*), from Yaquina Bay, Oregon. Also in 1969, Farley (1969a) described a probable neoplastic disorder in which abnormal hemocytes invaded the normal oyster tissue in two species of oysters, *Crassostrea virginica* and *C. gigas*. Six cases were gathered over a seven year span from approximately 30,000 oysters that were being screened for pathogens in an epizootiological study concerned with determining factors contributing to oyster mortality. The single report of DN in *C.gigas* was from an animal collected in Matsushima Bay, Japan, and the remaining 5 cases occurred in *C. virginica*, four were collected in Maryland, and one was collected in Long Island, New York. A second publication by Farley (1969b) described a sarcomatoid proliferative disease in mussels (*Mytilus edulis*) from Yaquina Bay, Oregon. It appeared that the prevalence of the condition was much higher than in the *Crassostrea* species; ten cases were identified in 100 animals sampled. In the past 28 years, several other species of bivalves have been found to have similar disorders; names for the condition have attempted to characterize the nature of the disease, with specific terms such as 'sarcoma', or 'leukemia'. In a large portion of the literature, the terms 'hemic' or 'hematopoetic' neoplasia are used because the neoplastic cells are found in the circulatory system, and presumably arose from hemocytes. In this thesis, the most general term suggested by Elston et al. (1992), disseminated neoplasia (DN), will be used; it implies that the neoplastic cells are spread throughout the animal, and that their origin remains unclear at this time.

Since the initial publications recognizing the condition, varying prevalence levels of disseminated neoplasia have been identified in at least 15 species of bivalves over a wide range of locations. Reviews have been prepared by Mix (1986), Peters (1988), and Elston et al. (1992) detailing these reports. Soft shell clams on the Northeast Atlantic coast have been shown to have prevalence levels up to 90% (Reinisch et al. 1984), while up until 1983, Chesapeake Bay *M. arenaria* were free of the condition (Farley et al., 1986). At that time, DN was reported at six locations with prevalence levels reaching 78% in 1990 in one area. Currently, the disease in Chesapeake Bay soft shell clams continues to be detectable at low levels (0-3%)

with occasional seasonal peaks (McLaughlin et al. 1996). DN has not been detected in *M. arenaria* in Yaquina Bay (0/>250), Nestucca Bay (0/>200), and Alsea Bay (0/>1000), (House and Reno, unpublished observations), or in Coos Bay (0/360), Oregon (Mix 1986).

The patterns of the occurrence of disseminated neoplasia in populations appear to depend on several factors. Different subspecies of the bay mussel, *M. edulis*, appear to have differing susceptibility to DN (Elston et al. 1992). *M. edulis (edulis)* is found in the Atlantic Ocean, and is not severely affected by the disease, with prevalence levels of less than one percent occurring on the East Coast of the United States (Hillman 1990). There have been no epizootics reported in *M. edulis (galaprovincialis)*, which occurs in the southern range of the mussel from central to southern (Elston et al. 1992). The bay mussel that inhabits the Pacific Northwest, *M. edulis (trossulus)*, has been found to have prevalence levels of greater than 40% in Puget Sound (Elston et al. 1988b), and up to 29.9% in British Columbia (Cosson-Mannevy et al. 1984). Mix (1983) found that there were differences in the prevalence of DN in populations of bay mussels within Yaquina Bay, OR. Over the course of a five year study, he found that one site had an average prevalence of 9.8%, while a site one km across the bay had a prevalence level of 0.3%, and no DN was found at the third site upriver. Temperatures and salinity were similar for all of the sites, with one difference between the sites being the proximity to anthropogenic contaminants, although no causal relationship was established or implied.

Several surveys and studies examining the relationship between pollutants, particularly petroleum products, and the occurrence of DN and other bivalve tumors, especially those of gonadal origin, have been reported (Yevich and Barszcz 1977, Brown et al. 1977, Harshbarger et al. 1979, Appeldoorn et al. 1984, Reinisch et al. 1984). In a comprehensive review, Mix (1986) concluded that many of the reports that were available lacked sufficient analytical data for evaluating the relationship between pollution and neoplasia in bivalves, and the results that were available were generally not supportive of a pollution-neoplasm correlation.

In addition to the differences seen in populations at varying locations, Mix (1983) also found a seasonal pattern in prevalence, but not in the severity, of DN. In the five year study of neoplasia in mussels in Yaquina Bay, he found that prevalence of DN peaked in the winter months (January through March), declined through the spring and summer, then increased at the end of fall and early winter. His study employed a more intensive sampling regime than a study by Farley and Sparks

(1970), and is supported by their earlier results, with the exception that the earlier work concluded that DN develops in the autumn and continues to progress throughout the spring. Mix (1983) found that there was no relationship between the severity of disease and the season.

Studies in the soft shell clam also indicate that there is a seasonal pattern in the prevalence of DN. Brousseau (1987) and Farley (1987) reported peak prevalence of DN in the fall through the spring when water temperatures are cool. Appeldoorn et al. (1984) reported a peak in the fall and in the spring, and Leavitt et al. (1990) reported two patterns at two sites in Buzzard's Bay, Massachusetts. The site at Little Buttermilk Bay showed a single peak prevalence and the site at New Bedford Harbor showed two peaks, one in the fall, and the second in the late winter. It has been suggested that these patterns may be related to the spawning cycle (Elston et al. 1992).

Other influences that have been considered in the patterns of neoplasia in bivalves are the water flow of the area that animals have settled, and the density of the local population. Rasmussen (1986) noted in a study in mussels that at Lyngs Odde, Denmark, a location with an DN incidence rate of 0.2%, the water current was stronger at the site at Hindsgavl, where the incidence rate of DN was 0.6%. In field surveys, Brooks and Elston (1991) found that there was a relationship between the occurrence of DN in mussels populations, the density of the population, and the degree of water circulation. In laboratory studies designed to examine this relationship, Brooks (1991) housed mussels from a population known to have DN with naive animals in four combinations of high and low population density (2000 DN affected mussels/m^2, and 200 DN affected mussels/m^2 respectively) and high and low water circulation (approximately 20 water exchanges per hour, and approximately one water exchange per hour respectively). He found that over a five month period the naive mussels exposed to low density and high water circulation conditions showed no signs of advanced disease. The naive mussels in the low circulation conditions, at both the low and high population densities showed high levels of DN prevalence. The mussels exposed to high population density with high water circulation showed intermediate levels of DN prevalence.

The complexities of the occurrences of DN suggest that aspects of the physiology of the animal, the environmental conditions, water flow and population density, in addition to the nature of the disease causing agent which will be discussed below, may be involved in determining the presence and severity of DN in a population.

DISEASE DESCRIPTION

In the first extensive descriptions of disseminated neoplasia, six oysters, *C.virginica* and *C. gigas* , (Farley, 1969a) and ten *M. edulis* (Farley, 1969b) at various stages of the disease were examined histologically. Although there were no gross lesions recognizable in any of the animals, more severely effected individuals demonstrated emaciated, or watery, condition with pale organs, atrophy of the gonad, and recession of the mantle. In tissue sections abnormally large, undifferentiated cells were found invading vascular spaces, and overtaking the normal tissues. In less severe cases, there appeared to be primary foci with localized disseminations of the abnormal cells. As the disease progressed, dissemination of the abnormal cells increased. In the acute stage there was extensive invasion of normal tissue. Yevich and Barszcz (1977) described the condition in the soft-shell clam, *Mya arenaria*, finding the abnormal cells throughout the connective tissue of the animal. Twomey and Mulcahy (1984) discovered the condition in the common cockle, *Cerastoderma edule*, in Europe, in which there was "infiltration of the muscle bundles, gonadal follicles, digestive diverticula and the gills with a pleiomorphic population of large, mitotically active, loose undifferentiated cells".

Initial recognition of this condition was based on the observations that abnormal cells with a disproportionally enlarged nucleus were overtaking the circulatory system and invading normal organ tissues (Jones and Sparks 1969, Farley 1969 a and b). These cells frequently showed mitotic figures which are rarely seen in normal bivalve hemocytes. Since that time, many cellular characteristics have been analyzed in an attempt to understand the nature and origin of the disease cells.

Kent et al. (1989) performed *in vivo* studies examining the ability of mussels with a range of levels of the disease to clear a bacterial suspension. In addition to these studies, the authors performed *in vitro* experiments and found that the neoplastic cells were not phagocytic, as the normal hemocytes were. Working with the soft-shell clam, Beckmann et al. (1992) compared lysosomal enzymes and surface receptors that are involved in phagocytosis. It was shown that neoplastic cells, unlike normal hemocytes, were unable to phagocytize yeast cells. The neoplastic cells had increased levels of acid phosphatase, nonspecific esterases, and B-glucuronidase compared to normal hemocytes, and lacked B-*N*

-acetylglucosaminidase found in the normal cells. It was also shown that there was no difference found in the cell surface staining with concavalin A, and the binding was inhibited by D+ glucose and eliminated by D+ mannose in both types of cells. With no apparent differences in cell surface receptors, the inability to phagocytize was thought to be due to differences in the cytoskeletal organization. Moore et al. (1992) found that neoplastic cells had less actin than normal hemocytes with altered actin filament patterns. The disrupted cytoskeletal structure in the neoplastic cells would "account for the inability of the cells to adhere to glass, clump and phagocytose foreign materials". Each of these papers, however, makes the unproven assumption that the DN cells are transformed hemocytes, and describes the differences detected between the normal hemocytes and the DN cells as changes.

In 1983 Reinisch et al. used monoclonal antibodies to explore antigenic similarities and differences between normal hemocytes and neoplastic cells in the soft-shell clam. Ten specific antibodies were produced, nine reacted with the DN cells and one was specific to the normal hemocytes. The variation in the reactivity patterns seen among the nine neoplastic cell specific antibodies convinced the authors that there were at least two classes of antigens unique to the DN cells. It was determined that one of the antibodies was raised to a 200 kDa protein unique to the membrane of the neoplastic cells (Miosky et al. 1989). Smolowitz et al. (1986) used another of the neoplastic cell specific antibodies to develop an indirect immunoperoxidase staining (IP) assay that was shown to be more sensitive and accurate than the already established method of examination of fresh hemocytes, and quicker to analyze than the Feulgen Picromethyl Blue method.

Monoclonal antibodies have been utilized in immunohistochemical studies designed to determine the tissue origins of the neoplastic cells. Using the immunoperoxidase method, Smolowitz et al.(1989) detected an antibody that reacted with a subpopulation of normal hemocytes as well as the neoplastic cells. Additionally, Noel et al.(1991) described the preparation and characterization of monoclonal antibodies raised against neoplastic hemolymph in *M. edulis*. Monoclonal antibodies specific for DN cells were characterized and subsequently used in work done by Moore (1993). The results of the work done in both mussels and soft shell clams will be discussed in detail below.

As previously noted, researchers have observed relatively high numbers of mitotic figures in the neoplastic cells; mitotic figures are rarely observed in normal bivalve hemocytes. Farley and Sparks (1970) describe the mitotic abnormalities as tripolar figures, chromosomes displaced from the mitotic spindle, and polyploidy.

These observations were based on histological sections; since then several techniques have been employed to gain more exact information concerning the DNA content and chromosome complement of the DN cells. Lowe and Moore (1978) used microdensitometry to determine that the neoplastic cells from a Plymouth, England population of *M. edulis* had 3.8 to 7.2 times as much DNA per cell as normal hemocytes. Elston et al. (1990) employed a flow cytometric technique to follow the progression of DN in *M. edulis* in two experiments involving repeated sampling of individuals over time. Animals with no signs of disease had three populations of circulating cells, 2.0n. 2.25n, and 4.03n. The first plody number indicates normal hemocytes in G_1 phase, designated G_{1a}. The second value was termed G_{1b}, and the authors were unable to determine the significance of this population, but it did not seem to have any relationship to the development of DN. The final population represents normal hemocytes dividing in circulation. This event appears to be rare, with only 3% of the population having 4n. As the disease progressed, fourth (5.08n) and then fifth populations (10.07n) of cells appeared. As the severity of the condition increased, these polyploid cell populations increased, and the proportion of cells with normal concentrations of DNA decreased. In addition to the pentaploid abnormal cells, two of the animals showed signs of an alternative pathological condition, showing an increase in the 4.0n population of cells. Late in the experiment, one of the animals developed a small 7.7n population. With further investigation, Moore et al.(1991) found that these populations represent two forms of neoplasia. The pentaploid (5n) form was found to be prevalent (66%) in the four Washington populations of mussels that were examined. The tetraploid (4n) form of the disease occurred in 29% of the animals, with 5% of the animals having both forms of the disease simultaneously. The tetraploid form is more mitotically active than the pentaploid form. Additionally, there were subtle morphological differences in the cells; the 5n cells are generally larger and less likely to have double nucleoli than the 4n form cells.

Most recently, Reno et al. (1994) published information on the chromosome complement and DNA content of DN cells from *M. arenaria.* Normal clam cells had a modal chromosome number of 34 (Allen et al. 1982) with a range of 26-39, whereas DN cells had chromosome numbers ranging from 44-80 with a mode of 69. The DN cells had acrocentric and telocentric chromosomes which were absent from normal cells. By flow cytometric analysis, DN cells had a mean of 1.76 (1.25 to 2.05) times more DNA than normal cells. These values indicate a tetraploid condition, and there was no evidence of a pentapolid population as seen in the

mussel. A significantly higher proportion of the aneuploid cells were in the S phase of the cell cycle than the normal cells. Aneuploid cells in G_0/G_1 were also larger in size than their diploid counterparts. Both the flow cytometry and the chromosome analysis indicate that there is a quantitative difference in the DNA content of DN cells as compared to normal cells.

Since the early recognition of these cells, most authors identified them as abnormal or neoplastic. In 1976, Mackin and Schlicht proposed that a parasite, *Labrinthomyxa patuxent*, could be the etiologic agent. Speculation as to the nature of these abnormal cells continues to the present day, although advances have been made to confirm the suspicion that the DN cells are, in fact, of host cell origin. In 1994, Gee et al. analyzed sequences of 16S-like rRNA from both the pentaploid and the tetraploid DN forms in *M.edulis*, as well as normal mussel hemocytes. This work showed a high degree of similarity between both forms (similarity coefficient, 0.982), as well as to the normal hemocytes (similarity coefficient, 0.990 and 0.992 respectively). When these were compared to other known sequences, there was a high degree of similarity to *Crassostrea virginica* sequences (similarity coefficient, 0.895-0.927). Additionally, a large phylogenetic distance was found between all three mussel cell sequences and those of several representative protists (similarity coefficient, 0.702-0.761). It was concluded that the DN cells were not unicellular parasites, but proliferative host cells.

The idea that the neoplastic cells result from host tissue was generally accepted even prior to Gee et al. (1994), but the tissue of origin is an unresolved issue to date. This topic was addressed briefly earlier in the discussion of terminology. Much of the difficulty arises from the fact that there is limited knowledge of normal bivalve hemocyte generation. In Mix's general model for leukocyte renewal (1976), a possible stem cell was identified as a large undifferentiated cells that labeled within hours of injection of the tritiated thymidine in both *Ostrea lurida* (now *Ostreola conchaphila)* and *Margaritifera margaritifera*. These cells were frequently found in close proximity to labeled hyalinocytes and found throughout the loose connective tissue, most commonly in areas underlying the mantle. No hematopoetic tissue was designated; the model went on to propose a scheme for the differentiation of mature hemocytes. In a study using the same radioisotopic labeling techniques, Mix (1975) found large numbers of abnormal cells labeled with the tritiated thymidine dispersed uniformly "throughout the connective tissue underlying the gills, mantle, stomach, gut, digestive diverticula, kidney and gonad". Based on the high labeling index, number of mitotic figures and the undifferentiated appearance of the cells, it was

concluded that this population of cells turned over more rapidly than the normal oyster cell population.

Work employing immunohistochemistry and other methods, suggest that the DN cells may have a connective tissue origin. As mentioned above, Smolowitz et al.(1989) found a monoclonal antibody that was reactive to both normal and neoplastic cells in *M. arenaria*. When this antibody was used to stain histological sections of animals in various stages of disease progression, it showed that the connective tissue cells became increasingly reactive to the antibody as the disease level increased. The relationship was thought to indicate that the connective tissue may be involved in the generation of the neoplastic cells, but the role of the reactive normal cell subpopulation was unclear. There did not seem to be a relationship between these cells and the development of neoplasia.

Moore (1993) used a variety of techniques to gain information about the origin of the neoplastic cells in *M. edulis*. In ultrastructural examinations of the cells, he found them to have characteristics consistent with vertebrate neoplasms such as "prominent nucleoli, abundance of ribosomes and polyribosomes, swollen mitchondria, multiple centrioles, bizarre nuclear configurations, nuclear inclusion and paucity of rough endoplasmic reticulum". In earlier work, Mix (1979) found similar characteristics in diseased *M. edulis* from Yaquina Bay. Additionally, Moore failed to observe epithelial cell characteristics such as " the presence of desmosomes or hemidesmosomes, microvilli with an associated terminal web, clusters of cells with an underlying basal lamina, and synthesis of glandular or characteristic secretory products", and concluded their absence supported the connective tissue origin of the DN cells. When he employed antibodies raised against mammalian intermediate filaments proteins and other tissue markers, no specific reactivity was identified in *M. edulis*. It was concluded that there was lack of shared epitopic identity between the mussel and the mammalian antigens, and that antibodies with a broader species specificity would be useful to pursue the work. The antibodies prepared by Noel et al. (1991) against DN hemolymph in *M.edulis* showed three patterns of reactivity when used to stain sections of normal and diseased animals. One group did not react with DN cells, and bound only to a subset of granular and hyaline hemocytes. The second group of antibodies reacted with both the tetraploid and pentaploid neoplastic cell, hemocytes and several connective tissue cell types. The third group of antibodies reacted only with a subset of the pentaploid form of DN cells and a limited set of connective tissues. This final group was also found to react with a DN serum protein, suggesting that the antigen may be on a cell secreted

protein. The patterns of reactivity suggest a connective tissue origin for the DN cells. This was further supported in an examination of lectin binding in tissue sections. Although the patterns were more variable than the monoclonal antibodies, wheat germ agglutinin and peanut agglutinin bound connective tissue and DN cells, while others did not bind to DN cells and did bind epithelial tissue. Moore (1993) concluded that based on the evidence, the DN should be designated a neoplasms of connective tissue origin, or sarcomas.

Several authors have demonstrated that DN is a progressive condition. Cooper et al.(1982a) developed a technique that provided a nonlethal sampling method for diagnosis of the neoplasia. They performed a comparative study of the number of neoplastic cells in a hemolymph sample drawn from either the posterior adductor muscle sinus or the pericardial region, and the degree of severity of the disease in histological sections. Stages of the disease were arbitrarily assigned according to the number of neoplastic cells per milliliter of hemolymph using a log progression to set the limits (stage 1, $<10^4$ neoplastic cells per milliliter, up to stage 5, $>10^7$ neoplastic cells per milliliter). The number of neoplastic cells per milliliter was positively correlated with the severity of the histopathological lesions. Using this sampling method in further work, they were able to follow 36 *M. arenaria* with various levels of severity of disease over a ten month period (Cooper et al. 1982b). At termination of the experiment, 50% of the animals had either increased in severity of the disease or died, 40% had developed a chronic condition in which the level of DN had stabilized, and 10% exhibited a decrease in the level of severity, or remission. Other authors have noted remission of the condition, and this will be discussed in more detail below. It was concluded that if the disease was at level 3 or greater ($>10^6$ neoplastic cells per milliliter), it would progress and lead to the death of the animal. At low severity levels the condition can become stable and chronic, or diminish and show remission.

Other authors have corroborated these findings, but have chosen to develop their own staging methods. To avoid confusion, the staging method employed in this study will be defined based on the percentage of DN cells in a sample (the number of DN cells/ total number of cells in sample x 100). In 1986, Farley held ten *M. arenaria* in the laboratory for three months and found that each individual progressed to the advanced stage of the disease. Farley (1987) performed a more detailed laboratory study in which 30 *M. arenaria* were monitored over a 6 month period, and observed that the disease progressed from early (stage 1=0.01%-0.09%) to advanced (stage 5= 90-100%) with a fatal outcome within 5 months for

all of the affected animals. Elston et al.(1988a), working with 40 mussels over a four month period, found that 50% of the animals developed a more severe condition or died, 20% showed remission, and 25% of the animals remained disease free. Brousseau and Baglivo (1991) showed that progression and remission occurs in the field as well as in the laboratory, and found that the lab conditions that they used modified the overall results of the studies. It appears that these authors did not provide any substrate for the animals in the laboratory which could contribute to the stress the animals experienced, and therefore increase the effects of the disease. Regardless of the various staging methods and scales have been adopted by other authors, the conclusion that DN can be a progressive or regressive condition is common to all of the work.

As presented above, Cooper et al.(1982a) Elston et al.(1988a), and Brousseau and Baglivo (1991) have documented the occurrence of a decrease in the severity of the neoplasm in individual animals. In a six month mark and recapture field trial involving 900 *M. arenaria*, Brousseau and Baglivo (1991) found that 16% of the animals initially diagnosed with less than 50% DN cells in circulation cleared the disease completely. Only one of 41 surviving initially diagnosed with "high severity level, HSN" (greater than 50% DN cell in circulation) completely cleared the disease. In the 18 week laboratory study, 67.5% (27/40) of the surviving animals (40/66) initially diagnosed as "low severity level, LSN", underwent remission. This high percentage of remission could be an artifact of the method of detection. The initial work done using the "histocytology" method described in Cooper et al.(1982a) did not detect any signs of neoplastic cells in the individuals that underwent remission. In a shorter study, Elston et al.(1988a) demonstrates in histological sections that there may be a cellular defense of walling off or engulfing the DN cells that could go undetected in the histocytological method, and states that histological sections are required to determine the complete remission of the neoplasia. Brousseau and Baglivo (1991) rely completely on the histocytological method, and may therefore be overestimating the number of individuals that show remission.

It has been documented repeatedly that the progression of DN leads to the death of the animal. In the two laboratory studies by Cooper et al.(1982a) demonstrating progression of DN over a 10 month period, it was also found that there was statistically significant higher mortality rate in the diseased animals compared to the normal animals. They found that the least severe cases (stage 1) had a 60% survival rate, stage 2, 54%; stage 3, 25% and stages 4 and 5, 0%. In the 1986 description of

a new occurrence of epizootic sarcoma in Chesapeake Bay soft-shell clams (Farley et al.), ten animals were monitored in the laboratory from December 1983 until May 1984. These animals showed progression from early stages (0.01%-0.9%) to advanced stages (50%-100%) with 100% mortality. Farley (1987) continued his work with *M. arenaria* and later documented that animals in with 50%-100% DN cells in circulation were three times as likely to die as animals with 0-49% DN cells in circulation over a three month period in the laboratory. In Elston et al.(1988a) studies in *M. edulis*, the authors concluded that "in cases in which the disease progressed to an advanced level, it resulted in the death of the mussels". Finally, in Brousseau and Baglivo's (1991) six month field study of *M. arenaria*, it was shown that animals initially diagnosed with greater than 50% neoplastic cells in circulation were more likely to die than animals with less than 50% or disease free animals. Additionally, those with less than 50% neoplastic cells were more likely to die than disease-free animals. Under laboratory conditions, they found that the higher severity group had significantly higher levels of mortality (61.1%) than the low severity (39.4%) or the disease free (28.3%) groups when held for 18 weeks. Again, the high level of mortality in the normal control group indicates that the laboratory conditions may have been less than optimal.

With overwhelming evidence that advanced DN kills the animal, the following mechanisms that lead to death have been proposed. Cooper et al.(1982b) suggested that the cause was the displacement of normal organ tissue by neoplastic cells, and Appeldoorn et al.(1984) cited the complete disruption of normal tissue architecture. Leavitt et al.(1990) measured the ratio of the dry weight of the soft tissue of the animal to the dry weight of the shell (condition index, CI) to gain information about the overall health of the animals; a higher CI indicates a healthier animal. Once a level of greater than 71% DN cells in circulation is reached, the CI of the clam decreased dramatically. It was found that animals with 71% to 100% neoplastic cells in circulation had a significantly lower CI compared to normal animals, with animals having 16% -70% DN cells following the same trend, and no significant difference between normal and animals with less than 15% DN cells. It appears that the massive infiltration of the neoplastic cells into normal tissues exhausts the animals reserves before death. Kent et al. (1989) observed bacterial septicemia in mussels dying of the disease. These observations promoted *in vitro* and *in vivo* studies exploring the ability of the diseased animals to clear bacteria from their systems. It was found that the neoplastic cells were not phagocytic as normal bivalve hemocytes are. Animals with no disease or that were lightly affected were able to clear greater

than 90% of a bacterial suspension injected into the posterior adductor muscle sinus, while animals with advanced disease cleared 44-83% of the bacteria. Considering this information in combination, the overall health of an animal with advanced levels of neoplasia is severely compromised. Nutritional reserves are depleted as indicated by decreased CI and defense mechanisms appear impaired; these factors clearly could lead to an inability to endure the stressful conditions to which estuarine animals are exposed.

TRANSMISSION OF DISSEMINATED NEOPLASIA

In the laboratory, a healthy animal injected with DN cells from a highly affected individual of the same species will develop the disease (Elston et al. 1992). Transplantation of DN by injecting cells from a highly affected animal into the circulatory system of a naive animal has been demonstrated in soft-shell clams (Appeldoorn et al. 1984, Farley et al. 1986), cockles (Twomey and Mulcahy 1988) and mussels (Elston et al. 1988b). The agent appears to be species-specific. In a transmission study completed by Kent et al. (1991), it was shown that injection with a homogenate prepared from DN cells from mussels, *Mytilus edulis*, caused DN to occur in mussels, but not in soft shell clams, *M. arenaria*, flat oysters, *Ostrea edulis*, or the native oyster treated with the same material.

Results of experiments by Brown et al. (1980) and Appeldoorn et al. (1984) indicated that DN could be passed to healthy *Mya arenaria* via the seawater effluent from tanks containing diseased clams, and also from ambient seawater drawn from Narragansett Bay, RI. In one experiment, effluent from headboxes with an unspecified number of diseased animals was used to supply tanks with healthy clams. The healthy clams were held in sediments from a polluted area, a clean area, a mixture of the two, or no sediment. By the sixth month of the study, up to 72% of the exposed animals were positive for DN. The most severely affected groups were those held in the mixture of the sediments and the animals without sediment. The study was repeated to confirm the results a year later. It was successful again, but there were fewer diseased animals available for the headbox than in the first trial, and the resulting number of exposed animals that contracted DN was decreased compared to the original trial. Experiments designed to examine transmission by feeding diseased material to healthy clams were not successful. In the first of the

two trials, the authors fed naive clams the tissue from diseased clams daily for a one week period, and monitored the recipient animals for DN over a three month period, and in another trial, the healthy clams were fed once a week for three months. In studies with neoplastic and non-neoplastic clams maintained in the same tank, DN was not detected in the recipients in a three month study period.

It was shown in studies done on the west coast with bay mussels, *Mytilus edulis trossulus,* that naive animals held in tanks with animals with DN contracted the disease over a five month period (Brooks 1991). Additionally, Elston et al. (1988b) and Kent et al. (1991) performed transmission experiments and found that negative control mussels were becoming infected with DN, most likely as a result of DN affected animals in the water supply for the laboratory (Sequim Bay, WA). Elston et al. (1988b) housed 20 healthy mussels with 50 mussels from a diseased population. Within a 231 day period, 40% of the healthy animals developed DN. Additionally, 20% of the negative control animals developed DN. In 1986, Farley et al. reported the occurrence of DN in a Chesapeake Bay population of soft shell clams, *M. arenaria,* that had been previously found free of the disease in more than one decade of sampling. The new occurrence of DN in this area was thought to be a result of an introduction of potentially diseased *M. arenaria* from the New England area, where the disease is endemic.

Transmission of DN with a cell-free homogenate prepared from DN cells was shown to be successful in mussels, *M. edulis* (Elston et al. 1988b, Kent et al. 1991, Moore 1993). In 1981, Oprandy et al. reported that a virus with physical and morphological characteristics of a type B retrovirus was observed in a preparation from DN positive soft shell clams, *M. arenaria.* The study went on to report that transmission of DN with a cell-free filtrate prepared from DN positive clams had been successful, with Koch's postulates completed. In a later study, Oprandy and Chang (1983) reported inducing viral production and DN in healthy clams drawn from a population known to have the disease by exposing the animals to 5-bromodeoxyuridine (5-BrdUrd). This technique has been shown to induce expression of endogenous retroviruses in some cultured mammalian cells (Lowry et al. 1971). Although these results appeared to demonstrate that DN in *M. arenaria* was caused by a retroviral agent, there has been no success in attempts to repeat this work, putting the question of a retroviral etiology into question. Transmission was not accomplished by injection of cell-free filtrates prepared from DN materials in cockles, *Cerastoderma edule*, (Twomey and Mulcahy 1988), mussels (Moore

1993), and soft shell clams (McLaughlin and Farley 1992), all of which were demonstrated to be susceptible to transplantation of DN by whole cell injection.

DN has been extensively studied since it was first discovered in 1969. Information leading to characterization of the etiologic agent, and how transmission occurs in populations would be important tools in managing transport and production of shellfish affected by this disease.

CHAPTER 3

SUSCEPTIBILITY OF WEST COAST SOFT SHELL CLAMS TO DISSEMINATED NEOPLASIA AND DOSE DEPENDENCE OF TRANSPLANTATION

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ABSTRACT

The progressive and lethal disease disseminated neoplasia (DN) has been found in populations of the soft shell clam, *Mya arenaria*, from the east coast of the United States, but has not been detected in populations from the west coast of the United States. It is not known whether west coast *M. arenaria* are refractory to the disease, or alternatively had been transferred to the west coast in the early 1900's prior to the introduction of the inducing agent responsible for disease in east coast clams. Studies on the west coast at the Hatfield Marine Science Center in Oregon, and on the east coast at Woods Hole Oceanographic Institution in Massachusetts were undertaken to determine if west coast clams are susceptible to disease transmission with whole DN cell suspensions. *Mya arenaria* collected from two bays on the east coast and two bays on the west coast were inoculated with neoplastic cells pooled from east coast animals with high levels of DN, or inoculated with filtered sea water. The west coast clams collected from Alsea and Yaquina bays were found to be susceptible to DN, with similar numbers of these clams developing the condition as in the group of clams collected from Barnstable Harbor, Massachusetts. The clams collected from Little Buttermilk Bay, Massachusetts did not appear to be affected by injection of DN cells, as the other groups of clams were. The severity of DN in the clams from the west coast was significantly higher than that seen in the Barnstable Harbor clams. In this study, survival was not affected by injection of DN cells. A second study used clams collected from Alsea Bay to examine the effect of the dose level on the prevalence, onset, intensity of disease and survival of the clams. A pool of DN cells was collected from clams with high levels of DN, and serially diluted in sterile seawater, and used for inocula. It was shown that the time of onset of DN was directly related to the concentration of DN cells administered, with the highest dose level showing earliest onset, a significantly more rapid development of high DN levels, and significantly decreased survival.

INTRODUCTION

Disseminated neoplasia (DN) is a proliferative disorder that occurs in the circulatory system of bivalves. This disease was first described in the native oyster (*Ostreola conchaphila*), in Yaquina Bay, Oregon (Jones and Sparks 1969). Since that time, the condition has been described in more than 15 species of bivalves around the world (Peters 1988) including soft-shell clams (*Mya arenaria*) (Yevich and Barszcz 1977), bay mussels (*Mytilus edulis*) (Farley 1969b), and cockles (*Cerastoderma edule*) (Twomey and Mulcahy 1984).

The earliest report of DN in populations of *M. arenaria* was found in clams from Searsport, Maine in 1976 (Yevitch and Barscz 1976). Since that time, DN has been studied extensively in east coast *M. arenaria*, with the disease occurring over the range of the animal, from Maine (Yevich and Barszcz 1976) to Maryland (Farley et al. 1986). Prevalence levels are variable depending on the location. In 1984, Reinisch et al. reported that the prevalence levels of DN ranged from 10-90% in populations of soft-shell clams in New Bedford Harbor, Massachusetts.

Soft-shell clams are not indigenous to the west coast of the United States, but are thought to have been introduced to the San Francisco Bay area more than 100 years ago with the importation of oysters (Stearns 1885). These stocks originated from the east coast. The spread of *M. arenaria* northward was assisted by intentional planting of the clams in areas that would support populations of the shellfish.

DN has not been found in *M. arenaria* on the west coast of the U.S. In one survey of 50 animals, Farley (1976) detected 3/50 animals with a non neoplastic proliferative disorder that resembled DN, but this appeared to be associated with a parasite. Surveys of this species revealed no DN in Oregon soft shell clams (Mix 1986 (0/>360), House and Reno unpublished observations (0/>1300)). The purpose of this study was to determine if the absence of the disease was due to a natural resistance of *M. arenaria* on the west coast of the U.S. to the condition, or the import of the animals before the agent appeared on the east coast, or to the absence of the DN causing agent in west coast *M. arenaria.*

Transplantation of DN by injecting neoplastic cells from a highly affected animal into the circulatory system of a naive animal has been demonstrated in soft-shell clams (Appeldoorn et al. 1984, Farley et al. 1986), cockles (Twomey and Mulcahy 1988), and mussels (Elston et al. 1988b). A study was conducted to determine if

such a transplantation was possible with DN cells from highly affected soft shell clams from Cape Cod, Massachusetts to west coast soft shell clams. This was followed by a second experiment designed to address the question of dose dependence of the transplantation.

METHODS

Susceptibility of West Coast *Mya arenaria* to DN

Animal Collection

Mya arenaria measuring between 40-120 mm valve length were collected from two sites on the Pacific Coast and two sites on the Atlantic Coast in Spring 1992. Clams were collected from Alsea Bay (AB) and Yaquina Bay (YB), Oregon (44° N, 124° W), and Barnstable Harbor (BH) and Little Buttermilk Bay (LB) , Massachusetts (41° N, 70° W). The experiments described below were carried out in duplicate on the west and east coast. Therefore, half of the animals collected at each location were shipped to the research facility on other coast. Prior to experimental use, clams were housed for less than one week of time at the Laboratory for Fish Disease Research at the Hatfield Marine Science Center, Newport, Oregon (HMSC), or the Coastal Research Laboratory at Woods Hole Oceanographic Institution, Woods Hole Massachusetts (WHOI).

Detection of Disseminated Neoplasia

Animals to be used in the infectivity studies were screened for the presence of DN prior to use. The condition was detected by drawing approximately 100 microliters of hemolymph from the anterior adductor muscle sinus of each individual. The hemolymph was placed into a 24 well microtitration plate which contained a 12mm glass coverslip previously coated with 0.05% poly-L-lysine (Sigma Scientific, St Louis, MO), rinsed with distilled water and air dried. The cells were allowed to adhere to the coverslip for 30 minutes at room temperature, and

fixed with 1% glutaraldehyde and 4% formaldehyde in filtered seawater. The sample was evaluated for the presence of DN cells under phase contrast microscopy at 200x. Samples were stored at 4°C in sealed bags until staining with anti- neoplastic clam cell monoclonal antibody by immunocytochemical techniques (Smolowitz and Reinisch, 1986) could be completed. Individuals that were negative by the initial evaluation were used in the experiment described below.

Experimental Design

Ninety DN negative clams from each of the four bays (two east coast locations and two west coast locations) were randomly divided into a treatment group and a control group. At HMSC, circulating cells from nine clams with high levels of DN collected from Little Buttermilk Bay, were pooled and the total number of cells per milliliter was determined by counts on a hemocytometer to be $10^{7.8}$ cell/ml. At WHOI, cells were collected from six clams from Little Buttermilk Bay and Barnstable Harbor to yield a pool with $10^{7.3}$ cell/ ml. Cells were held briefly on ice until the inocula was injected into recipients using a 25 gauge needle fitted onto a 1 ml syringe. The treatment group from each bay consisted of 45 animals injected in the adductor muscle sinus with an inoculum of 0.1 ml of hemolymph from clams with greater than 95% DN cells in circulation. Filtered seawater (0.2 micron pore size) was injected into the adductor muscle sinus of negative control animals.

Within each group 30 animals were tested at weeks 2, 4, 6, 8, 10, 12, 14, and 18 post-injection at HMSC, and at weeks 2, 4, 7, 9, 15, and 19 post-injection at WHOI, for the presence of DN as described above. The remaining 15 animals were sampled only at the end of the experiment (week 18 at HMSC and week 19 at WHOI) in order to provide information on the effects of repeated sampling on the disease condition of the animals.

At HMSC, the animals were distributed into 0.5 x 1.5 m tanks supplied with sand-filtered, ultraviolet-irradiated running seawater at ambient temperature (range 10-18°C during the course of the experiment). Frequent sampling of the incoming water indicated few or no bacteria entering the system. Effluent water was treated by chlorination (> 2 mg/L chlorine with a residence time of at least 1 hour). Each tank contained either 6 or 3 (4L) buckets filled with clean sand for housing the animals in a substrate that emulated natural conditions. Separate tanks were used for west and

east coast clams to avoid the possibility of cross contamination. Additionally, control animals were separated from clams injected with DN cells, and those sampled repeatedly were separated from those which were sampled only once. Clams were fed daily by interrupting the water flow for two hours and adding a 50/50 mixture of algal culture of *Isochrysis galabania* and *Cheatosorous calcitrans*.

At WHOI, the clams were held in sand filled containers in two concrete raceways (2.6 x10 x1.3 m) supplied with ambient, untreated Nantucket Sound seawater which flowed through the raceway at a rate of 19 L min^{-1}. One raceway contained only west coast clams, and the other only east coast clams. Control animals were held at the inlet of the raceway and DN injected animals were held at the outlet, approximately 10m downstream from the controls. All animals were held under ambient light, temperature, and salinity conditions. The food source was that which was available in the inflowing seawater. The water temperature ranged from 14-22°C during the course of the experiment.

Immunocytochemistry and Sample Evaluation

Analysis for the presence and intensity of DN in hemocyte samples using immunocytochemistry was executed as follows. The coverslips were washed three times for 5 minutes in phosphate buffered saline (PBS) to remove fixative, and nonspecific binding sites were blocked by incubating with 1:500 normal goat serum (Gibco, Grand Island, NY) in PBS for 20 minutes at room temperature. Samples were incubated with anti-neoplastic cell monoclonal antibody (Smolowitz and Reinisch 1986) for a minimum of 1 hour at room temperature. Unbound antibody was removed with three-5 minute PBS washes. The bound antibody was detected by using the Vectastain ABC-AP Kit (Vector Laboratories, Burlington, CA), according to the instructions. Following amplification of the signal by utilizing an avidin-biotin-antibody complex and staining with the chromagen Fast Red^{T}, the samples were counterstained with hematoxylin (Sigma Scientific, St. Louis MO) for 10 minutes, rinsed with distilled water followed by ammonia water (0.5%) for 10 seconds. After a final wash with distilled water, the coverslips were allowed to dry and were mounted on slides with Cytoseal mounting medium (Fisher, Pittsburgh, PA).

Ten fields per sample containing a total of at least 1,000 cells were examined at a magnification of 200x using brightfield microscopy. DN positive cells stained red, and normal hemocytes were counterstained blue.

Dose Response:

Experimental Animals

Mya arenaria were collected in Alsea Bay (150 animals, mean valve length = 60 mm, SD = 8 mm, range = 42-77 mm) and screened for the presence of DN by phase contrast microscopy. No DN was detected. The animals were randomly divided into five groups of 30, individually numbered and treated as described below.

Protocol

All inocula were kept on ice until injected into the adductor muscle sinus of the recipient animals. A pool of DN cells was collected from five clams with 99% DN cells in circulation. Four cell concentrations were prepared and administered as follows. The high dose treatment consisted of the undiluted hemolymph with $2.2x10^7$ cell/ml ($10^{7.3}$cells /ml). A series of four-fold dilutions of the hemolymph was made in sterile seawater to obtain the remaining three treatments which had $4.0x10^6$ cell/ ml ($10^{6.6}$cells /ml), $1.4x10^6$ cell/ml ($10^{6.1}$cells /ml), and $4.0x10^5$ cell/ml ($10^{5.6}$cells /ml), as determined by hemocytometer counts. One tenth of a milliliter of the suspension was delivered to each of thirty animals in each group, for dose levels administered at $10^{6.3}$cells /clam, $10^{5.6}$cells /clam, $10^{5.1}$cells /clam, and $10^{4.6}$cells /clam. The animals in the negative control group were injected with 0.1 ml of filtered seawater.

The treatment groups were divided into three groups of 10 animals each, and each subgroup was placed in an isolated 3 gallon tank half filled with clean sand, and independently supplied with 0.2L/min pathogen free seawater. The flow was interrupted daily for 3 - 4 hours to allow for feeding 1L of a 50/50 mixture of *Cheatoserous calcitrans* and *Isochrysis galabanea.* The animals were monitored for mortalities over the next 12 months, with hemolymph samples drawn monthly from

the adductor muscle. The samples were fixed and analyzed using the anti-neoplastic cell monoclonal antibody as previously described.

Statistical Analysis

In comparing the prevalence of DN over the course of the experiment, Kaplan-Meier survival analysis (StatView 4.5, Abacus Corporation, Berkeley, CA) with the event being the initial detection of the disease, was employed. A logrank test (Mantel-Haentszel test, StatView 4.5, Abacus Corporation, Berkeley, CA) was used to determine if there were significant differences among the different groups. The same series of analyses was used to compare survival, with the event being the time of death.

Intensity was defined as the percent of neoplastic cells in circulation, with the lowest value of 0.01%, to the highest value of 100%, in the most severe cases. Since the intensity is dependent on the detection of at least one DN cell by immunocytochemistry, the time of initial detection of DN in the individual was considered as month one for the purpose of comparing intensity between animals. A generalized linear model analysis (GLM, GLMSTAT, Ken Beath, Perth, Australia) was utilized to determine the drop in deviance for the parameter of interest. These drops in deviance were tested for significance, and if the parameter was found to be significant, post hoc testing to determine which means were different was performed using Fisher's Protected Least Significant Difference (PLSD) test (StatView 4.5, Abacus Corporation, Berkeley, CA).

RESULTS

Susceptibility of West Coast *Mya arenaria* to DN:

The results of the study indicate that west coast soft shell clams are susceptible to DN, and the disease was successfully transplanted to both the Alsea and Yaquina Bay clams used in this study.

Experiments performed at Woods Hole Oceanographic Institution

Table 3.1 is a compilation of the number of DN positive clams of those surviving at the times that samples were taken. Within the first 2 weeks of initiation of the experiment, greater than 50% of all of the animals, both east and west coast, controls and treated groups, had died. In four weeks, 80% mortality had occurred, with only 15% of the animals surviving 19 weeks. The mortalities were spread throughout the groups, confounding statistical analysis of the occurrence of DN in this study. The Alsea Bay animals had a higher survival than clams from other bays, although the reason for this was not investigated. The cause of the mortalities was not identified.

By week 2 post injection, 67% of the surviving Alsea Bay clams and 44% of the surviving Yaquina Bay clams had developed DN. In week 7 post-injection, 100% of the Alsea Bay and 83% of the Yaquina Bay clams that were surviving were DN positive. Additionally, west coast animals that were not injected also developed DN while held in the facilities at WHOI. In week 4 of the experiment, 14% of the Yaquina Bay animals were DN positive and in week six, 50% of the Alsea Bay clams were DN positive.

Experiments performed at the Hatfield Marine Science Center

None of the west coast clams at the HMSC in the negative control groups became positive for DN over the course of the 18 week study. Table 3.2 is a compilation of the number of DN positive clams surviving at each sample time. The negative control clams from the east coast showed evidence of DN in animals injected with seawater. Based on the initial phase-contrast evaluation of the hemolymph samples, only clams that were negative for the presence of DN were selected for use in the transmission study. When the more sensitive immunocytochemical technique became available to evaluate the samples, it was found that three of the thirty Barnstable Harbor clams in the negative control group
initially had low levels of DN (from 0.04% to 0.12% DN cells in circulation). Additionally, four of the Little Buttermilk Bay clams in the negative control group, and three from the DN cell injected group also had low levels of DN (from 0.02% to 0.45% DN cells in circulation). These clams were removed from analysis of the study.

Table 3.1 Prevalence of DN in the experiment performed at Woods Hole Oceanographic Institution. Soft shell clams were collected from four bays: Alsea Bay, Oregon (AB), Yaquina Bay, Oregon (YB), Barnstable Harbor, Massachusetts (BH) and Little Buttermilk Bay, Massachusetts (LBB). The clams from each bay were divided into two groups; each clam was injected with either 0.1ml of sterile seawater (SW) or $10^{6.3}$ cells in 0.1 ml of hemolymph which was collected from clams with > 95% DN cells (DN). Hemocyte samples were analyzed for the presence of DN using immunocytochemistry. The number of DN positive clams per the number of surviving clams are provided. NS indicates that all clams in that group had died and no sample was taken.

		Week post-injection						
Bay	**Group**	**0**	**2**	**4**	**7**	**9**	**15**	**19**
A B	**SW**	0/30	0/25	0/26	12/25	0/20	0/16	5/13
	DN	0/29	8/12	10/11	11/11	5/5	2/2	2/2
Y B	**SW**	0/29	0/10	2/14	0/10	0/4	0/3	0/2
	DN	0/30	4/9	6/10	5/6	NS	NS	NS
B H	**SW**	0/28	0/13	0/4	0/5	1/4	2/2	2/2
	DN	0/25	0/15	0/8	1/10	1/10	1/8	2/8
L B B	**SW**	0/18	0/8	0/7	1/3	0/3	0/3	0/3
	DN	0/20	1/11	0/5	0/3	2/2	2/2	2/2

Table 3.2 Prevalence of DN in clams at the Hatfield Marine Science Center. Soft shell clams were collected from four bays: Alsea Bay, Oregon (AB), Yaquina Bay, Oregon (YB), Barnstable Harbor, Massachusetts (BH) and Little Buttermilk Bay, Massachusetts (LBB). The clams from each bay were divided into two groups; each clam was injected with either 0.1ml of sterile seawater (SW) or $10^{6.8}$ cells in 0.1 ml of hemolymph collected from clams with > 95% DN cells in circulation (DN). Hemocyte samples were analyzed for the presence of DN using immunocytochemistry. The number of DN positive clams per the number of surviving clams are provided.

		Week post-injection								
Bay	**Group**	**0**	**2**	**4**	**6**	**8**	**10**	**12**	**14**	**18**
A B	**SW**	0/30	0/30	0/29	0/27	0/27	0/27	0/27	0/27	0/27
	DN	0/30	5/30	9/28	11/28	11/27	11/26	8/23	12/23	11/20
Y B	**SW**	0/30	0/28	0/25	0/25	0/24	0/23	0/23	0/23	0/23
	DN	0/30	12/30	17/28	15/28	16/28	15/25	14/22	14/22	13/18
B H	**SW**	0/27	0/27	1/19	1/13	0/12	2/11	2/11	1/11	1/11
	DN	0/30	3/30	12/28	13/21	8/14	5/7	1/4	3/4	3/4
L B B	**SW**	0/26	0/26	2/12	2/7	0/5	0/5	0/4	0/4	0/3
	DN	0/27	4/26	1/21	1/17	1/6	0/4	0/4	0/3	0/3

Also, over the course of the study, two of the negative control animals in the Little Buttermilk Bay group developed DN (less than or equal to 3.67% DN cells in circulation) during the course of the experiment. In the Barnstable Harbor negative control group, three animals developed DN (less than or equal to 0.09% circulating cells). Using Kaplan-Meier analysis in combination with a logrank test, a comparison of the occurrence of DN in the negative control group to that in the DN cell injected group from the same bay showed that for the Barnstable Harbor clams, the occurrence of disseminated neoplasia was significantly higher in the DN cell injected group of clams than in the negative control group of clams that had been injected with sterile seawater (chi square = 16.992, p-value <0.0001). In the Little Buttermilk Bay groups, there was no significant difference between the seawater injected group and the DN cell injected group (chi square = 0.051, p-value = 0.8209).

Rate of Development of DN:

Clams from all four bays in the treatment groups had detectable levels of DN at two weeks post-injection (see Table 3.2). The Alsea Bay group had 5 DN positive clams /30 (16.6%), Yaquina Bay 12/30 (40.0%), Barnstable Harbor 3/30 (10%), and Little Buttermilk Bay 4/26 (15.4%). As depicted in Figure 3.1, the number of clams that developed DN increased over the course of the experiment for all groups except Little Buttermilk Bay. The total percentage of the initially injected clams that developed DN during the 18 week course of the experiment was 47% for Alsea Bay, 63% for Yaquina Bay, 60% for Barnstable Harbor, and 15% for Little Buttermilk Bay. There was no significant difference in the time of onset of disseminated neoplasia in the DN cell injected groups when the west coast bays were compared to the east coast bays (p-value = 0.1203). Comparisons between animals from different bays showed that Alsea Bay, Yaquina Bay and Barnstable Harbor groups had significantly different times of onset of DN than the clams from Little Buttermilk Bay (see Table 3.3 for p-values).

Figure 3.1 Prevalence of DN in clams from the east and west coasts exposed to DN by injection. The percent of soft shell clams that developed disseminated neoplasia in the DN cell treated groups are shown over the course of the 18 week study at the Hatfield Marine Science Center, Newport, Oregon. Soft shell clams from two Oregon bays (Alsea: black bar, and Yaquina: striped bar) and two Massachusetts bays (Barnstable Harbor: stippled bar, and Little Buttermilk Bay: white bar) were used in this study.

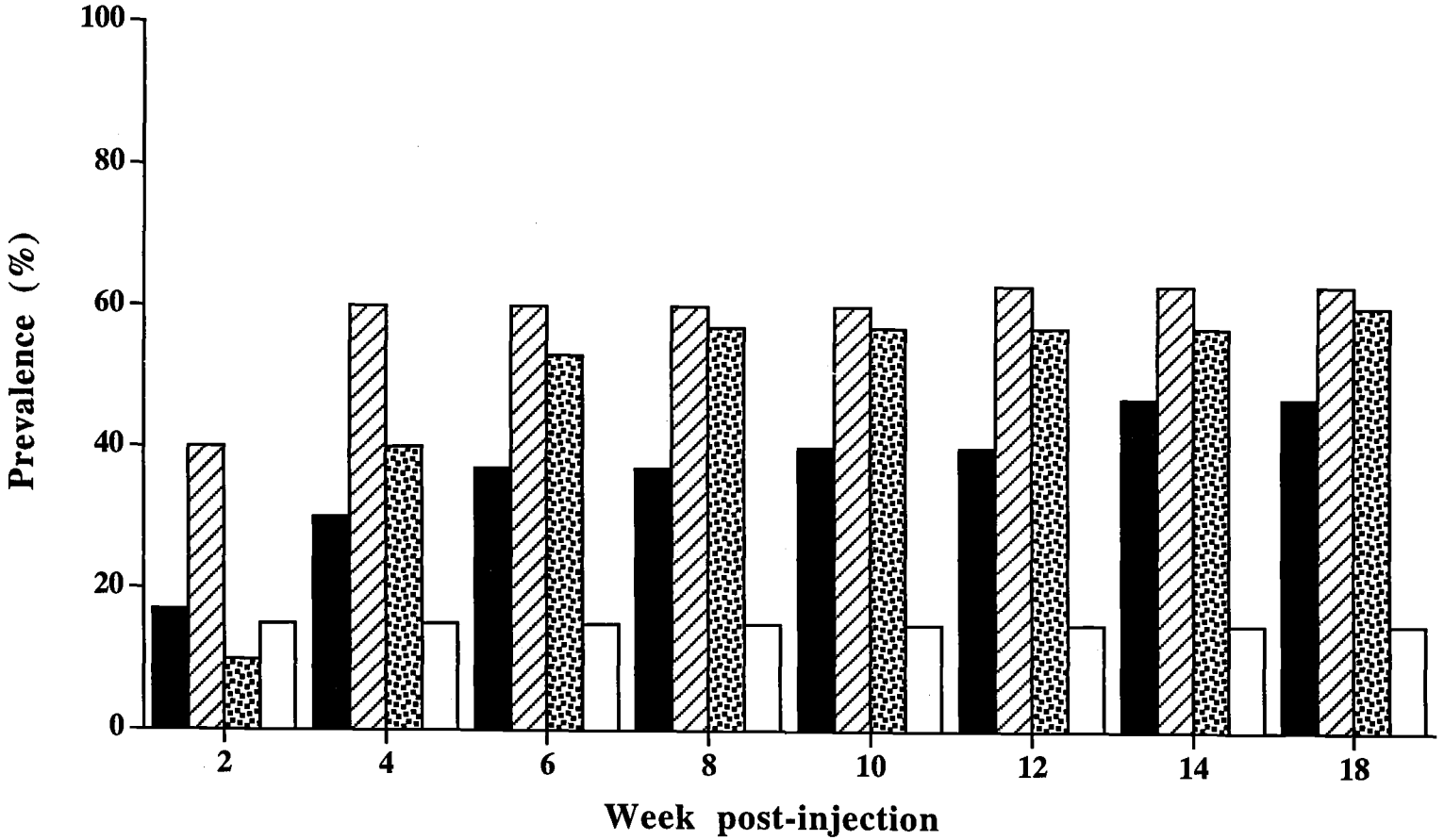
100
80
60
40
20
0
Prevalence (%)
2
4
6
8
10
12
14
18
Week post-injection

FIGURE 3.1

Table 3.3 Comparison of the rate of development of disseminated neoplasia at the Hatfield Marine Science Center. Alsea Bay and Yaquina Bay are west coast estuaries in Oregon, and Barnstable Harbor and Little Buttermilk Bay are east coast estuaries in Massachusettes. Listed below are the two groups compared, the chi-square value from the logrank test, the degrees of freedom for the test (Df), and the resulting p-value. Significant differences at the 95% confidence level are denoted by *.

Comparison	Chi-square	Df	P-value
East to West	2.413	1	0.1203
Alsea to Yaquina	0.855	1	0.3553
Alsea to Barnstable Harbor	2.053	1	0.1520
Alsea to Little Buttermilk Bay	7.656	1	0.0057*
Yaquina to Barnstable Harbor	0.053	1	0.8172
Yaquina to Little Buttermilk Bay	13.709	1	0.0002*
Barnstable Harbor to Little Buttermilk Bay	12.706	1	0.0004*

Intensity of DN in positive clams:

The intensity of DN in the animals also increased over time, again with the exception of Little Buttermilk Bay clams (Figure 3.2). The differences in intensity over time among bays were significant (F statistic = 23.97, p-value = <0.0001), with the Alsea Bay animals developing the highest intensities of DN. Individuals in this group had up to 90% DN cells in circulation. Yaquina Bay clams reached up to 69% DN cells in circulation. When the differences between bays was further investigated, it was shown that Alsea Bay had significantly higher levels than Yaquina Bay (p-value = 0.0129, see Table 3.4 for Fisher PLSD values and p-values). Additionally, both of the west coast bay groups had significantly higher levels of DN than the east coast bay groups, with no significant difference detectable between Barnstable Harbor and Little Buttermilk Bay animals. In both of the DN cell injected east coast groups, the levels of DN reached during the course of the experiment remained under 1% DN cells in circulation, with the exception of one Little Buttermilk Bay clam in the negative control group. This animal was injected with sterile seawater at the initiation of the study, and at week six PI, was found to have 3.67% neoplastic cells in circulation.

Survival:

West coast clams had significantly lower mortality rates than east coast clams in this experiment (chi square = 92.830, p-value >0.0001). However, there was no significant difference in mortalities among clams injected with DN cells and those injected with sterile seawater for any of the treated groups compared to its corresponding negative control group, with the exception of the Little Buttermilk Bay animals that showed a significantly higher level of mortality in the negative control group than in the treated group. Therefore, the mortalities that occurred cannot be attributed to the effects of the injection of DN cells. Table 3.5 summarizes the percentage of surviving clams for the negative control and the DN cell injected groups for each bay, as well as the values obtained in the survivorship analysis and logrank comparison of these groups for each bay. Figures 3.3 A and B show

Figure 3.2 Intensity of disseminated neoplasia in DN positive soft shell clams. The log of the average intensity for DN positive clams from each bay (Alsea: square, Yaquina: diamond, Barnstable Harbor: circle, and Little Buttermilk Bay: triangle) is shown over the course of the study at the Hatfield Marine Science Center Newport, Oregon.

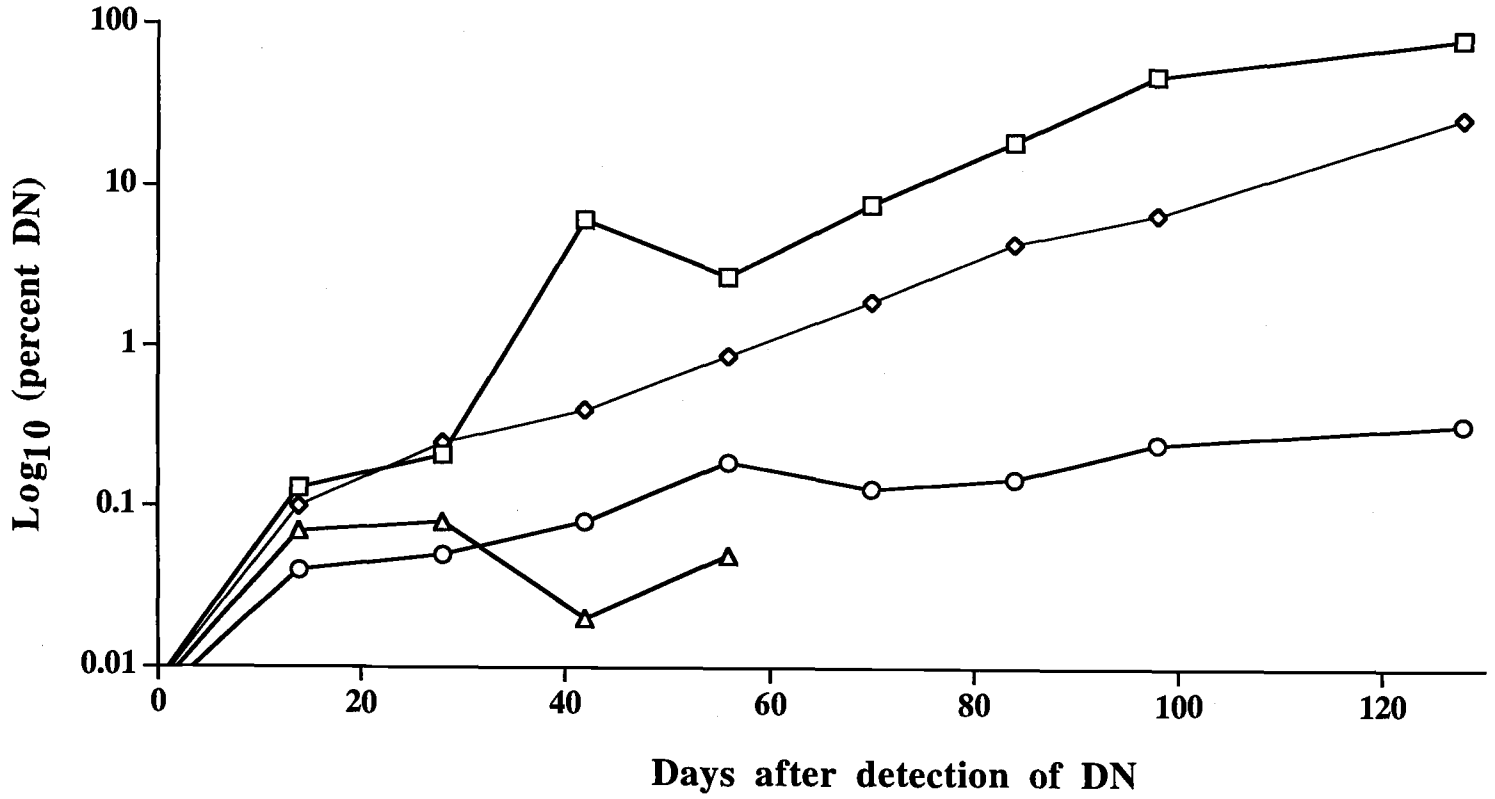
100
10
1
0.1
0.01
Log10 (percent DN)
0
20
40
60
80
100
120
Days after detection of DN

FIGURE 3.2

Table 3.4 Comparison between the intensity levels of disseminated neoplasia (DN) in DN positive soft shell clams at the Hatfield Marine Science Center. Intensity of DN was determined by immunocytochemical analysis of hemolymph samples taken at 2, 4, 6, 8, 10, 12, 14, and 18 weeks following injection with $10^{6.8}$ drawn from clams with > 95% DN cells in circulation (DN). Significant differences at the 95% confidence level are denoted by *.

Comparison	Mean difference	Critical difference	P-value
Alsea to Yaquina	1.185	0.643	0.0129*
Alsea to Barnstable Harbor	3.264	0.806	<0.0001*
Alsea to Little Buttermilk Bay	1.587	1.234	0.0009*
Yaquina to Barnstable Harbor	2.079	0.767	<0.0001*
Yaquina to Little Buttermilk Bay	0.401	1.209	0.0112*
Barnstable Harbor to Little Buttermilk Bay	1.677	1.303	0.6997

Table 3.5 Comparison of survival of soft shell clams in negative control seawater (SW) injected and DN cell injected groups. Alsea Bay and Yaquina Bay are west coast estuaries in Oregon, and Barnstable Harbor and Little Buttermilk Bay are east coast estuaries in Massachusetts. The percentage of clams that survived the 18 week course of the study at the Hatfield Marine Science Center are listed for each bay by treatment group. Listed below are the chi-square values from the logrank test, the degrees of freedom for the test (Df), and the resulting p-value. Significant differences at the 95% confidence level are denoted by *.

	% Mortality				
Bay	**SW Injected**	**DN Cell injected**	**Chi-square**	**Df**	**P-value**
Alsea	9	12	2.028	1	0.1544
Yaquina	31	19	0.185	1	0.6672
Barnstable Harbor	60	69	0.314	1	0.5752
Little Buttermilk	80	61	4.842	1	0.0278*

Figure 3.3 Cumulative mortality of soft shell clams. The cumulative mortality of clams is shown for A. the negative control seawater injected group for each bay (Alsea: black bar, Yaquina: striped bar, Barnstable Harbor: stippled bar, and Little Buttermilk Bay: white bar) and B. the DN cell injected groups for each bay over the course of the study at the Hatfield Marine Science Center Newport, Oregon.

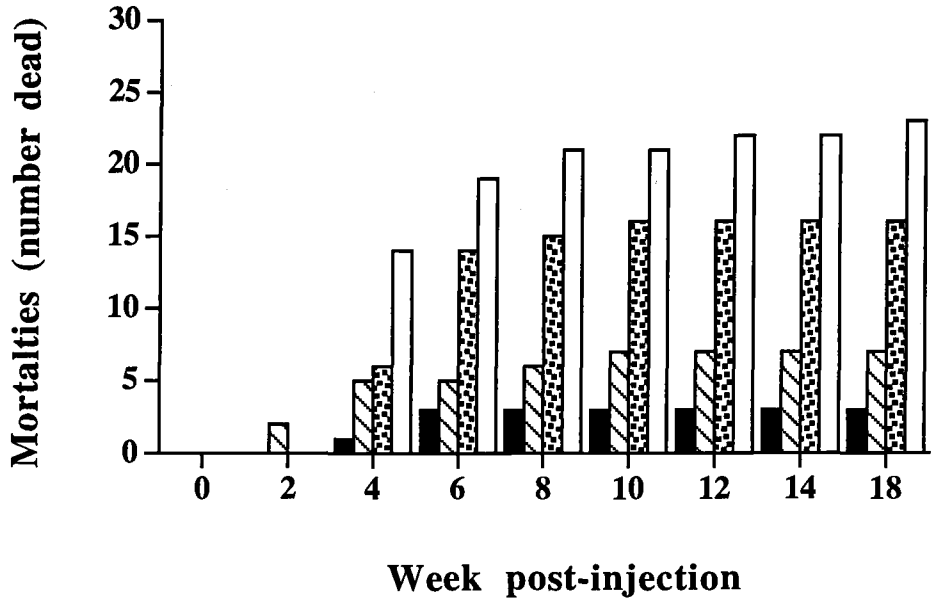

FIGURE 3.3A

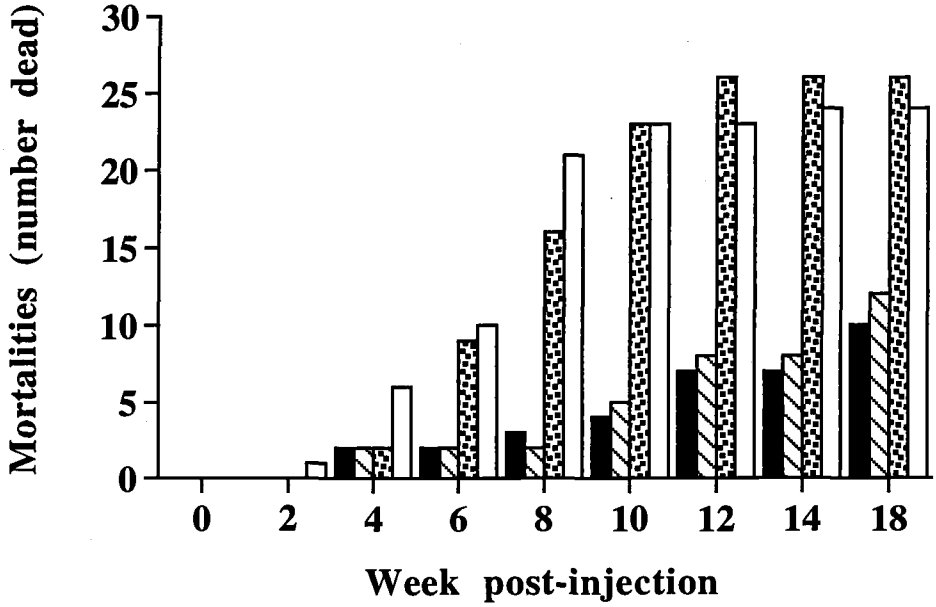

Figure 3.3B

mortalities by bay over time for the negative control groups and the DN cell injected groups respectively.

Effect of repeated sampling:

There was no significant effect of repeated sampling as compared to one-time sampling for DN intensity (F-statistic = 1.84, p-value = 0.1758). Survival was significantly higher in the groups of animals that were sampled only once (see Table 3.6 for number of survivors per group), except for the Yaquina Bay animals, which had unexplained elevated losses in the negative control group that was sampled only once (see Table 3.7 for p-values).

Dose Response:

Rate of Development of DN

Over the 12 month course of the study, none of the negative control animals that had been injected with sterile seawater in the dose response experiment developed DN. As shown in Figure 3.4, clams in all four of the treatment groups that were injected with DN cells developed disseminated neoplasia. The earliest onset was in the treatment group that received the highest dose of DN cells ($10^{6.3}$ cells/ animal). In this group, there were 6/26 surviving individuals with detectable levels of DN at one month post-inoculation (PI) (see Table 3.8), and all surviving animals (23/23) had developed the disease by five months PI. The high dose treatment group had a significantly earlier onset of DN when compared to all of the other treated groups (see Table 3.9 for p-values). The treatment group that was injected with $10^{5.6}$ cells/clam had DN positive clams (2/26) in month 2 PI. The remaining treatment groups that were injected with $10^{5.1}$ cells/clam and $10^{4.6}$ cells/clam had DN positive clams, 3/27 and 1/26 respectively, at month 3 PI. At the termination of the study at 12 months PI, all of the surviving clams in each of the four DN cell injected groups had developed DN. There were no treatment groups in which surviving clams did not develop DN, except for the negative control group; therefore the treatments that were used in this study all contained at least one infectious dose.

Table 3.6 Number and percent of mortalities for each bay grouped by repeated or one time sampling. One group was repeatedly sampled at weeks 2, 4, 6, 8, 10, 12, 14, and 18 weeks post-injection. The other group was sampled only at 18 weeks post-injection. The number and percent of mortalities at the final time point out of the initial number of clams in the groups are listed by bay of origin in the table below.

Bay	Repeated Sampling		One time Sampling	
Alsea	13/60	22%	1/30	3%
Yaquina	19/60	32%	8/30	27%
Barnstable Harbor	45/60	75%	14/30	47%
Little Buttermilk	54/60	90	14/30	47%

Table 3.7 Comparison of repeated vs one-time sampling effects on survival of at the Hatfield Marine Science Center. Listed below are the chi-square values from the logrank test, the degrees of freedom (Df) for the test, and the resulting p-value. Significant differences at the 95% confidence level are denoted by *.

Bay	Chi-square	Df	P-value
Alsea	4.308	1	0.0379*
Yaquina	0.309	1	0.5781
Barnstable Harbor	4.906	1	0.0268*
Little Buttermilk	14.449	1	0.0001*

Figure 3.4 Prevalence of disseminated neoplasia. The cumulative percent of originally injected soft shell clams that developed disseminated neoplasia in the DN cell treated groups are shown over the course of the 12 month study. No neoplasia was detected in the negative control group, which was injected with sterile seawater. The treatment groups are listed by number of DN cells injected at the initiation of the study. The lowest dose group received $10^{4.6}$ cells: stippled bar, the remaining groups received $10^{5.1}$: striped bar, $10^{5.6}$: black bar, and $10^{6.3}$: stippled bar ,DN cells per clam.

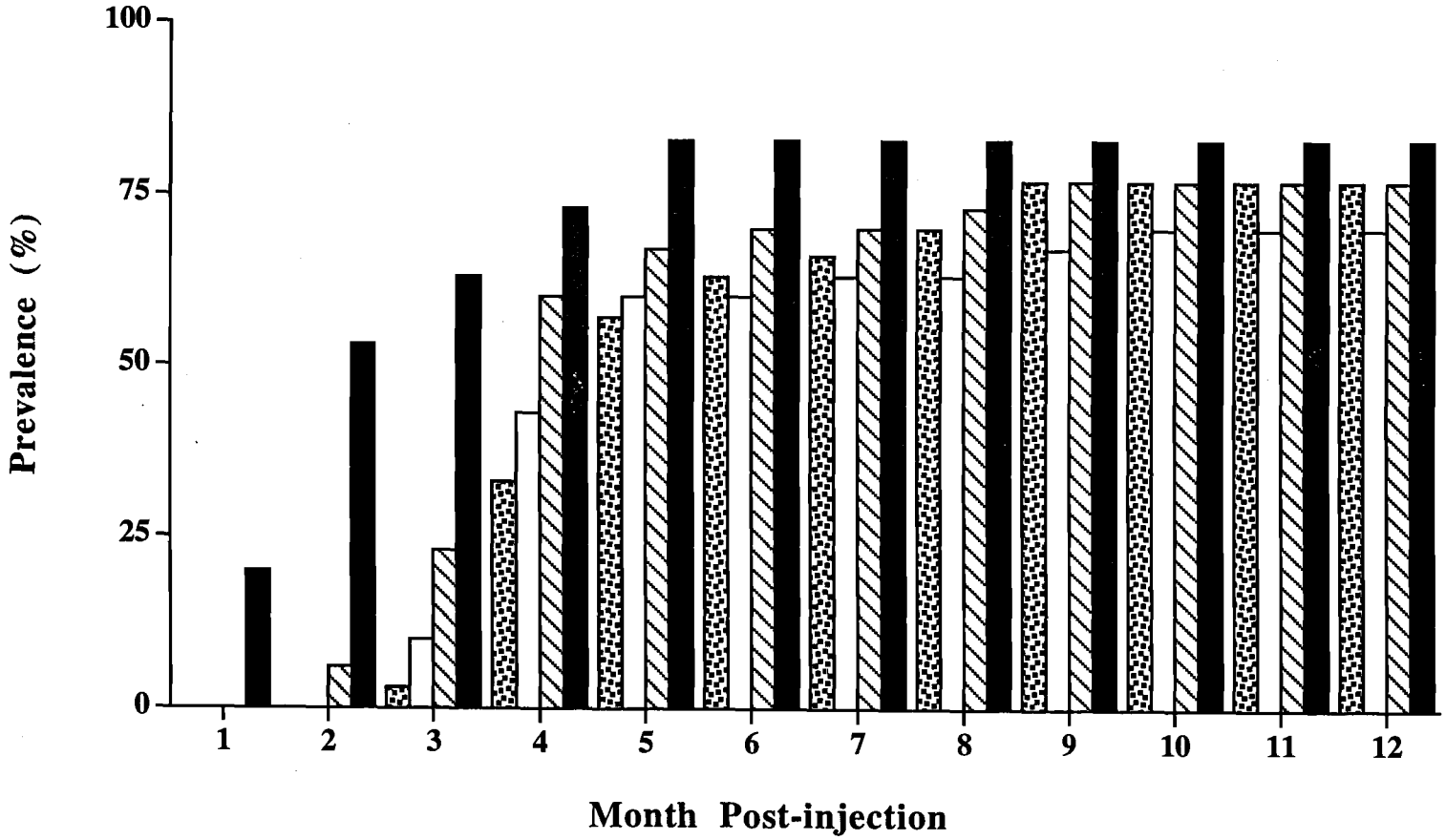
100
75
50
25
0
Prevalence (%)
1
2
3
4
5
6
7
8
9
10
11
12
Month Post-injection

FIGURE 3.4

Table 3.8 Prevalence of DN in surviving clams in the dose response study. Soft shell clams were collected from Alsea Bay, Oregon. In the negative control group, sterile seawater was injected into the adductor muscle sinus.The other groups of clams were each injected with 0.1 ml of undiluted hemolymph which was collected from clams with 99% DN cells in circulation, or a dilution of this hemolymph in sterile seawater. The number of cells that each of the clams in the treatment was injected with are listed under treatment. Monthly hemocyte samples were analyzed for the presence of DN using immunocytochemistry. The number of DN positive clams per the number of surviving clams is provided.

	Month post-injection												
Treatment	**0**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Seawater	0/30	0/30	0/29	0/28	0/28	0/27	0/27	0/27	0/26	0/23	0/22	0/17	0/16
$10^{4.6}$	0/30	0/27	0/27	1/26	10/25	16/24	18/24	19/24	18/22	19/21	14/16	9/11	9/10
$10^{5.1}$	0/30	0/29	0/29	3/27	13/22	16/19	16/19	17/19	17/19	17/18	15/15	13/13	10/10
$10^{5.6}$	0/30	0/26	2/26	7/26	18/26	18/23	19/22	19/22	20/22	18/19	17/18	16/17	8/9
$10^{6.3}$	0/30	6/26	16/26	18/25	21/24	23/23	21/21	19/19	16/16	14/14	8/8	6/6	4/4

Table 3.9 Comparison of time of onset of disseminated neoplasia in soft shell clams from four DN cell treated groups. The groups are listed by the number of cell that were injected into each clam in the group. Listed below are the two groups compared, the chi-square value from the logrank test, the degrees of freedom for the test (DF), and the resulting p-value. Significant differences at the 95% confidence level are denoted by *

Comparison	Chi-square	P-value
$10^{4.6}$ to $10^{5.1}$	1.817	0.1776
$10^{4.6}$ to $10^{5.6}$	2.369	0.1238
$10^{4.6}$ to $10^{6.3}$	26.685	<0.0001*
$10^{5.1}$ to $10^{5.6}$	0.264	0.6076
$10^{5.1}$ to $10^{6.3}$	20.294	<0.0001*
$10^{5.6}$ to $10^{6.3}$	13.744	0.0002*

Intensity of DN in positive clams

The average intensity for each of the treated groups is illustrated in Figure 3.5. For each DN cell treatment group there was an increase in the intensity of DN over time once neoplastic cells were detected in the animal. When this data was analyzed using a generalized linear model (GLM), it was found that the number of cells that the clam was injected with had a significant effect on the intensity of disease that developed over the course of the experiment (F-statistic = 7.79, p-value <0.0001). This finding was further analyzed with comparisons among treatment groups using Fisher's PLSD, and it was shown that the intensity of DN in the group of clams treated with the lowest dose ($10^{4.6}$ cells) was significantly different than that of the other groups. Additionally, the clams in the group that received $10^{5.1}$ cells had a significantly different intensity of DN than that of the group receiving the highest dose in the study ($10^{6.3}$ cells per clam). Differences between other treatment groups were not significant (see Table 3.10 for p-values).

Survival:

Figure 3.6 illustrates the pattern of increasing mortality in all of the groups through the course of the 12 month study. At the conclusion of the experiment, 53% of the clams in the negative control group had survived, with the mean time to death of 9.5 months (see Table 3.11). In the treatment group in which the clams received the lowest dose of cells ($10^{4.6}$ cells) there was 33% survival, with the mean time to death equal to 8.3 months. There was 30% survival in the group of clams that were injected with $10^{5.1}$ cells, and the mean time to death was 7.7 months. The treatment group that received $10^{5.6}$ cells had 33% survival, with the mean time to death at 8.4 months. The treatment group that was injected with the highest dose of cells ($10^{6.3}$ cells) had the highest levels of mortality with 13% surviving, and the mean time to death at 7.1 months. This data was analyzed by Kaplan-Meier survivorship analysis, followed by a logrank test; Table 3.13 summarizes the comparisons between the survival times of the treatment groups. The highest dose treatment group ($10^{6.3}$ cells) showed significantly higher mortality levels than the negative

Figure 3.5 Intensity of disseminated neoplasia in DN positive soft shell clams in the dose response study. The log of the average intensity for DN positive clams from each treatment group ($10^{4.6}$: square, $10^{5.1}$: diamond, $10^{5.6}$: circle, and $10^{6.3}$: triangle DN cells per clam) over the 12 month course of the study.

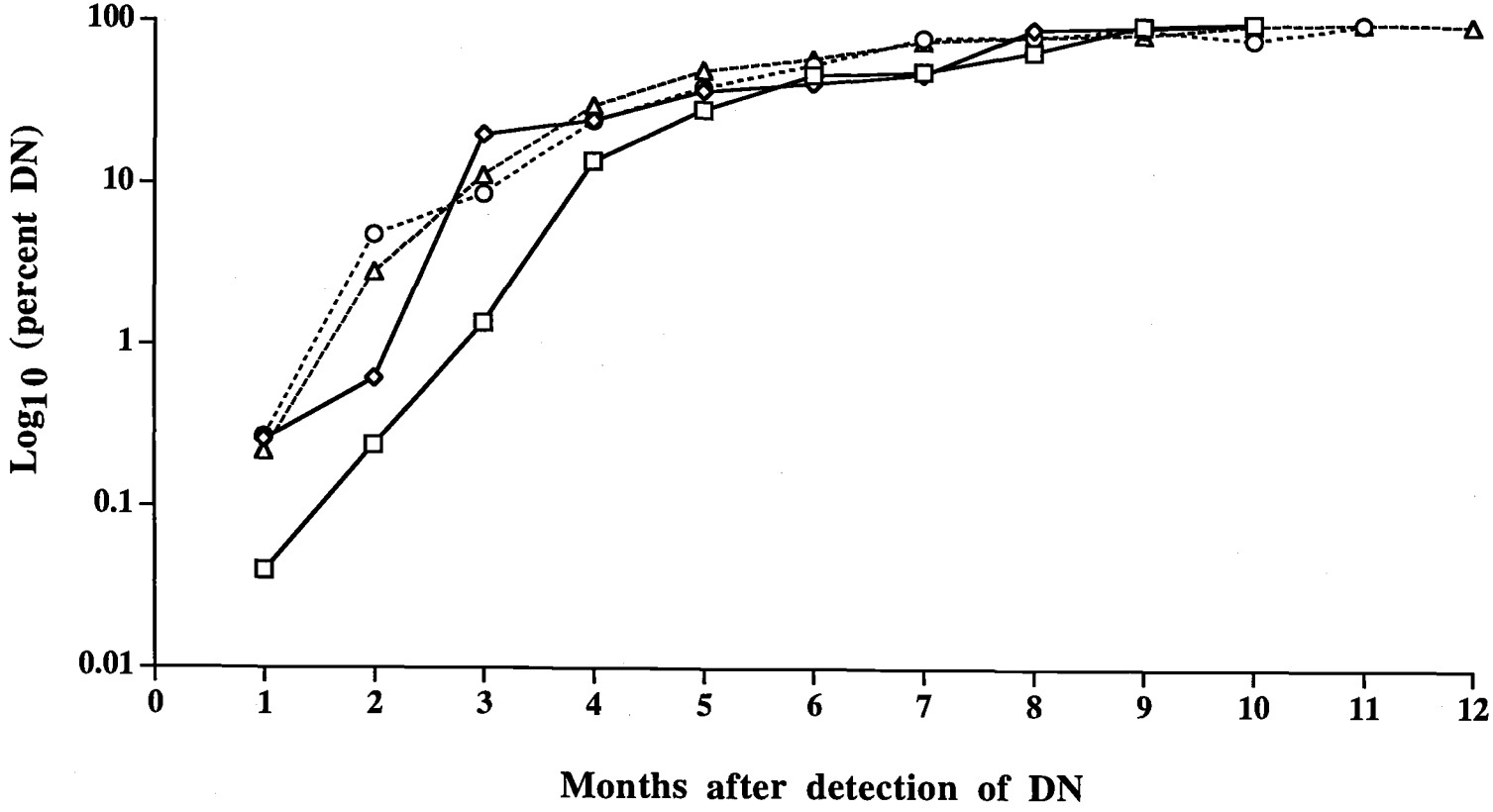
100
10
1
0.1
0.01
Log10 (percent DN)
0
1
2
3
4
5
6
7
8
9
10
11
12
Months after detection of DN

FIGURE 3.5

Table 3.10 Comparison between the intensity levels of disseminated neoplasia in DN positive soft shell clams in the dose response study. Hemolymph samples taken monthly following injection with the number of cells listed below. Results from analysis by Fisher's PLSD test for differences in DN intensity in between dose levels of DN cells are listed below. Significant differences at the 95% confidence level are denoted by *

Comparison	Mean Difference	Critical Difference	P-value
$10^{4.6}$ to $10^{5.1}$	-1.413	1.081	0.0105*
$10^{4.6}$ to $10^{5.6}$	-2.024	1.027	0.0001*
$10^{4.6}$ to $10^{6.3}$	-2.461	1.012	<0.0001*
$10^{5.1}$ to $10^{5.6}$	-0.611	1.038	0.2483
$10^{5.1}$ to $10^{6.3}$	-1.048	1.023	0.0447*
$10^{5.6}$ to $10^{6.3}$	-0.437	0.966	0.3748

Figure 3.6 Cumulative mortality of soft shell clams. The number of dead clams in each treatment group ($10^{4.6}$: stippled bar, $10^{5.1}$: white bar, $10^{5.6}$: striped bar, and $10^{6.3}$: black bar, DN cells per clam) as well as the seawater injected negative control group (dashed bar) over the course of the study are shown.

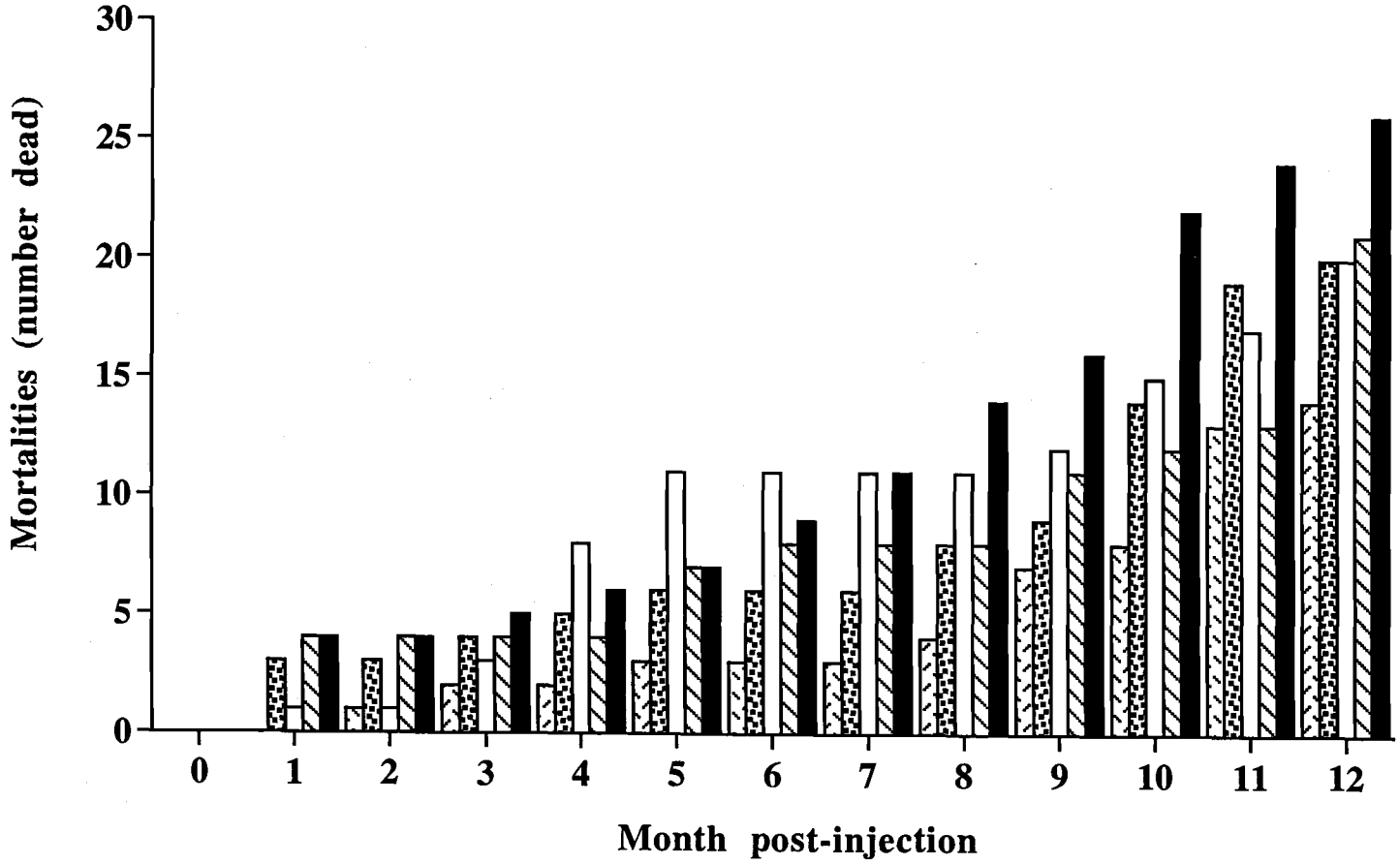
Mortalities (number dead)
30
25
20
15
10
5
0
0
1
2
3
4
5
6
7
8
9
10
11
12
Month post-injection

FIGURE 3.6

Table 3.11 Mortalities of soft shell clams in the negative control and DN cell treated groups in the dose response study. The negative control group was injected with sterile seawater and the DN cell injected groups were injected with the number of cell listed below by treatment group. The mean time to death for each group is listed, in addition to the total percent mortality at the termination of the 12 month study.

Treatment Group	Mean time to death	Total Mortality
Seawater	9.5 months	47%
$10^{4.6}$	8.3 months	67%
$10^{5.1}$	7.7 months	70%
$10^{5.6}$	8.4 months	67%
$10^{6.3}$	7.1 months	87%

Table 3.12 Comparison of survival of soft shell clams in negative control seawater injected (seawater) and the four DN cell injected groups. The number of DN cells that each clam in the treatment group was injected with is used to denote that treatment group. Listed below are the chi-square values from the logrank test, the degrees of freedom (DF) for the test, and the resulting p-value. Significant differences at the 95% confidence level are denoted by *

Comparison	Chi-square	Df	P-value
Seawater to $10^{4.6}$	2.682	1	0.1015
Seawater to $10^{5.1}$	3.085	1	0.0790
Seawater to $10^{5.6}$	2.436	1	0.1186
Seawater to $10^{6.3}$	13.181	1	0.0003*
$10^{4.6}$ to $10^{5.1}$	0.024	1	0.8766
$10^{4.6}$ to $10^{5.6}$	0.025	1	0.8748
$10^{4.6}$ to $10^{6.3}$	4.316	1	0.0377*
$10^{5.1}$ to $10^{5.6}$	0.068	1	0.7950
$10^{5.1}$ to $10^{6.3}$	2.740	1	0.0979
$10^{5.6}$ to $10^{6.3}$	4.698	1	0.0302*

control group, the lowest dose treatment group ($10^{4.6}$ cells), and the group that received $10^{5.6}$ cells (see Table 3.12 for chi square values and p-values). The remaining comparisons between groups showed no significant differences.

DISCUSSION

It has been demonstrated at both experimental sites (HMSC and WHOI) that west coast soft shell clams, which were originally introduced from the east coast, are susceptible to disseminated neoplasia by transplantation of neoplastic cells injected into the circulatory system.

Based on the results of the studies presented here, the absence of disseminated neoplasia in west coast, specifically Oregon, populations of soft shell clams (Mix 1986, House and Reno unpublished observations) does not appear to be due to disease resistance in these animals, but may be due to the absence of the causative agent of DN. It is possible that the soft shell clams that were introduced to the west coast at the turn of the century (Stearns 1881) were drawn from a population of clams that were not affected by disseminated neoplasia, and/or the agent of DN was not present at that time. The first published record of DN in *M. arenaria* on the east coast was made by Yevich and Barszcz in 1976. In 1977, these authors reported finding 'hematopoietic neoplasms' in soft shell clams collected at Long Cove, Maine in samples taken in 1972. Other samples collected in 1975 from the same site, and 1974 and 1975 samples from nearby Harpswell Neck also contained animals with these neoplasms. There is an indication that the condition was observed in archived histological materials from soft shell clams collected at Newburyport, Massachusetts as early as twenty years prior to the Yevich and Barzscz publication (personal communication, Stuart Sherburne, Department of Marine Resources, Boothbay Harbor, Maine). In a field survey that took place from 1976-1978 (Appeldoorn et al. 1984), DN was found to be most prevalent in southern New England, with prevalence north and south of this region decreasing to zero. The present day west coast *M. arenaria* may have originated from stocks of soft shell clams that were naive to disseminated neoplasia.

At the Hatfield Marine Science Center in Newport, Oregon, none of the west coast (Alsea Bay and Yaquina Bay) clams in the negative control groups, which were injected with sterile seawater, developed DN. Some of the clams from the east

coast bays included in the study had subclincal levels of DN before initiation of the experiment. Disseminated neoplasia in these animals was undetectable at the beginning of the study. Hemolymph samples were initially evaluated using phase-contrast microscopy. When the samples were re-examined using the more sensitive immunocytochemistry technique, individuals that started the study with levels of DN below detection by the previous method were removed from consideration. Two of the clams in each of the negative control groups from Barnstable Harbor and Little Buttermilk Bay developed DN in the course of the study. In the Barnstable Harbor group that was injected with DN cells, there was a significant elevation in the development of DN compared to that of the negative control group, therefore the DN cell injection effectively transmitted the disease. In the Little Buttermilk Bay animals, there was no significant difference in occurrence of DN between the negative control group and the DN cell treated group. In this case, the injection of DN cells does not appear to have induced DN.

Over the course of the study, the development of disseminated neoplasia in clams that were injected with DN cells was not significantly different between animals from the two coasts. However, the comparison of the time of onset of DN among DN injected animals from different bays showed that the occurrence of neoplasia in the Little Buttermilk Bay clams was lower than in those from the other bays. The reason for the lack of development of neoplasia in the Little Buttermilk Bay clams after being injected with a dose of DN cells sufficient to induce DN in clams from other bays is not clear. Other researchers have transplanted DN successfully by injection in soft shell clams (Appeldoorn et al. 1984, Farley et al. 1986), mussels (Elston et al. 1988b) and cockles (Twomey and Mulcahy, 1988), as was seen in the animals from the other locations in this study. One exception in the available literature is a brief introductory reference by Reinisch et al. (1983) stating that the condition "cannot be transferred to normal clams even at high tumor cell doses". Further information was not available. Because the objective of the present study was concerned with the susceptibility of west coast soft shell clams to DN, no further efforts were pursued involving the Little Buttermilk Bay clams.

In the DN cell injected groups, the intensity of neoplasia increased over time for Alsea Bay, Yaquina Bay and Barnstable Harbor clams, with the increase in the latter group being statistically lower than those of the first two groups. The Little Buttermilk Bay clams did not show an increase in intensity of DN. In the group of Alsea Bay clams, levels of 90% DN cells in circulation were reached, and levels of 69% were reached in the Yaquina Bay animals. The progression of neoplasia in

these groups of clams is similar to that seen in studies of DN in *M. arenaria* reported by Cooper et al. (1982a) in Rhode Island and Farley et al. (1986) in Maryland, as well as in *M. edulis* by Elston et al. (1988a) in Washington. The Barnstable Harbor and Little Buttermilk Bay clams DN intensity levels remained less than 1% DN cells in circulation through the 18 week course of the study. The results presented here indicate that the animals collected from different bays in this study responded differently to injection with DN cells, with both of the west coast bay clams developing higher intensity levels of DN than the animals collected from the east coast bays used. Future studies expanding the number of locations clams are collected from would be important in determining if this pattern extends beyond the populations sampled in this study. Variables that could be considered may include increasing the number of animals in the treatment groups and increasing the duration of the study in order to determine if low intensity of DN is maintained over a more extended length of time.

Survival over the course of the first experiment was not correlated with exposure to DN cells. There were differences between the east and west coast animals, although the negative control groups for each bay experienced similar losses, and in some cases more severe losses than the DN exposed groups. The cause of mortality was not identified, but it did not appear to be related to the DN cell injection. Factors that may have contributed to the increased mortality of the east coast clams compared to the west coast clams are possibly the handling stress of shipping, or exposure to unidentified pathogens or environmental stresses at their original location. In three of the four bays, groups of clams that were repeatedly sampled had an increased mortality rate compared to the groups that were sampled only at the termination of the study, indicating that increased handling may affect survival adversely.

The second experiment, which investigated the effect of dose of neoplastic cells injected showed that prevalence, onset, development of neoplasia and survival were directly correlated with dose. DN occurred in all of the groups of clams that were injected with DN cells. The lowest dose of $10^{4.6}$ cells per animal contained sufficient numbers of DN cells to cause the condition to occur in the recipient clams. Because all of the treatment groups in this study exceeded a dose of neoplastic cells that failed to cause development of the disease, it was not possible to determine the minimum effective dose.

The onset of disseminated neoplasia occurred most rapidly in the group of clams that received the highest dose of $10^{6.3}$ DN cells per clam. Disseminated neoplasia was detectable in the sample taken at one month post-injection. The clams in the

remaining groups developed neoplasia in the months to follow, with $10^{5.6}$ cells inducing disease at two months, and the lower doses ($10^{5.1}$ and $10^{4.6}$ cells) at three months post-inoculation. The intensity of the neoplasia that developed in the recipient clams showed that once the animal had detectable levels of DN, there were significant reduction in the level of disease in the groups of animals that received the lowest dose of neoplastic cells and those that received higher numbers of cells. The clams that were injected with a lower dose of neoplastic cells had a slower progression of disease in the early stage of development of DN (see Figure 3.5), but did develop elevated levels of DN as time progressed.

In a histological examination of mussels that showed a decrease in the percentage of neoplastic cells in circulation as determined by prior examination of hemolymph samples, Elston et al. (1988a) observed that the neoplastic cells were apparently entrapped in an extracellular matrix that appeared to be secreted by normal hemocytes. Elston et al. (1992) later noted that the process of remission involved destruction of neoplastic cells in addition to the formation of this matrix. Cooper et al. (1982a) reported that remission of DN occurred in soft shell clams when the disease was at a low severity level, although no histological examination of the clams in remission was reported. No remission was observed in either of the present studies, but it is possible that the immune mechanisms that Elston et al. (1988a) reported in mussels played a role in how rapidly the DN developed.

Clams that were injected with high doses of DN cells developed the disease more rapidly than those that received lower doses. The latter may have initially been more successful in slowing the progression of the disease, possibly by using immune mechanisms, while higher dose of neoplastic cells may have overwhelmed the animals ability to resist development of DN. Histological examination over the time course of disease progression would be necessary to further explore if these mechanisms are in fact involved.

In the dose response study, the mortality rates in the high dose group were significantly higher than the lower dose and the unexposed negative control groups. The development of DN in the high dose group is clearly related to the increased mortality. Cooper et al. (1982a), Farley et al. (1986) and Elston et al. (1988a) have reported that DN is a lethal condition. The extended time course of the dose response experiment allowed the differences in survival to be detected.

Finally, the results of the WHOI portion of the horizontal transmission study suggested that water-borne transmission occurred fairly quickly and effectively. West coast animals that were not injected with DN cells developed DN in contrast to

those at the west coast facility (Hatfield Marine Science Center). Because the water that was supplied to the WHOI animals was not treated to reduce potential contamination with waterborne agents capable of inducing DN, the source of infection could have been from the local bay where the disseminated neoplasia is enzootic. Elston et al. (1988b) and Kent et al. (1991), suggested that the development of DN in negative control mussels was the result of the water supply being contaminated with DN infected mussels. Further investigation of water-borne transmission will be discussed in an accompanying work.

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CHAPTER 4

WATER-BORNE TRANSMISSION OF DISSEMINATED NEOPLASIA IN SOFT SHELL CLAMS

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ABSTRACT

Disseminated neoplasia (DN) is a progressive disease that occurs in the circulatory system of bivalves. Transplantation of DN to healthy individuals has been achieved in the laboratory by injection of hemolymph containing a high number of DN cells from highly affected animals. Experiments were performed to establish water-borne transmission, and to determine if there was a dose-dependent response when clams were exposed to known numbers of neoplastic cells. Soft shell clams, *Mya arenaria*, were exposed to a suspension of algae and DN cells at two treatment levels, 10^6 and 10^7 DN cells. One negative control group was exposed to algae only; a second negative control group was exposed to algae and 10^7 normal clam hemocytes. No negative control clams developed DN at any time in these experiments. Over the next 9 months, individuals were monitored for the development of DN in monthly hemolymph samples. After 5 months, there were low levels of DN found in the group fed the high dose. After 6 months, the low dose group was also developing DN. A second experiment was designed to determine how effectively one diseased clam could pass DN to healthy clams. Nine groups of 5 healthy animals were placed into isolated tanks supplied with sand-filtered UV-irradiated sea water with 1 DN positive animal. Transmission of DN to healthy animals was detected 8 months after initiation of the experiment, with seven of the ten groups showing transmission over the 14-month course of the study.

INTRODUCTION

Disseminated neoplasia (DN) is a progressive and lethal disease that has been described in at least 15 species of bivalves (Peters 1988, Elston et al. 1992). The condition was initially described in the native oyster, *Ostreola conchaphila* (formerly *Ostrea lurida)*, in Yaquina Bay, Oregon in 1969 (Jones and Sparks). The neoplastic cells, found in the circulatory system, are larger than normal hemocytes, and have a relatively enlarged nucleus (Farley 1969). DN can be chronic, with low numbers of DN cells in circulation, or develop into a more severe disease with high numbers of neoplastic cells flooding the animal's circulatory system, leading to death. In the laboratory, healthy animals injected with neoplastic cells from highly affected individuals of the same species will develop the disease (Elston et al. 1992). The agent appears to be species-specific; in a transmission study completed by Kent et al. (1991), it was shown that injection with a homogenate prepared from DN cells from mussels (*Mytilus edulis*) caused DN to occur in mussels, but not in soft shell clams (*Mya arenaria*), flat oysters (*Ostrea edulis*), or the native oyster treated with the same material. Remission has been observed in laboratory studies in soft shell clams (Cooper et al. 1982), and mussels (Elston et al. 1988) and on soft shell clam flats (Brousseau and Baglivo, 1991).

Water-borne transmission studies with DN have been completed, but the development of neoplasia in negative control animals has made studies of this process in the laboratory difficult. Results of experiments by Brown (1980) and Appeldoorn et al. (1984) indicated that DN could be passed to healthy soft shell clams via the seawater effluent from tanks containing diseased clams within months, and also from ambient seawater drawn from Narragansett Bay, RI within 6 months. Transmission was not observed in further experiments in which healthy clams were fed minced tissue from clams with high levels of DN. In the first of the two trials, the authors fed naive clams the tissue from diseased clams daily for a one week period, and monitored the recipient animals for DN over a 3 month period. A second trial involved feeding the healthy clams once a week for 3 months. Additionally, in studies with neoplastic and non-neoplastic clams maintained in the same tank, horizontal transmission of DN to the naive clams was not detected in the three month study period.

Other evidence for water-borne transmission has been reported from laboratory work at other facilities. In a 231 day cohabitation experiment, Elston et al. (1988) found that in addition to the healthy mussels housed with DN positive mussels developing DN, negative control animals exposed only to ambient seawater became positive for DN, most likely as a result of DN affected animals in the water supply for the laboratory (Sequim Bay, WA). On other transmission studies at the same facility, Kent et al. (1991) reported that a negative control mussel developed DN over the 152 day course of the study. This situation is similar to that which occurred at the Woods Hole Oceanographic Institute (WHOI) facility in Massachusetts (House, Reno and Leavitt unpublished observation). In that case, *M. arenaria* from Oregon in the negative control group had not been exposed to DN by injection, and developed DN, probably from the ambient seawater. Clams from the same source concurrently held at the Hatfield Marine Science Center in Newport did not develop DN. In 1986, Farley et al. reported the occurrence of DN in a Chesapeake Bay population of soft shell clams, *M. arenaria.* The clams in this area had previously been found DN-free over more than a decade of sampling. The appearance of DN in this population was thought to have been due to the exposure of the local population of clams to newly introduced, potentially DN positive, *M. arenaria* from the New England, where the disease is enzootic. The evidence for water-borne transmission of DN is strong, but the presence of DN positive animals in laboratory water supplies has hindered studies in the laboratory.

In Yaquina Bay, since the initial finding of DN in the native oyster (Jones and Sparks 1969), mussels (Farley 1969) and *Macoma* sp. (Farley 1976) have also been found to have DN, but the condition has not been found in *M. arenaria* (0/>250) (House and Reno, unpublished observations). Furthermore, DN has been undetected in other populations of soft shell clams from Oregon estuaries that have been examined, namely Coos Bay (0/>360) (Mix 1986), Nestucca Bay (0/>200), and Alsea Bay clams (0/>1300) (House and Reno, unpublished observations). Based on this information, there appears to be no contaminating source of DN for *M. arenaria* in the water supply for Hatfield Marine Science Center (Yaquina Bay), the site of the current study. The aim of these experiments presented here was to determine if DN could be transmitted to susceptible west coast *M. arenaria* by a controlled water-borne exposure, and gather information on dose related response and cohabitation.

METHODS

Water-Borne Transmission:

Experimental Animals

Soft-shell clams (valve length 27-52 mm, average = 39 mm, SD = 5 mm) were collected from Alsea Bay, Oregon. Each animal was examined for the presence of neoplastic cells (DN) in the following manner. Hemolymph was drawn from the anterior adductor muscle sinus, and placed on a 0.5% poly-L-lysine coated coverslip. The cells were allowed to adhere for 30 minutes at room temperature, then the sample was fixed in 1% glutaraldehyde and 4% formaldehyde in sterile seawater (Smolowitz and Reinisch 1986). The samples were examined for the presence of DN using phase contrast microscopy. Samples were stored at 4°C and later re-examined as described below, using an immunocytochemical assay with a monoclonal antibody recognizing an epitope specific to DN cells in *M. arenaria* (Smolowitz and Reinisch 1986, generously provided by Dr. Carol Reinisch). No DN positive animals were found.

Ten-10 liter containers filled halfway with clean quartz sand, and were independently supplied with 0.2 L/min. pathogen-free seawater at ambient temperature (8°C-16°C). Barriers were placed between tanks to insure that no water splashed from tank to tank. The clams were fed 1 liter of a 50/50 mixture of *Isochrysis galabanea* and *Cheatoserous calcitrans* ($7x10^5$ - $1.2x10^6$ cells per ml) on a daily basis. The water flow was turned off for 3 hours following addition of algae to allow time for the animals to filter the algae. All effluents from the system were treated by chlorination before being released.

Experimental Design

The animals were randomly divided into the five treatment groups listed below.

Negative control 1:	no clam cells
Negative control 2:	$10^{6.9}$ normal clam hemocytes
High dose DN cells:	$10^{7.0}$ DN cells
Low dose DN cells:	$10^{6.0}$ DN cells
Positive control:	injected $10^{5.3}$ DN cells into adductor muscle sinus

The first four groups each consisted of a total of thirty clams (divided into three containers of ten clams each). The fifth group consisted of 15 animals held in one container. Each individual was marked with a number to allow it to be monitored throughout the course of the experiment.

The following treatment protocol was repeated daily over 5 consecutive days.

A 50/50 algal mixture of *Isochrysis galabanea* and *Cheatoserous calcitrans* ($1x10^6$ cells per ml) was mixed with an equal volume of sand-filtered UV-treated pathogen free seawater and divided into four 3.1L portions. The following additions were made to each of the treatment batches.

Negative control mixture 1: No additions were made.

Negative control mixture 2: A 35 ml aliquot of hemolymph was collected from a pool of 45 Alsea Bay animals with no evidence of DN. The hemolymph was kept on ice until added to the 3.1 L algae mixture. A sample was removed from the hemolymph, and the number of cells was determined by a count on a hemocytometer.

High dose and low dose DN mixture: Four ml of hemolymph was collected from a pool of 15 clams with 95-99% DN cells in circulation. The hemolymph was kept on ice, as above. A sample was removed from the pool, and the number of cells was determined by a count on a hemocytometer. A 3.5 ml aliquot of this pool was added to 3.1 L of the algae mixture for the high dose treatment, and a 0.35 ml aliquot was added to 3.1 L of the algae mixture for the low dose treatment.

Each treatment mixture was allocated into thirty 100 ml portions in 250 ml containers. One clam was placed into each container, exposing it to the appropriate treatment. These static containers were held within a large water bath of flowing ambient seawater to maintain the ambient temperature (11-12°C) during the 5 hour feeding periods. This amount of time was sufficient for the animals to clear the algae from the water, although there were day to day differences in the feeding habits of individuals, as determined by the clarity of the remaining seawater following feeding. Over the 5 day treatment period, the clams in the normal hemocyte group were exposed to a total of $10^{6.9}$ cells, those in the low and high DN cell groups were exposed to a total of $10^{6.0}$ and $10^{7.0}$ respectively.

Positive control group: During the five days of feeding treatments, these clams were maintained on the normal feeding regime. On the final treatment day, an additional 1.5 ml of hemolymph was collected from the DN positive animals. The

15 positive control animals were each injected with 5.3 x10^6 DN cells in 0.1 ml of hemolymph, into the adductor muscle sinus, and returned to the 10 L container.

Sampling

Tanks were monitored for mortalities over the next 9 months. Hemolymph samples were drawn from each individual monthly. The sample was placed onto a coverslip and fixed as previously described. Immunocytochemistry, detailed below, was used to stain the samples, which were then examined for the presence of DN cells, and their proportion related to normal hemocytes. Prevalence was defined as the percent of DN positive clams in a group, and intensity as the percentage of DN cells in relation to the total number of cells in the sample.

Immunocytochemistry

Analysis for the presence and intensity of DN using immunocytochemistry was executed as follows. The samples were washed 3 times for 5 minutes in PBS to remove fixative, then nonspecific binding sites were blocked by incubating with 1:500 normal goat serum (Gibco, Grand Island, NY) for 20 minutes at room temperature. Samples were then incubated with an anti-neoplastic cell monoclonal antibody (Smolowitz and Reinisch 1986) for at least 1 hour at room temperature. Unbound antibody was washed away with 3 five minute PBS washes. The bound antibody was detected by using the Vectastain ABC-AP Kit (Vector Laboratories, Burlington, CA), according to the instructions. Briefly, a biotin-labeled anti-murine antibody, followed by an avidin-biotin-peroxidase complex were incubated with the sample, and unbound material was removed with PBS washes, as described above. The bound complex was exposed to the chromagen Vector Red^T. Finally, samples were counterstained with hematoxylin (Sigma, St. Louis, MO) for 10 minutes, rinsed with distilled water then ammonia water (0.5%) for 10 seconds. Following a final wash with distilled water, the coverslips were allowed to dry and were mounted on slides with Cytoseal mounting media (Fisher, Pittsburgh, PA).

Samples were examined at a magnification of 200x using light microscopy. DN positive cells stained by the antibody were red, and normal hemocytes were

counterstained blue. The total number of cells per field, and the number of DN cells in each field was counted and recorded. This was repeated for 10 fields per sample, and the percent DN cells in the sample was calculated.

Cohabitation Transmission:

Experimental Animals

Mya arenaria (mean valve length 33-98 mm, average = 61 mm, SD = 17 mm) were collected from Alsea Bay, Oregon and screened for the presence of DN as described previously. No DN was detected. One hundred animals were randomly divided into twenty groups of five as healthy "recipients". Each of the ten control groups received a sixth healthy clam to serve as a control for the "donor."

Ten DN "donor" clams were selected based on their level of DN as initially determined by phase contrast microscopy and later confirmed by immunocytochemistry. The intensity levels of DN ranged from 0% to 99.9% circulating DN cells (Table 4.1). Seven of the ten DN positive animals were collected from Bar Harbor, Maine (Paul S. Anderson, Maine Department of Maine Resources, Ellsworth, ME), and three were Alsea Bay, OR animals that had been injected with DN cells 6 months before initiation of this study.

The animals were maintained in 10L tanks as described above.

Experimental Design

The objective of this experiment was to determine if DN could be transmitted from one positive animal to healthy animals under controlled conditions. Experiments were designed to determine how long transmission would take and what intensity of DN was necessary for the donor to transmit the disease. Ten experimental tanks with one DN positive clam and five randomly selected naive healthy clams, and ten control tanks with six randomly selected healthy animals each were established.

Protocol

Each of the twenty groups of five healthy animals was placed into a tank, like those described above, with a donor animal, either a DN positive for experimental tanks or a DN negative for the control tanks. When a donor animal died, it was left buried in the sand until the valves were clean, thus ensuring equal treatment of donors among tanks. Hemolymph was sampled and analyzed for the presence of DN by immunocytochemistry over the next fourteen months.

Differences in the time to onset of disease for the low and high dose treatments in the feeding transmission experiment were compared using a Kaplan-Meier analysis with a logrank test (Statview 4.5, Abacus Corporation, Berkeley, CA), with the first sample detecting DN as the event of interest. Survival was compared between groups in the same manner, with death of the animal as the event. The difference between DN intensity in treatment groups was analyzed in a general linear model (GLM, GLMStat version 1.5, Ken Beath, Perth, Australia). The cohabitation study survival data were analyzed using the Kaplan-Meier analysis with the logrank test as described above.

RESULTS

Water-Borne Exposure:

No animals from the negative control groups that were exposed to algae only or algae and normal clam hemocytes became DN positive (0/60). DN cell injected animals used as positive controls first developed DN within one month post injection (PI)(2/15), and the number of animals with DN increased over the course of the experiment (10/15) (Figure 4.1). The intensity of the disease in the positive control animals progressed from less than 1% to greater than 90% DN cells in circulation (data not shown).

Disseminated neoplasia was first detected in the high dose treatment group (exposed to a total of $1.0x10^7$ DN cells) at five months post exposure, with 50% (15/30) of the animals in that group contracting DN by the ninth month of the experiment. The low dose group in which the clams were each exposed to a total of $1.0x10^6$ DN cells had one animal with a detectable level of DN at 6 months post

Figure 4.1 Cumulative prevalence of disseminated neoplasia. The percentage of soft shell clams that developed DN over the 9 months following exposure to DN cells is shown. No negative control animals developed DN over the course of the study. The prevalence of DN in the positive control group, which were injected with DN cells, is shown by line graph. The percentage DN positive clams in the treatment groups that were exposed to 10^7 DN cells (black bars) or 10^6 DN cells (white bars) in algae suspension are illustrated in the bar graph.

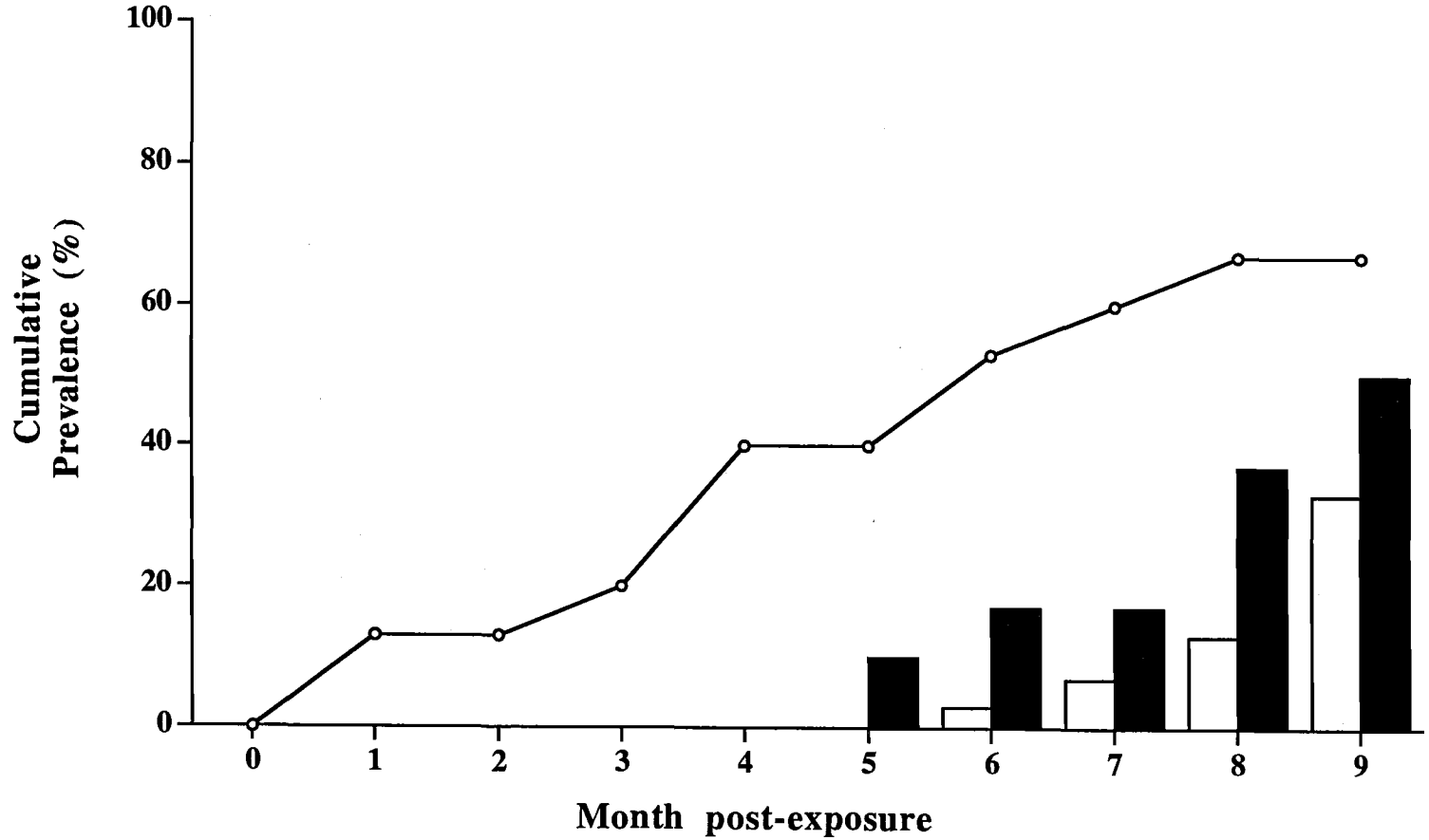

Cumulative Prevalence (%)
100
80
60
40
20
0
0
1
2
3
4
5
6
7
8
9
Month post-exposure

FIGURE 4.1

exposure. By the end of the experiment at 9 months, 33% (10/30) of the exposed animals had developed the disease (Figure 4.1). The high dose group developed DN at a mean time of 8 months, which was significantly earlier than the 9 month mean time to DN onset in the low dose group (logrank chi-square value: 7.622, p-value = 0.0058).

Intensity of DN increased in positive individuals over the course of the study. There was no significant difference in the intensity of DN between animals that developed disease in the low level treatment and those in the high level treatment group (F-statistic = 1.50, p-value = 0.225). In both groups there were clams that had greater than 90% DN cells in circulation at the termination of the study. There was a high degree of variation between individuals in both groups, with intensity of disease sharply increasing from one month to the next in many individuals.

Figure 4.2 illustrates the cumulative mortality for this study. In the negative control group in which animals were exposed to algae and no clam cells, 13% of the clams died over the 9 month course of the experiment, with a mean time to death at 8 months. In the group that was exposed to normal clam hemocytes, 7% of the clams in the group died, with a mean time to death at 8 months, and the group that was exposed to the low dose of DN cells had a 10% mortality. The mean time to death for the low DN dose group was 4 months. The group of clams exposed to the high dose of DN cells had significantly higher mortalities (27%) than the group exposed to normal hemocytes (chi square = 4.342, p-value = 0.0372), but no other significant differences at the 95% confidence level were detected.

Cohabitation:

At no time during the 14 month course of the cohabitation study did any of the clams in the negative control groups, donors or recipients, develop DN (0/60). Transmission of DN from donors to recipients occurred in seven of the ten groups of exposed animals. The first detection of DN in the recipient clams was eight months after initiation of the experiment, with individuals in 3 of the 10 groups showing low numbers of DN cells in circulation. Table 4.1 gives the number that developed DN during the 14 month study out of the number of clams that survived past 8 months in each of the ten groups, information about the donor animal, and the initial time of detection of DN in recipient animals in that group.

Figure 4.2 Mortality of soft shell clams in the feeding study. The cumulative percentage mortality for each of the water-borne exposure groups: Algae (stippled bars), Normal hemocytes (striped bars), 10^6DN cells (white bars), and 10^7 DN cells (black bars) is shown over the course of the 9 month study.

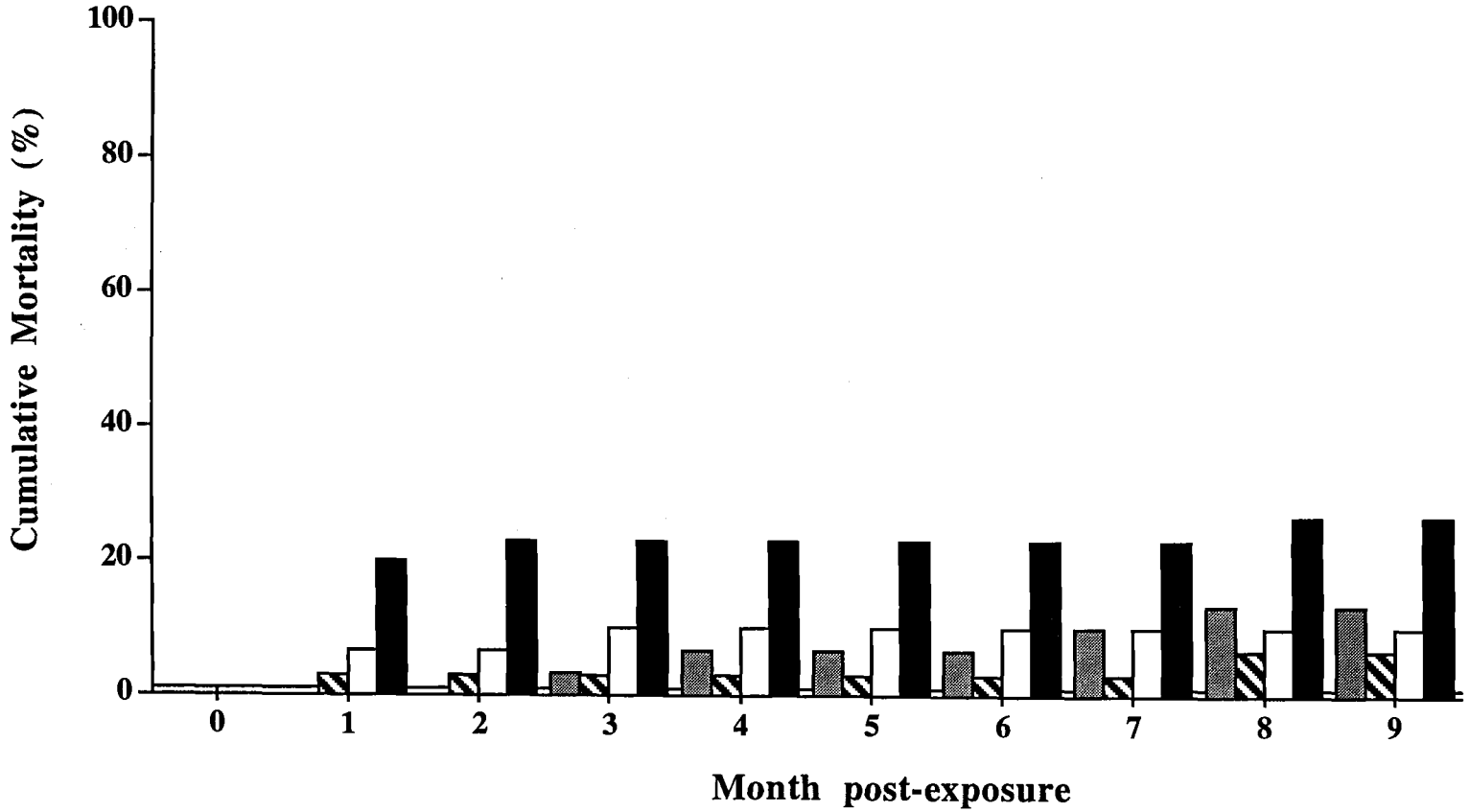

Cumulative Mortality (%)
100
80
60
40
20
0
0
1
2
3
4
5
6
7
8
9
Month post-exposure

FIGURE 4.2

Table 4.1 Summary of DN intensity, survival times of donor soft shell clams, and detection of DN in the hemolymph of recipient clams. Group numbers designate ten tanks that contained one DN donor and five healthy animals. DN positive recipient animals are presented as the number of DN positive animals per the number of potential recipients surviving at least 8 months. The number of months at least one potential recipient survived, as well as the number of months in the study until DN was detected in a recipient clam are provided.

Group	Initial DN intensity of donor	Survival time of donor in months	DN positive recipient clams	Months until last recipient died	Months until initial detection of DN
1	99.4	< 2	0/0	7	not detected
2	0.0	< 13	0/3	> 14	not detected
3	54.2	< 5	2/2	> 14	12
4	66.0	< 4	1/5	> 14	14
5	95.9	< 2	1/1	> 14	10
6	96.7	< 1	1/1	> 14	8
7	95.5	< 1	2/5	> 14	8
8	99.9	< 1	1/3	> 14	12
9	78.9	< 3	0/3	> 14	not detected
10	99.9	< 2	3/3	> 14	8

The three groups that failed to show transmission of DN had the following characteristics. In group 1, the donor animal survived more than one month into the experiment with a DN intensity level at 99.4% neoplastic cells in circulation. This is similar to the conditions in groups 5 and 10. The difference between the groups in which transmission was successful and group 1 was that there were no surviving recipient animals past seven months into the study for group 1. The earliest initial detection of DN was at the 8 month sampling for any other groups. In group 2, the donor animal thought to have very low levels of DN when initially screened by phase contrast microscopy, however, the animal did not have detectable levels of DN throughout the experiment, survived past 12 months of the study, and did not transmit DN. The donor in group 9 survived past the two month sampling period, and had DN levels ranging from 78.9% at the initiation of the study and 84.6% in the second month of sampling. Three of the five potential recipient animals survived past 12 months of the study, but no DN was detected.

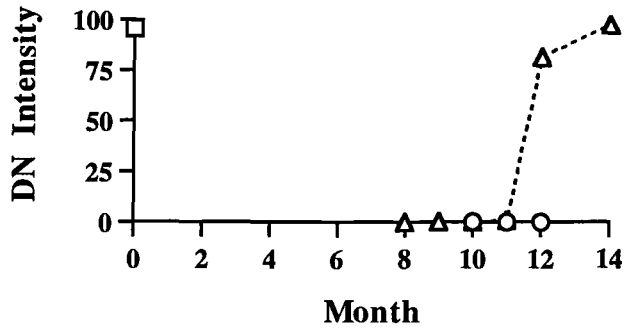
There were three general outcomes for this cohabitation experiment that are represented by the three graphs of the intensity DN of the donor, and those of the recipients over the course of the experiment in Figure 4.3. Figure 4.3A shows the results for group 7. In this group, as well as in groups 6 and 8, the donor animals died within the first month of the study. The levels of DN of donor clams were greater than 95% neoplastic cells in circulation for all three of these groups, and 2/5 of the recipient animals developed DN as listed in the table above.

Figure 4.3B shows the results from group 10. The donor animal had 99.9% DN cells in circulation and prior to the second month of the study; DN was detected in three of the five recipient animals. The group 5 donor clam also survived through the first month of the study, and the recipient animal that developed DN also reached a high disease intensity (92.67%) by the termination of the experiment.

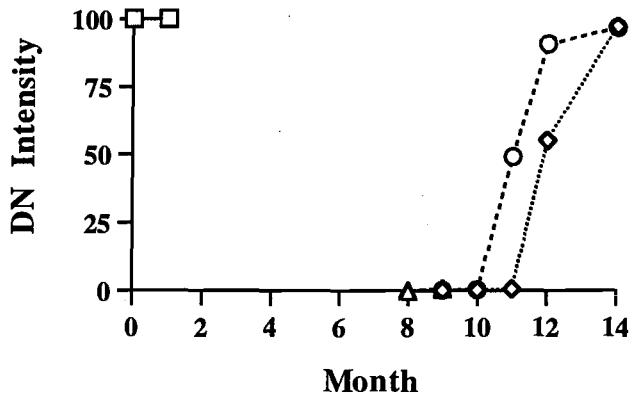
Figure 4.3C shows group 3 results, in which the donor clam died after the fourth month of sampling. This animal started the experiment with 54.2% DN cells in circulation, and had progressed to a level of 98.6% DN cells by the fourth month of the study. The recipient animals developed low levels of DN 12 months after the initiation of the experiment. The donor animal in group 4 followed a similar pattern, surviving through 3 months of the study with 66.0% DN cells in circulation in the beginning, and ending with 96.9%. DN was detected in the fourteenth month of the study for this group. A total of 11/45 of the recipient clams exposed to DN donors developed the condition over the course of the experiment.

Figure 4.3 DN intensity over time in the cohabitation experiment. The intensity levels in the monthly samples for the donor in the group and the recipients that became DN positive are shown. The lack of data points for the donor animals indicates the death of the animal. The lack of data points for the recipient animals prior to the first detection of DN indicates the absence of DN, while a discontinuation of data for an individual indicates the death of the animal before termination of the 14 month study. A. Group 7, B. Group 10, and C. Group 3.

A.



B.



C.

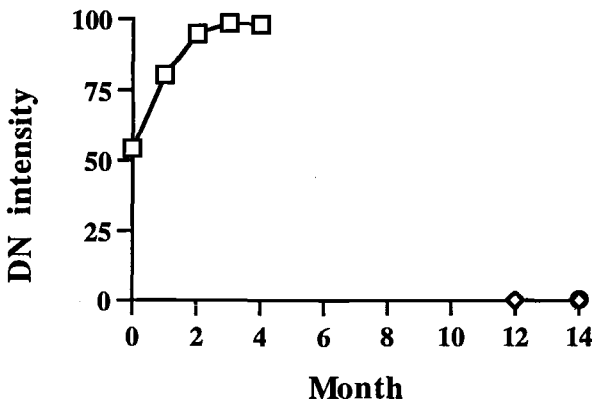


FIGURE 4.3

When the mortality rate of the exposed recipient clams was compared to that of the negative control recipient animals, no significant difference was found (logrank chi-square value: 0.676, p-value = 0.4111). The mean time to death for the exposed animals was 9 months and the control animals was at 9 months. The final percent mortality was 66% in the exposed groups and 58% for the control groups.

DISCUSSION

These experiments have clearly shown that DN can be transmitted to soft-shell clams by feeding neoplastic cells drawn from clams with high levels of DN to healthy clams, and by cohabitation of healthy animals with diseased individuals. The lack of DN in *M. arenaria* in Yaquina Bay (House and Reno, unpublished observations), the availability of sand-filtered UV-treated seawater in the laboratory, and the ability to maintain algal cultures as a food source for the clams allowed this work to be completed without experiencing the difficulty of outside sources of DN in the water supply experienced in some other laboratory situations. Early work showed that DN could be passed to healthy animals held in the effluent of (Brown et al. 1980, Appeldoorn et al. 1984), or shared with (Elston et al. 1988), DN positive animals, but negative control animals also developed DN.

In the water-borne exposure experiment, it was demonstrated that an exposure time of as little as 5 days can lead to efficient transmission of DN to healthy animals. Appeldoorn et al. (1984) performed similar experiments, but did not detect the development of DN. In two trials, these authors monitored the recipient animals for DN over a three month period. In the work reported here, the clams were monitored for DN for 9 months, with the initial detection of low intensity levels of DN at 5 months post exposure. The mean time to developing DN in the high dose group was 8 months, and the low dose group was 9 months. Therefore, the detection of transmission of DN by water-borne exposure to DN cells was accomplished here because the time course of the development of DN is extended, and was longer than the time period that previous investigators had allowed.

The clams were exposed to a known number of neoplastic cells, allowing us to determine that the level of exposure to DN cells has an effect on the time of onset of DN. We have shown that onset of disease is more rapid, and that a higher number of animals develop DN in the group that was exposed to tenfold more DN cells

compared to the group that was exposed to the lower dosage. Brown (1980) and Appeldoorn et al. (1984) demonstrated that DN was transmissible through water by exposing healthy clams to the effluent from headboxes with an unspecified number of DN positive animals. By the sixth month of the study, up to 72% of the exposed animals were positive for DN. In a repeated study, there were fewer diseased animals available for the headbox than in the first trial, and the resulting number of exposed animals that contracted DN was decreased compared to the original trial. The authors felt that this indicated that there may be a dose-dependent relationship. This is corroborated by the water-borne exposure experiment presented here. No significant differences were detectable in the intensity of disease as it developed in animals that were exposed to different doses of DN cells. The intensity of disease that developed in individual recipient animals increased over time at both exposure levels. In some cases, the proportion of neoplastic cells circulating in the hemolymph elevated rapidly within one month.

The results of the cohabitation study suggest that animals with high levels of DN are releasing an infectious agent, either the whole DN cell or a smaller particle, into the water. This may occur in the late stages of disease or as the dead animal is decaying. In earlier work, Elston et al. (1988) showed that transmission by cohabitation was possible by housing 20 healthy mussels with 50 mussels from a diseased population. Within a 231 day period, 40% of the healthy animals developed DN. Additionally, 20% of the negative control animals developed DN. It was discovered that the mussels in the water source for the laboratory had DN, and thought that the negative control mussels were developing DN as a result of being exposed to low levels of DN in the water supply. The current study confirms that DN is transmissible by cohabitation, and indicates that a single DN positive animal in close proximity to healthy individuals can effectively transmit the disease.

It has been demonstrated by Sunila and Farley (1987) that the DN cells can survive at least 48 hours in seawater. Appeldoorn et al. (1984) examined neoplastic cells from *M. arenaria* using phase-contrast microscopy, and found that the neoplastic cells averaged 12 microns in diameter (range 9-15 microns). In an electron microscopy study of the neoplastic cells in *M. edulis*, Mix et al. (1979) reported that the cell diameter averaged 15 microns. These cell sizes are within the upper limit of the 2-15 micron particle size range that bivalves can filter and ingest (Langdon and Siegfried 1984). Exposure of epithelial membranes and gills to DN cells during filtration of particulate matter in the water, or ingestion of DN cells may

provide a possible route of entry into the animal for the causative agent of disseminated neoplasia.

The results presented here indicate that this disease can require a period of approximately a year (8-14 months) to be detectable as antigenically distinct neoplastic cells in the hemolymph. The length of time to developing detectable levels of DN and prevalence levels in a population could depend on factors including the level of exposure, the susceptibility of the recipient animals, and environmental conditions. In some areas, prevalence levels have been reported to reach up to 90% (Reinisch et al. 1984), although lower prevalence levels are more commonly reported (for review see Elston et al. 1992). Elston et al. (1992) noted that the density of shellfish and water circulation in an area may be correlated to the prevalence of DN. The density of the clams used in the cohabitation study was similar to that of local clam flats, although density and other aspects of population structure vary greatly depending on location. In a natural situation on a clam flat, susceptible clams could be continuously exposed to the cell or agent capable of transmitting the disease if DN was enzootic at that location. Although the period between exposure and disease is long, a constant exposure rate, possibly for the lifetime of the clam which can be as long as 10-12 years (Brousseau 1978), could insure that the condition was maintained in the population.

A better understanding of this disease process may be obtained by investigating the size of the infectious agent, the fate of DN cells in the recipient animal, and the pathogenesis in animals infected through water-born transmission. Additional studies determining if the agent is shed before and/or after death of the diseased animal, the numbers of neoplastic cells shed by an animal, and further work on how population densities effect transmission could elucidate how this disease is established and maintained in populations under natural conditions.

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CHAPTER 5

SOFT SHELL CLAMS, *MYA ARENARIA*, WITH DISSEMINATED NEOPLASIA (DN) DEMONSTRATE REVERSE TRANSCRIPTASE ACTIVITY, BUT UNAFFECTED CLAMS DO NOT

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ABSTRACT

Disseminated neoplasia (DN), a proliferative cell disorder of the circulatory system of bivalves, was first reported in oysters in 1969. Since that time the disease has been determined to be infectious, but the etiological agent has not been unequivocally identified. In order to determine if a viral agent, possibly a retrovirus, could be the causative agent of DN, two transmission experiments, one using a cell-free filtrate and the other a sucrose gradient purified preparation of a cell-free filtrate of DN positive materials were performed. Additionally, a PCR enhanced reverse transcriptase assay was employed to determine if reverse transcriptase, was present in tissues or DN cells from DN positive clams, *Mya arenaria.* DN was transmitted to healthy soft shell clams by injection with whole DN cells, but not with cell-free filtrates prepared from DN positive tissue or DN cells. The cell-free preparations from DN positive tissues and hemolymph with high levels of DN cells in circulation exhibited positive reactions in the PCR- enhanced reverse transcriptase assay. Cell-free preparations from hemolymph from clams with low levels of DN (< 0.1% of cells abnormal) hemocytes from normal soft shell clams and normal soft shell clam tissues did not produce a positive reaction in the PCR enhanced reverse transcriptase assay.

INTRODUCTION

Disseminated neoplasia (DN) is a progressive and lethal condition of bivalves in which neoplastic cells are found in the animal's circulatory system and occlude tissue spaces. This disease was first described in native Pacific oysters (*Ostreola conchaphila*) in 1969 (Jones and Sparks). Since then, the condition has been described in at least 15 species of bivalves over a wide geographic distribution (Peters 1988, Elston et al. 1992). Prevalence levels of DN in certain locations have been reported to reach up to 90% (Reinisch et al. 1984). The disease is transmissible by water-borne exposure of healthy animals to DN positive animals (Brown 1980, Appeldoorn et al. 1984, Elston et al. 1988, House 1997). Laboratory experiments in several species have shown that DN can be transplanted to healthy animals by the injection of hemolymph taken from animals with high numbers of DN cells in circulation (Farley et al. 1986, Twomey and Mulcahy 1988, Elston et al. 1988). Transmission of the disease with an unfiltered cell homogenate prepared from DN cells from mussels, *M. edulis*, was successful within the same species (Elston et al. 1988, Kent et al. 1991, Moore 1993).

In 1981, Oprandy et al. reported that a virus with the physical and morphological characteristics of a type B retrovirus was observed in tissue from DN positive soft shell clams, *M. arenaria when* observed in a negatively-stained preparation by transmission electron microscopy. They reported that transmission of DN with a cell-free filtrate prepared from DN positive clams had been successful, and that Koch's postulates had been fulfilled by recovering virus from animals that developed the disease as a result of the initial injection, and re-infecting disease-free clams with this material. Although these results suggested that DN in *M. arenaria* was caused by a retroviral agent, attempts to repeat this work have not been successful (Elston et al. 1992), and therefore, the evidence for a retroviral etiology remains unsubstantiated. Furthermore, transmission with cell-free filtrates has been attempted, but was not successful, in mussels (Moore 1993) and soft shell clams (McLaughlin et al., 1992), which were demonstrated to be susceptible to transplantation of DN by whole cell injection.

Early attempts to determine if RT was present in DN tissues provided equivocal results (Brown, 1980), and more recent work presented by Medina et al. (1993) and Holzshcu et al. (1995) reported that RT was present, but normal soft shell clam tissues and hemocytes were not tested as negative controls.

The fact that the logistics of such transmission experiments with bivalves are difficult was recognized by Elston et al. (1992), specifically citing the ability to provide the animals with food and water free of potential infectious agents over the possibly extended course of the experiment. The capacity to provide high quality filtered seawater and cultured algae as a food source at the Hatfield Marine Science Center in Newport, Oregon enabled us to maintain soft shell clams in the laboratory for more than a year (House, 1997), which encompassed the duration of the experiments reported here, thereby addressing these concerns.

The objectives of the work presented here were to determine if DN could be transferred by injection of cell-free filtrates from DN positive tissues or DN cells from *M. arenaria and* to determine by if retroviral elements, specifically reverse transcriptase, are present in clams with DN.

METHODS

Experimental Animals:

Soft shell clams, *Mya arenaria*, were collected from Alsea Bay, Oregon. Hemolymph samples were drawn from the anterior adductor muscle sinus and placed on coverslips coated with 0.05% poly-L-lysine and allowed to settle for 30 minutes at room temperature before fixation with 1% glutaraldehyde/4% formaldehyde in seawater. These samples were screened for DN cells as described below, and none were found to have DN at the initiation of the experiment.

Determination Of DN Level:

Hemolymph samples fixed to a microscope slide were stained with a monoclonal antibody against a neoplastic cell epitope (Smolowitz and Reinisch, 1986), generously provided by Dr. Carol Reinisch, Tufts Veterinary College, Grafton, Massachusetts, and bound antibody was detected by using the Vectastain ABC-AP Kit (Vector Laboratories, Burlington, CA); samples were counterstained with hematoxylin and mounted. Cells staining red were considered DN positive while normal cells stained blue. At least 1,000 cells per animal were examined at 400 x.

Transmission Experiments:

Mya arenaria collected from Alsea Bay, Oregon negative for DN were divided into treatment groups of 20 clams each: a negative control group injected with sterile seawater, a positive control group injected with $10^{5.5}$ whole DN cells, and 2 groups injected in the anterior adductor sinus with a cell-free homogenate preparation, either unfiltered or filtered, as described below.

The inocula for the latter three groups were prepared from hemolymph drawn from a pool of 20 animals with 99% DN circulating cells (6.5 x 10^6 cells/ml). The hemolymph was sonicated on ice for five 30 second intervals at 50% duty cycle with 2 minute rests between intervals, then centrifuged at 10,000 x g for 30 minutes at 4°C to remove whole cells & microsomes. A subsample of the supernatant was examined under phase-contrast microscopy, and no intact cells were detected. A portion was reserved and was injected as the cell-free homogenate treatment. The remaining supernatant was filtered through a 0.45 μmeter pore size filter, and 0.1 ml injected as the filtrate treatment.

Following inoculation, clams were held in tanks supplied with 0.2 micron filtered seawater, (temperature range: 10°-17°C) throughout the course of the experiment. The clams were fed 1 liter of a 50/50 mixture of cultured *Isochrysis galabanea* and *Cheatoserous calcitrans* ($7x10^5$ - $1.2x10^6$ cells per ml) on a daily basis. Tanks were monitored for mortalities over the next 12 months. Hemolymph samples were taken at 0, 4, 7, 8, 10, 11 and 12 months and analyzed for the presence of DN cells using immunocytochemistry.

A second transmission experiment was established to test the transmissibility of DN using a sucrose gradient purified preparation of a cell-free filtrate. A positive control group of 15 clams was injected with whole DN cells, and a negative control group of 15 clams was injected with sterile Tris-buffered saline with EDTA (TNE: 10 mM Tris pH 7.4, 400 mM NaCl and 1 mM EDTA, Oprandy et al. 1981). Treatment inocula consisting of bands from sucrose gradients prepared from DN cells, DN tissue, normal cells and normal tissue by the method of Oprandy et al. (1981) as described below. Each treatment was injected into the adductor sinus of 20 clams in a volume of 0.1 ml/ animal.

Following injection with the treatment inocula, the animals were treated as in the filtrate experiment. Mortalities were monitored over the next ten months. Hemolymph samples were drawn at 0, 3, 5, 6, 8, 9 and 10 months and analyzed for the presence of DN cells.

Preparation of transmission study inocula and PCR enhanced reverse transcriptase (PERT) was performed in the following manner. Hemolymph was collected from 15 animals with >95% DN cells in circulation ($8x10^6$ cells/ml) The DN tissue sample used for the PERT assay consisted of the body with the siphon removed of two animals with >95% DN cells in circulation.

The normal cells and tissues were collected from 20 animals that were free of DN as shown by immunocytochemistry. The normal hemocytes were centrifuged at 1,500 g for 5 min, and the soft pellet of hemocytes was resuspended in 8.5 ml normal hemolymph to provide a cell concentration approximately equal to that of the DN sample ($2.1 x10^6$).-The soft tissue, minus the siphon, of two clams was used in the tissue sample.

Tissue and hemolymph samples were frozen at -70°C then thawed at 4°C three times. The samples were maintained at 4°C for the rest of the procedure. TNE was added to the samples at a ratio of 1:4 (cells v/v, and tissue w/v). The samples were then homogenized in a blender and clarified by centrifugation at 10,000 x g for 30 min filtered through a 0.45 micron filter, and centrifuged at 100,000 g for 90 minutes. The pellet was resuspended in 5% sucrose in TNE, and placed onto a discontinuous gradient of 15%, 35%, and 50% sucrose in TNE then centrifuged at 100,000 x g for 90 minutes. The interface between the 35% and 50% sucrose was removed and diluted 1:10 with TNE, and centrifuged at 100,000 g for 90 minutes. The resulting pellet was resuspended in 5% sucrose in TNE, and placed onto a 10% to 60% continuous sucrose gradient. The gradient was centrifuged at 100,000 x g for 90 minutes. Fractions (0.5 ml) were taken from the gradient and the average density of the fractions was determined. Three fractions in the range of 1.17 g/ml (1.14 g/ml to 1.20 g/ml) were used as the inoculum for each group. A 125 microliter (µl) portion of each of these fractions was removed and processed for analysis in the PERT assay described below. For each treatment, the volume of the combined fractions was brought to 2 ml with sterile TNE. Each of the twenty animals in the treatment groups was injected with 0.1 ml of the appropriate inoculum.

The samples that had been set aside for reverse transcriptase (RT) analysis by the PERT assay were diluted with 5.4 ml of TNE, and centrifuged at 100,000 x g

for 90 minutes. The supernatant was discarded, and the pellet was resuspended in 20 µl glycerol-based sample buffer (Pyra et al. 1994) (50 mM KCl, 25 mM Tris-HCl, pH 7.5, 5 mM dithiothreitol, 0.25 mM EDTA, 0.025% Triton X-100, and 50% glycerol), and stored at -20°C.

Additional samples were prepared for RT analysis in the same manner, with the exceptions that the material was homogenized with a Dounce homogenizer on ice, and the pellet resulting from the discontinuous sucrose gradient was resuspended in the glycerol-based sample buffer for use in the PERT assay. In addition, hemolymph samples for PERT assay were drawn from three recipient clams in each of the transmission study groups 5 months after injection.

Also, additional continuous sucrose gradients were prepared using material from DN cells and normal hemocytes. Fractions with the buoyant density from 1.17-1.19 g/ml were divided for analysis with the PERT assay and examination with transmission electron microscopy (TEM). The TEM samples were negatively stained with phosphotungstic acid.

In order to determine the sensitivity of the PERT assay, two ml hemolymph samples were collected from animals with 100%, 1% and 0.1% DN and processed in the same manner described above. Additionally, a series of samples of varying DN concentrations of cells were prepared. Samples of DN cells were prepared by combining hemolymph from clams with >99% DN cells diluted with hemolymph from normal clams to give concentrations of 100%, 50%, 10%, 1%, 0.1% and 0% percent DN cells. The samples were treated as those in the preparation of transmission study inocula and other PERT samples.

PCR Enhanced Reverse Transcriptase (PERT) Assay:

A sensitive, PCR-based assay described by Pyra et al. (1994) was used to determine the presence of reverse transcriptase activity. Briefly, the PCR enhanced reverse transcriptase (PERT) assay uses a single stranded RNA template, MS2, and an MS2 specific primer to detect the presence of reverse transcriptase. The sample, prepared as described above, was added to the first strand synthesis reaction, incubated at 15°C for 5 hours, then RNase was then added to degrade the RNA in the RNA/DNA hybrid. Next, an additional pair of primers was added along with *Taq* polymerase and PCR was performed on the sample. After the PCR

products were run on an agarose gel and transferred to nitrocellulose by Southern blotting (Southern 1975), an internal probe is used to show the specificity of the product. The presence of a band in the Southern blot indicates that RT was present in the sample.

RESULTS

Transmission Experiments:

Samples from clams injected with 10^6 DN cells at four month post-injection (PI) showed that 100% (14/14) of the surviving clams were positive for DN. None of these animals survived until the next sampling time at 7 months PI.

No other clams in this experiment developed DN. Neither the negative controls nor the animals injected with the cell-free homogenate, nor the cell-free filtrate prepared from DN cells showed detectable DN through the 12 month course of the experiment. Survival was greater than 50% in each of these groups at termination of the experiment.

In experiments using sucrose gradient purified material, *Mya arenaria* in the group injected with whole cells developed DN. At three months PI, 44% (4/9) of the clams were positive for DN. In the samples taken at months 5, 6, and 8 PI, 83% (5/6), 100% (6/6) and 100% (4/4), respectively, were positive for DN. No clams in this group were alive at 9 months post injection.

No clams injected with material purified from highly DN positive clam tissues, DN cells, normal soft shell clam tissues, or normal soft shell clam hemocytes developed DN. At the completion of the ten month experiment, survival was 70% or greater in groups that were injected with the sucrose gradient purified products, and 60% in the negative control group.

PCR Enhanced Reverse Transcriptase (PERT) Assay:

When samples prepared from DN positive clams were tested for the presence of enzymes that resemble vertebrate reverse transcriptase (RT), this activity was detected.

As shown in Figure 1, a 112 bp PCR product was produced using samples from the 35/50% sucrose interface of the discontinuous gradients prepared from soft tissues of animals with DN (lane 2), as well as DN cells in the hemolymph (lane 4). The fractions from the continuous sucrose gradients buoyant densities of 1.17-1.19 g/ml used in the transmission experiment also indicated the presence of RT in DN tissue (lane 5), and in hemolymph containing DN cells (lane 7). No PCR products were detected in material prepared from normal clam tissues collected from the interface of the discontinuous gradient (lane 3), or from fractions of the continuous gradient in material prepared from normal clam tissues and normal clam hemocytes (lanes 6 and 8, respectively).

When hemolymph samples were taken from animals with known levels of DN determined by immunocytochemistry, the PERT assay detect RT activity in animals with-100% DN cells and 1% DN cells, but not 0.1% DN cells (lanes 2, 3, and 4, respectively) (Figure 2). Similarly, samples prepared by diluting hemolymph from a clam with >99% DN cells with normal hemocytes, to DN concentrations of 50%, 10 %, and 1% DN cells (lanes 5, 6, 7 and 8 respectively), PCR products indicative of RT activity were detected, but at 0.1% DN cells (lane 9) there was no detectable RT. The 112 bp PCR products were also absent in the sample from normal hemocytes (lane 10).

Hemolymph samples taken from the clams in the sucrose gradient transmission study at 5 months post injection had no detectable PCR products that would indicate RT activity present due to the presence of a retrovirus injected into these animals.

Fractions from sucrose gradients in which PCR products were detected were examined by transmission electron microscopy after negative staining with photsphotungstic acid. No structures resembling virus particles were observed.

DISCUSSION

The results of this study indicate that disseminated neoplasia could be transmitted to healthy soft shell clams by direct injection of whole DN cells, but not by injection of cell-free preparations of either DN cells or tissues of DN affected clams. The results of the transmission studies presented here, in addition to those also using soft shell clams (McLaughlin et al., 1992) and mussels (Moore 1993) in which transmission was not successful, do not support earlier evidence that a

Figure 5.1. Detection of PCR products in the PERT assay. Lane 1. 100 bp ladder (BRL) Lanes 2-5 contain PERT assay products prepared from the 35/50% sucrose gradient interface of material from: lane 2. DN tissue, lane 3. normal soft shell clam tissue, lane 4. DN cells. Lanes 5-8 contain PERT assay products from material in continuous gradient fractions prepared from: lane 5. DN tissue, lane 6. normal soft shell clam tissue, lane 7. DN cells, lane 8. normal soft shell clam hemocytes.

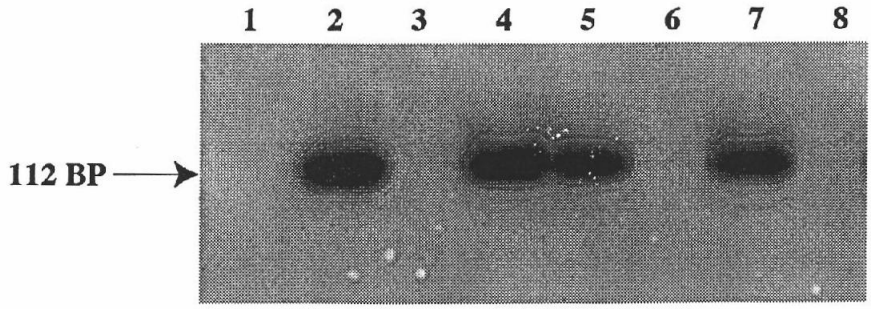

FIGURE 5.1

Figure 5.2. Sensitivity of PERT assay using hemolymph samples. Lane 1. 100 bp ladder (BRL). Lanes 2-4 contain PERT assay products from samples prepared from hemolymph collected from animals with known levels of DN: lane 2. 100 % DN, lane 3. 1 % DN, lane 4. 0.1 % DN. Lanes 5-10 contain PERT assay products from hemolymph preparations with decreasing proportions of DN cells diluted in normal soft shell clam hemocytes: lane 5. 100 % DN, lane 6. 50% DN, lane 7. 10 % DN, lane 8. 1 % DN, lane 9. 0.1 % DN, lane 10. normal soft shell clam hemocytes.

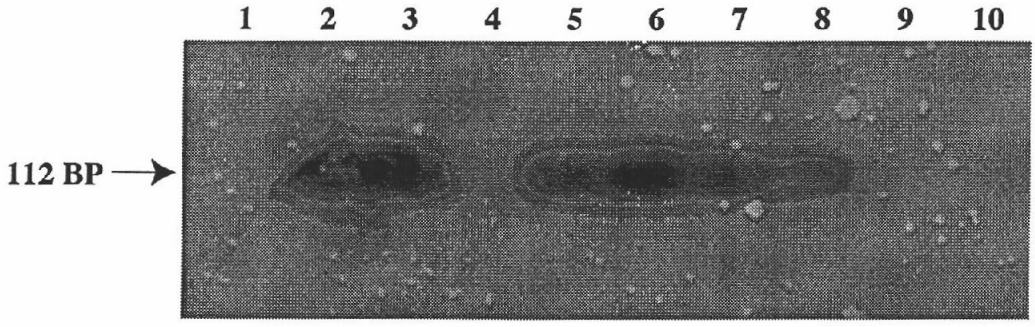

FIGURE 5.2

filterable virus is the etiological agent of DN. Previous studies (House, 1997) indicated that the disease could be transmitted to susceptible clams at a dose of ≤ $10^{4.6}$ whole DN cells injected into the adductor sinus. The centrifugation step would have removed not only whole cells, but also the microsomal fraction of lysed cells. This may have reduced the concentration of virus below the threshold necessary for infection, in spite of the fact that RT activity was associated with this fraction. Alternatively, the process of purification itself may have inactivated any viral agent present, in spite of the fact that the methods are know to retain the infectiousness of other retroviruses. It is known that retroviruses can be strongly cell-associated, and the removal of membranous material by centrifugation may have removed virus also. Supporting this concept, Elston et al. (1988) successfully transmitted DN in *M. edulis* with a cell-free homogenate that included membranous material. If a virus was not able to be freed from the cell membrane by the methods utilized here, it could have been lost in the preparation of the inoculum. In contrast to the results reported here, Oprandy et al. (1981) reported transmission of disseminated neoplasia in soft shell clams with a cell-free filtrate.

PCR products indicative of reverse transcriptase activity were detected in samples prepared from both DN tissue and DN cells, including the material that was used as the inocula in the sucrose gradient purified product transmission study. No PCR products indicating RT activity were detected in samples prepared from normal tissue or hemocytes, or from hemolymph samples from clams with low levels of DN. Early attempts to detect RT in bivalves with DN were equivocal (Brown 1980, Appeldoorn et al. 1984), but the more sensitive methods used here indicate that RT activity is present in DN cell. In an abstract, Medina et al. (1993) reported detection of RT in association with viral particles in soft shell clams. The enzyme was reported to be active at 6°C and inactive above 25°C, but only limited information was available concerning the methods of detection that were used. In our studies, the temperature for the PERT assay was 15°C. Holzschu et al. (1995) identified RT in DN positive clams, but negative controls were not tested for RT activity, as were normal tissues in this study. Thus, this study provides the first unequivocal evidence that RT is present in DN cells and tissues of affected clams, but not in clams which are known to be DN negative. The lack of the disease in softshell clams from Oregon and the availability of an UV-irradiated seawater source provide the confidence needed that the host clams were indeed DN negative.

The PERT assay did not detect RT in hemolymph samples from animals with a low proportion of DN cells in circulation (<1%), or in samples that were prepared

with 0.1% DN cells or less. Although the animals used in the PERT study were clearly DN positive by immunohistochemical and morphological evaluation, they may be below the level of detection of RT by this method, even though this is quite a sensitive assay in other systems. It may well be that the parameters of the assay, which was designed for mammalian systems (Pyra et al. 1994) are not optimal for invertebrate systems.

Other possible sources of the reverse transcriptase activity could be an endogenous retrovirus, or mycoplasma (Maramorosch and Korprowski, 1977). If an endogenous retrovirus unrelated to the occurrence of DN was the source of the RT activity, similar activity in normal tissues and cells would be expected; this was not the case in these studies, and therefore, an endogenous retrovirus is unlikely to be the source of the RT activity detected here. Further studies should be performed to determine the source of the RT activity in the DN positive materials.

The single report by Oprandy et al. (1981) of a negatively stained type B retrovirus in DN positive tissue from *M. arenaria* has not been confirmed by other researchers utilizing thin sections to examine the cellular structure of DN cells. As reviewed in Elston et al. (1992), several electron microscopy studies did not detect the presence of viral particles associated with DN in *M. arenaria* (Appeldoorn et al. 1984, Farley 1976), *Mytilus edulis* (Mix et al. 1979, Moore 1993), or *Cerastoderma edulis* (Auffret and Poder 1986), despite the intensity of such research efforts. The samples that were examined by negative staining techniques in this study were likewise negative.

In summary, the cell-free preparations from DN materials exhibited positive reactions in the PCR enhanced reverse transcriptase assay, indicative of the presence of a retroviral-associated enzyme. This PCR product was not detectable in samples prepared from normal soft shell clam hemocytes or tissues, or from hemolymph of clams with low proportions of DN cells. Although DN was not transmitted via cell-free filtrate, cell membrane-bound material, potentially containing a retrovirus, may contain the etiological agent of disseminated neoplasia. Future studies pursuing identification of the source of the reverse transcriptase activity in the DN positive materials are essential to determining if a retrovirus is the etiological agent of this condition. Additionally, testing mussels and cockles with DN for reverse transcriptase activity using the methods described here could determine if similar mechanisms are responsible for this disease condition in other bivalve species.

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CHAPTER 6

THESIS SUMMARY

SUSCEPTIBILITY OF WEST COAST SOFT SHELL CLAMS TO DISSEMINATED NEOPLASIA AND DOSE RELATED EFFECTS ON DEVELOPMENT OF DN

Disseminated neoplasia was successfully transplanted to soft shell clams collected from Alsea Bay and Yaquina Bay, Oregon by injecting whole DN cells from clams with >95% neoplastic cells in circulation. The lack of disseminated neoplasia in populations of clams in these bays does not appear to be due to the animals' resistance to the condition, but probably to the absence of the causative agent of the disease. The development of DN in negative control west coast clams at the Woods Hole Oceanographic Institute appears to indicate that water-borne transmission of DN occurred through the water supply to the laboratory facility. The dose response study demonstrated that onset, development of neoplasia, and survival were directly correlated with the number of DN cells injected into recipient animals. In this study, lowest dose of DN cells administered ($10^{4.6}$ cells per animal) contained sufficient numbers of DN cells to cause the condition to occur in the recipient clams. It was not possible to determine the minimum dose that would successfully transplant DN from the results of this work.

WATER-BORNE TRANSMISSION OF DISSEMINATED NEOPLASIA

The results of the feeding transmission study demonstrated that water-borne exposure to DN cells for 5 days allows the disease to be transmitted to healthy animals, and that neoplastic cells in hemolymph from animals with high levels of disseminated neoplasia contain the causative agent of DN. Onset of neoplasia was related to the level of exposure, with a more rapid development of DN observed in the treatment group that was exposed to higher numbers of DN cells. The cohabitation study results suggest that animals that are dying, or that have died of,

high levels of DN are releasing an infectious agent, either the whole DN cells or a particle, into the water.

SOFT SHELL CLAMS, *MYA ARENARIA*, WITH DISSEMINATED NEOPLASIA (DN) DEMONSTRATE REVERSE TRANSCRIPTASE ACTIVITY, BUT UNAFFECTED CLAMS DO NOT

Disseminated neoplasia was not successfully transmitted to recipient clams that were injected with cell-free filtrates prepared from DN cells or DN positive soft shell clam tissues. Cell-free preparations from DN materials exhibited positive reactions in the PCR enhanced reverse transcriptase assay, which yields a PCR product if RT, an essential enzyme of retroviruses, is present. Samples prepared from normal soft shell clam hemocytes or tissues, as well as those from hemolymph with very low proportions of DN cells, did not have any detectable PCR products. Although DN is not reliably transmitted via cell-free filtrate, it is possible that cell membrane-bound material, potentially containing a retrovirus, may contain the etiological agent of disseminated neoplasia.

FUTURE DIRECTIONS OF RESEARCH

The results of the research presented here indicate that disseminated neoplasia is an infectious disease effectively transmitted via the water-borne route. The nature of the etiologic agent remains unknown at this point, but valuable information could be gained by using molecular techniques to determine if retroviral sequences are associated with DN, and by examining the fate of DN cells in animals infected via water-borne transmission. Additionally, studies exploring how population density effects the efficiency of transmission would lead to a more complete understanding of how disseminated neoplasia is transmitted under natural conditions.

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