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Soft shell clams *Mya arenaria* with disseminated neoplasia demonstrate reverse transcriptase activity

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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 transmissible through water-borne exposure, 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, transmission experiments were performed, using both a cell-free filtrate and a sucrose gradient-purified preparation of a cell-free filtrate of DN positive materials. Additionally, a PCR-enhanced reverse transcriptase assay was used to determine if reverse transcriptase was present in tissues or hemolymph from DN positive soft shell clams *Mya arenaria*. DN was transmitted to healthy clams by injection with whole DN cells, but not with cell-free filtrates prepared from either tissues from DN positive clams, or DN cells. The cell-free preparations from DN-positive tissues and hemolymph having high levels of DN cells in circulation exhibited positive reactions in the PCR-enhanced reverse transcriptase assay. Cell-free preparations of hemolymph from clams having 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.

KEY WORDS: Soft shell clam · *Mya arenaria* · Disseminated neoplasia · Reverse transcriptase activity

INTRODUCTION

Disseminated neoplasia (DN) is a progressive and lethal condition of bivalves characterized by the presence of neoplastic cells in the animal's circulatory system and tissue spaces. This disease was first described in native Pacific oysters *Ostreola conchaphila* by Jones & Sparks (1969). 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 of DN in certain locations has been reported to reach 90% (Reinisch et al. 1984). The disease is transmissible to healthy animals by water-borne exposure to DN-positive animals (Brown 1980, Appeldoorn et al. 1984, Elston et al. 1988, House 1997). Laboratory experiments using several species of bivalves have shown that DN can be transplanted to healthy animals of the same species by the injection of hemolymph taken from animals with high numbers of DN cells in circulation (Farley et al. 1986, Elston et al. 1988, Twomey & Mulcahy 1988). Transmission of the disease via an unfiltered homogenate of DN cells from mussels *Mytilus edulis* was successful within the same species (Elston et al. 1988, Kent et al. 1991, Moore 1993).

Oprandy et al. (1981) reported that a virus with the physical and morphological characteristics of a type B retrovirus was observed by transmission electron microscopy of tissue from DN-positive soft shell clams *Mya arenaria*. They reported successful transmission of DN using a cell-free filtrate prepared from DN-positive clams, and that Koch's postulates had been fulfilled by recovering virus from clams that developed the disease following injection, and re-infecting disease-free clams with this material. Although these results suggested that DN in *M. arenaria* was caused

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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. Transmission of DN using cell-free filtrates has been attempted, unsuccessfully, 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.

The difficulty in conducting long-term transmission experiments with bivalves was recognized by Elston et al. (1992), who specifically cited the need to provide the animals with food and water free of potential infectious agents over the 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, USA, 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.

Reverse transcriptase (RT) is an enzyme essential to retroviral replication and has been used as an indicator for the presence of a retrovirus (Maramorosch & Koprowski 1977). Early attempts to determine if RT was present in DN tissues provided equivocal results (Brown 1980).

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

METHODS

Experimental animals. Soft shell clams *Mya arenaria* (average valve length = 43 mm) were collected from Alsea Bay, Oregon. Hemolymph samples (0.1 ml) 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 min at room temperature before fixation with 1% glutaraldehyde/4% formaldehyde in sea water. All clams were screened for DN cells, and were negative at the initiation of the experiment.

Detection of DN. Hemolymph samples fixed to a microscope slide were stained with a monoclonal antibody against a neoplastic cell epitope (Smolowitz & Reinisch 1986), generously provided by Dr Carol Reinisch, Tufts Veterinary College, Grafton, Massachusetts, USA. Bound antibody was detected using the Vectastain ABC-AP Kit (Vector Laboratories, Burlington, CA, USA); samples were counterstained with hematoxylin and mounted. Cells staining red were considered DN positive while normal cells stained blue. At least 1000 cells per clam were examined at 400×. The percentage of DN cells in the sample was recorded.

First transmission experiment. Disease-free *Mya arenaria* were divided into treatment groups of 20 clams each: a negative control group injected with sterile sea water, 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 3 groups were prepared from hemolymph drawn from a pool of 20 clams with 99% DN circulating cells (6.5×10^6 cells ml^{-1}). The hemolymph was sonicated on ice for five 30 s intervals at 50% duty cycle with 2 min rests between intervals, then centrifuged at $10\,000 \times g$ for 30 min at 4°C to remove whole cells and microsomes. A subsample of the supernatant was examined using 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 µm pore size filter, and 0.1 ml injected as the filtrate treatment.

Following inoculation, clams were held in tanks supplied with 0.2 µm filtered sea water throughout the course of the experiment (temperature range: 10 to 17°C). The clams were fed 1 l of a 50/50 mixture of cultured *Isochrysis galabana* and *Chaetoceros calcitrans* (7×10^5 to 1.2×10^6 cells ml^{-1}) on a daily basis. Tanks were monitored for mortalities over the next 12 mo. Hemolymph samples were taken at 0, 4, 7, 8, 10, 11 and 12 mo and analyzed for the presence of DN cells using immunocytochemistry.

Second transmission experiment. A second transmission experiment was performed 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 consisted of fractions from sucrose gradients containing material from DN cells, DN tissue, normal cells and normal tissue prepared 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 $clams^{-1}$.

Following injection, the clams were held as described in the first experiment. Mortalities were monitored over the next 10 mo. Hemolymph samples were drawn at 0, 3, 5, 6, 8, 9 and 10 mo and analyzed for the presence of DN cells.

Sucrose gradient purification. ***Transmission study inocula:*** Preparation of transmission study inocula and PCR enhanced reverse transcriptase (PERT) was per-

formed in the following manner. Hemolymph from 15 clams with >95% DN cells in circulation was collected and pooled (8×10^6 cells ml^{-1}). The DN tissue sample used for the PERT assay consisted of the soft tissue, with the siphons removed, of 2 clams with >95% DN cells in circulation.

Control inocula were prepared from cells and tissues collected from 20 clams that were free of DN as shown by immunocytochemistry. The pooled hemolymph was centrifuged at $1500 \times g$ 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 (2.1×10^6 cells ml^{-1}). The soft tissues, minus the siphons, of 2 clams were combined and used in the tissue sample.

Tissue and hemolymph samples were frozen at −70°C then thawed at 4°C, 3 times. The samples were maintained at 4°C for the rest of the procedure. Samples were diluted in sterile TNE to 20% v/v (cells) or w/v (tissue). The samples were then homogenized in a blender and clarified by centrifugation at $10\,000 \times g$ for 30 min, filtered through a 0.45 µm filter, and centrifuged at $100\,000 \times g$ for 90 min. 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 $100\,000 \times g$ for 90 min in an SW 50.1 rotor (Beckman, Palo Alto, CA). The interface between the 35 and 50% sucrose layers was removed and diluted to 10% v/v with TNE, and centrifuged at $100\,000 \times g$ for 90 min. 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 $100\,000 \times g$ for 90 min. Fractions (0.5 ml) were taken from the gradient and the average density of the fractions was determined. Based on the results of Oprandy et al. (1981), 3 fractions in the range of 1.17 g ml^{-1} (1.14 to 1.20 g ml^{-1}) were used as the inoculum. A 125 µl portion of each fraction was removed and processed for analysis in the PERT assay described below. To prepare inocula, the fractions were combined, and the volume was brought to 2 ml with sterile TNE. Each of the 20 clams in the treatment groups was injected with 0.1 ml of the appropriate inoculum.

Reverse transcriptase assay samples: Samples that had been set aside for RT analysis by the PERT assay were diluted with 5.4 ml of TNE, and centrifuged at $100\,000 \times g$ for 90 min. 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.

In addition to samples prepared from the inocula for the transmission study, samples were prepared from other clams 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 used in the PERT assay. Also, hemolymph samples were drawn from 3 recipient clams in each of the transmission study groups 5 mo post-injection (PI) and analyzed by the PERT assay.

Finally, continuous sucrose gradients were prepared using material from DN cells and normal hemocytes. Fractions with the buoyant density from 1.17 to 1.19 g ml^{-1} were divided for analysis with the PERT assay and examination with transmission electron microscopy (TEM). The TEM samples were negatively stained with phosphotungstic acid.

Reverse transcriptase assay. A sensitive, PCR-based assay described by Pyra et al. (1994), with modification of the temperature and incubation time of the first strand synthesis, was used to determine the presence of RT activity. Briefly, the PERT assay uses a single-stranded RNA template, MS2, and an MS2-specific primer (RT-1) to detect the presence of RT. The sample, prepared as described, was added to the first strand synthesis reaction, incubated at 15°C for 5 h, then RNase was then added to degrade the RNA in the RNA/DNA hybrid. If RT was present in the sample, MS2 cDNA was synthesized. Next, a second MS2 specific primer (RT-2) and an additional amount of RT-1 were added along with *Taq* polymerase and PCR was performed to amplify the MS2 cDNA. After the PCR products were run on an agarose gel and transferred to nitrocellulose by Southern blotting (Southern 1975), an internal probe (RT-3) was used to show the specificity of the product. The presence of a band in the Southern blot indicated that RT was present in the sample.

Sensitivity of PERT assay. In order to determine the sensitivity of the PERT assay, 2 ml hemolymph samples were collected from clams with 100%, 1% and 0.1% DN and processed as described above. Additionally, a series of samples of varying concentrations of DN cells were prepared by combining hemolymph from clams with >99% DN cells with hemolymph from normal clams to give concentrations of 100, 50, 10, 1, 0.1 and 0% DN cells. The samples were treated as those in the preparation of transmission study inocula and other PERT samples.

RESULTS

First transmission experiment

In the first experiment, samples collected at 4 mo post-injection (PI) from clams injected with $10^{5.5}$ DN cells 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 mo PI.

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

Second transmission experiment

In the second transmission experiment, *Mya arenaria* in the group injected with whole cells developed DN. At 3 mo PI, 44% (4/9) of the clams were positive for DN. In the samples taken at 5, 6, and 8 mo 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 mo post-injection.

No clams injected with gradient purified material 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 10 mo experiment, survival was 70% or greater in groups that were injected with the sucrose gradient purified preparations, and 60% in the negative control group.

Reverse transcriptase assay

Samples prepared from DN positive clams produced evidence for the presence of RT activity. As shown in Fig. 1, a 112 bp PCR product was obtained 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 from samples of DN cells in the hemolymph (lane 4). The fractions from the continuous sucrose gradients having buoyant densities of 1.17 to 1.19 g ml^{-1} that were 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 gradients containing material prepared from normal clam tissues or normal clam hemocytes (lanes 6 and 8, respectively).

When hemolymph samples were taken from clams with known levels of DN as determined by immunocytochemistry, the PERT assay detected RT activity in clams with high (100%) and moderate (1%), but not with a very low number (0.1%) of DN cells (Fig. 2, lanes 2, 3, and 4, respectively). Similarly, samples of hemolymph from a clam with >99% DN cells diluted with normal hemocytes to concentrations of 50%, 10%, and 1% DN cells (lanes 5, 6, 7 and 8 respectively), prouced PCR products indicative of RT activity, but at 0.1% DN cells (lane 9) there was no detectable RT activity. The 112 bp PCR product was absent in the negative control sample of normal hemocytes (lane 10).

Hemolymph samples taken at 5 mo post-injection from clams injected with the preparation from sucrose gradients in the second transmission study had no detectable PCR products. The absence of RT activity in these samples indicates that the RT activity was only associated with the presence of DN cells in clams that were successfully infected.

Samples from fractions of the sucrose gradients which were positive for RT activity by the PERT assay were examined by transmission electron microscopy after negative staining with phosphotungstic acid. No structures resembling virus particles were observed.

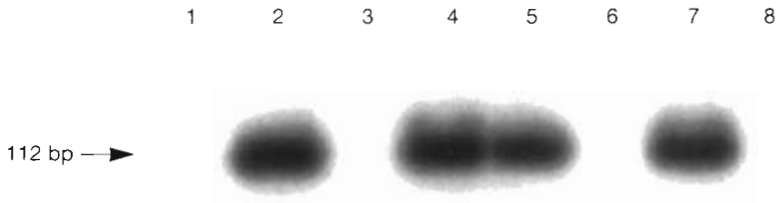

Fig. 1. Detection of PCR products labeled with ^{32}P in the PERT assay. Lane 1: unlabeled 100 bp ladder (BRL). Lanes 2–4: PERT assay products prepared from the 35/50% sucrose gradient interface of material from DN tissue (lane 2), normal soft shell clam tissue (lane 3), and DN cells (lane 4). Lanes 5–8: PERT assay products from material in continuous gradient fractions prepared from: DN tissue (lane 5), normal soft shell clam tissue (lane 6), DN cells (lane 7), and normal soft shell clam hemocytes (lane 8)

DISCUSSION

Disseminated neoplasia was 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 taken from DN-affected clams. The results of the transmission studies presented here, in addition to those using cell-free filtrates in soft shell clams (McLaughlin et al. 1992) and mussels (Moore 1993) in which transmission was not successful, do not support the results of Oprandy et al. (1981), who reported transmission of DN in soft shell clams with a cell-free filtrate.

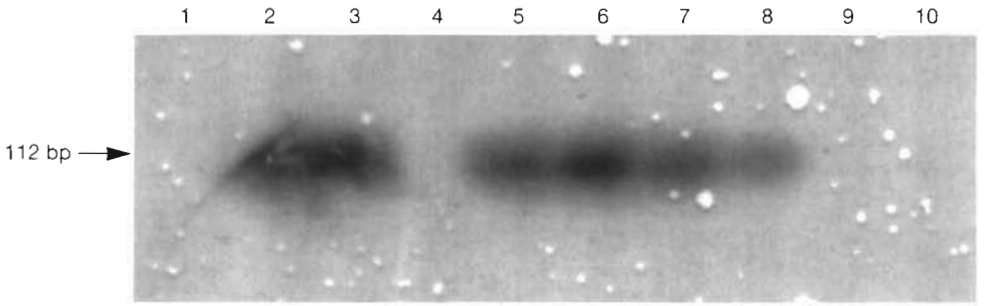

Fig. 2. Sensitivity of PERT assay using hemolymph samples labeled with ^{32}P. Lane 1: unlabeled 100 bp ladder (BRL). Lanes 2–4: PERT assay products from samples prepared from hemolymph collected from clams with known levels of DN: 100% DN (lane 2), 1% DN (lane 3), and 0.1% DN (lane 4). Lanes 5–10: PERT assay products from hemolymph preparations with decreasing proportions of DN cells diluted in normal soft shell clam hemocytes: 100 % DN (lane 5), 50% DN (lane 6), 10 % DN (lane 7), 1 % DN (lane 8), 0.1 % DN (lane 9), and normal soft shell clam hemocytes (lane 10)

In work done to determine if disease development was affected by the number of cells injected, House (1997) found that DN could be successfully transmitted by injection of as few as 10^{4-6} whole DN cells injected into the adductor sinus of susceptible clams. In the protocol used in the current study, centrifugation would remove not only whole cells, but also the microsomal fraction of lysed cells. This may have reduced the concentration of a putative virus to a level below the threshold necessary to initiate infection, although RT activity could still be detected. Alternatively, the procedures used during purification may have removed or inactivated a highly labile infectious agent. Retroviruses can be cell-associated (Evermann 1990), and the removal of membranous material by centrifugation may have effectively removed virus. Elston et al. (1988), Kent et al. (1991), and Moore (1993) successfully transmitted DN from DN-positive mussels to disease-free mussels with a cell-free homogenate that included membranous material. If a virus could not be freed from the cell membrane by the methods utilized here, it could have been lost in the preparation of the inoculum.

Early attempts to detect RT in bivalves with DN were equivocal (Brown 1980, Appeldoorn et al. 1984), but the more sensitive methods used here indicated that RT activity was present in DN cells. Medina et al. (1993) reported detection of RT 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. This study provides evidence that while RT is present in DN cells and tissues of affected clams, it is absent from clams which are known to be DN negative.

The PERT assay did not detect RT in hemolymph samples from clams with a low proportion of DN cells in circulation (<1%), nor in samples that were diluted with normal cells to contain 0.1% or fewer DN cells. Although the clams used in the PERT study were judged DN positive by immunohistochemical and morphological evaluation, they may have been below the level of detection by the PERT assay, even though the method has been shown to be quite sensitive in other systems (Pyra et al. 1994). Another possible source of RT activity could be mycoplasma (Maramorosch & Koprowski 1977); however, the absence of RT activity in negative clams suggests that RT activity was associated with the infectious process that results in DN. Further studies will be required to determine the source of the RT activity in the DN positive materials.

Oprandy et al. (1981) observed what they interpreted to be a type B retrovirus in a negatively stained sample of a sucrose gradient preparation from DN positive *Mya arenaria*. As reviewed in Elston et al. (1992), several other electron microscopy studies have failed to detect the presence of viral particles associated with DN in *M. arenaria* (Farley 1976, Appeldoorn et al. 1984), *Mytilus edulis* (Mix et al. 1979, Moore 1993), or *Cerastoderma edulis* (Auffret & Poder 1986). The samples that were examined by negative staining techniques in this study were likewise negative for virus-like particles.

Studies pursuing the source of the RT activity in DN positive materials will be essential to determining the etiological agent of this disease. RT assays alone are not enough to confirm that a retrovirus is the causative agent of DN, and transmission studies and visualization of viral particles using EM have not proven convincing. The development of marine invertebrate cell culture systems could advance the study of DN by potentially allowing isolation and characterization of a viral agent. Determining the temperature range of the RT activity using the PERT assay could lead to a better

understanding of the seasonal nature of the disease that has been reported by several authors (Cooper et al. 1982, Appeldoorn et al. 1984, Farley et al. 1986, Brousseau 1987, Leavitt et al. 1990). Additionally, testing mussels and cockles with DN for RT activity using the methods described here would determine if the same patterns of enzyme activity existed, and potentially, if similar mechanisms are responsible for this condition in different species of bivalves.

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