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Invertebrate Neoplasia: Initiation and Promotion Mechanisms

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U. S. DEPARTMENT OF COMMERCE
National Oceanic and Atmospheric Administration
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Invertebrate Neoplasia: Initiation and Promotion Mechanisms

Proceedings of an International Workshop, 23 June 1992, Washington, D.C.

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[d] Wilson, D.E.; Reeder, D.M. 1993. Mammal species of the world: a taxonomic and geographic reference. Washington, DC: Smithsonian Institution Press; 1206 p.

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Acronyms

ANF	=	[a]napthaflavone
B[a]P	=	benzo[a]pyrene
BRH	=	Black Rock Harbor (New Haven, CT)
ConA	=	concanavalin A
IGF	=	insulin-like growth factor
MPF	=	maturation-promoting factor
ODC	=	ornithine decarboxylase
PAH	=	polycyclic aromatic hydrocarbon
PCB	=	polychlorinated biphenyl
PCR	=	polymerase chain reaction
RTLA	=	Registry of Tumors in Lower Animals
3MC	=	3-methylcholanthrene
TIMP-2	=	tissue inhibitor of metalloproteinase-2

PREFACE

This compilation of summary reports is an outgrowth of the International Workshop on Invertebrate Neoplasia, sponsored by the Invertebrate Division of the Tissue Culture Association and the Society for Invertebrate Pathology. The workshop was a part of the World Congress of Cell and Tissue Culture, held in Washington, DC, during 20-25 June 1992. The workshop consisted of three parts: (1) a series of oral reports, each 15-20 min in length, describing progress of research on neoplasia in aquatic and terrestrial invertebrates; (2) brief comments and reports from a "panel" whose members summarized related talks they gave elsewhere during the Congress and/or presented material that broadened the scope of the subject; and (3) interesting and provocative dialog between speakers, panelists, and the audience that resulted in a clearer understanding of the initiation and promotion mechanisms behind neoplasia in specific invertebrates, as well as of the relationships such mechanisms have to related developmental abnormalities in other animals.

Because publication of these proceedings was delayed beyond the time frame expected, authors were given the opportunity to add to their reports. These comments are appended to their submissions in order to maintain the temporal integrity of the proceedings.

ACKNOWLEDGMENTS

We are pleased to acknowledge the support provided by the National Ocean Service's Office of Ocean Resources, Conservation, and Assessments, and by the National Marine Fisheries Service's Office of Protected Resources.

Dr. Clyde J. Dawe (School Street, Woods Hole, MA, formerly of the Comparative Oncology Section, Laboratory of Pathology, National Cancer Institute, Bethesda, MD) was inspirationally helpful over the years by participating in, as well as stimulating the development of, research programs, scientific forums, and workshops (including the present) aimed at greater understanding of neoplasias, their causes and effects on animal species (including humans), and their effect on the quality of human life. We also appreciate the valuable ideas and suggestions of Dr. John C. Harshbarger (Registry of Tumors in Lower Animals, Department of Pathology, George Washington Medical Center, Washington, DC, formerly of the Registry of Tumors in Lower Animals, Museum of Natural History, Smithsonian Institution, Washington, DC) on the theme of the workshop and the development of specific areas of discussion to be included. Dr. Harshbarger has been an incomparable source of knowledge and information that he unselfishly shares with all who have the need, including many of those who participated in this workshop.

Special recognition is due Ms. Karen Hayman, (Oxford Cooperative Laboratory, National Marine Fisheries Service, Oxford, MD) for valuable assistance during the organizational phase of the workshop.

ABSTRACT

These proceedings of the 1992 International Workshop on Invertebrate Neoplasia provide reports that summarize--comparatively--oncogenic events and processes as they occur in several invertebrate forms. It is now clear that some regulatory genes operating during development are common to a wide array of vertebrates and invertebrates, including *Drosophila*, mollusks, sponges, and yeasts. Furthermore, these evolutionarily conserved genes (oncogenes) can also play a critical role in carcinogenic processes and their consequences. The subject of field and controlled laboratory exposure of invertebrates (particularly mollusks) to environmental pollution received considerable emphasis, including discussions on the ability of some manufactured compounds and their derivatives to alter or activate genes that then become responsible for tumorigenesis. The role of exogenous viruses in invertebrate neoplasia was also discussed, as were retroviruses or RNA viruses and their involvement with carcinogenesis.

WORKSHOP INTRODUCTION

A. Rosenfield[1] and C.L. Reinisch[2], Convenors

Many perplexing issues confront cellular biologists, biochemists, and biophysicists when trying to understand the mechanisms behind the initiation and promotion of tumors in vertebrate forms. Those working in the various areas of pollution biology, comparative lower-animal oncology, and developmental biology, especially invertebrate pathologists, are similarly confronted--one could say confounded--by the same issues, perhaps even more so. Examples of these issues are:

1. **Some "exogenous" viruses or viral groups are oncogenic in vertebrate species.** Do these viruses or viral groups occur and behave in the same or parallel ways in invertebrate species? Have more recently discovered viruses (retrovirus)[3] been implicated as etiologic agents of neoplasia in invertebrates?

2. **Oncogenes and other regulatory genes apparently are associated with neoplasia, developmental abnormalities, and cellular transformations in vertebrate forms.**[4] These entities are also found in an unusual array of organisms belonging to many phyla, classes, and families, including invertebrates, even yeasts. What involvement do these molecules have in tumorigenic processes in invertebrates?[5]

3. **Certain natural toxins, heavy metals, and anthropogenic organic compounds, when applied or fed to vertebrate forms, are known to transform cells or induce neoplasia in specific tissues and organs.** Do such chemicals or their derivatives exert their effects in the same way in invertebrates? If so, under what conditions and levels of exposure?

4. **Some neoplasias in vertebrates collected from aquatic environments appear to be the result of unexplainable spontaneous transformation of differentiated or mature cells. Transformed cells: (1) are polyploid; (2) undergo uncontrolled growth and spread or metastasize to other tissues; and (3) can be transplanted into other animals of the same species.** Do these same phenomena occur under field conditions in invertebrates?[6] If so, under what environmental, nutritional, behavioral, and chronological (age) conditions? Can invertebrates be used as sentinels, and is the occurrence of disseminated neoplasia useful for assessing environmental quality? What hypotheses can we formulate to explain clustering and/or stochastic distribution of neoplasia in invertebrate stocks sampled from polluted and non-polluted environments? What can we say about the roles of contagious agents and genetically predisposed neoplasia in invertebrates? Do we have any understanding of remission mechanisms in invertebrates?

5. As noted, **exposure of vertebrates to certain types and levels of chemical carcinogens, irradiation, and biological agents (viruses, transfected DNA segments, or genes) can bring about tumor initiation and genetotoxic effects in these life forms.** What is known about mutagenesis, teratogenesis, and carcinogenesis in invertebrates, and what does current information tell us about tumorigenic and abnormal developmental and differentiation processes in invertebrates? Can we relate any of this to environmental perturbations, contamination, and pollution, as associated with maintenance of biological diversity, human health, disease prevention, and biological control? Can invertebrates be used as models in studies of mutagenesis and carcinogenesis? What role, if any, does cell transformation play in immortality or the perpetual in-vitro cultivation of insect/non-insect invertebrate cells? Why are cases of neoplasia rarely or never found in some taxa or groups of invertebrates?

This introduction outlines (admittedly in a simplistic way) a workshop program that intends to take on an enormously complex subject, or series of subjects, concerned with comparative oncology and developmental biology. There will be areas of agreement, disagreement, comfort, and discomfort with interpretations and understanding among participants, including the audience, of the mechanisms associated with initiation and promotion of neoplasia and related conditions, such as abnormal development in invertebrates. This is to be expected in a workshop that lasts only 3 hr. Therefore, the range of issues cannot be explored fully. Some points will be clarified, yet many important issues (applied and basic) will probably not even be raised--for example, risk assessment and risk management approaches to prevent or mitigate neoplasia as it affects resource sustainability and loss of biological diversity.

Eradication systems will not be major topics for inclusion in these workshop proceedings, yet these subjects were noted and do merit as much attention as those outlined. We do not apologize for limiting, as we have, the range of possible topics that might otherwise satisfy the curiosity and thirst for facts and information about certain aspects of neoplasia by those in attendance. We do trust, however, that this workshop will help generate other workshops, symposia, conferences, and meetings that will address issues of invertebrate neoplasia and comparative oncology, and that will explore more fully the subjects that we are able to cover in this workshop.

ENDNOTES

1. Oxford Cooperative Laboratory, National Marine Fisheries Service, Oxford, MD.

2. School of Veterinary Medicine, Tufts University, North Grafton, MA.

3. The role of retroviruses in vertebrates has been known for some time. Although such infective particles are suspected to be present in some neoplastic lesions in clams, their isolation has not been demonstrated in invertebrates. Recently, however, an abstract of a report given during an aquaculture seminar presents information on the successful isolation of particles from softshells, *Mya arenaria*, with hematopoietic neoplasia. The particles were shown to demonstrate reverse transcriptase--a characteristic of retrovirus (Medina et al. 1993).

4. Levine (1984) compared the symmetries of the biological processes of embryogenesis and early development with those of tumor formation. The report noted that: (1) in normal embryonic development, there is regulated cell growth and division, whereas in cancer, there is unregulated cell proliferation; (2) in early stages of normal development, there is orderly adhesion of cells to cells, whereas in cancer, there is disorderly cell mass with irregular adhesion; (3) normal cells recognize their neighbors, and when in-vitro, become confluent and demonstrate topo-inhibition, whereas cancer cells are not inhibited by contact and eventually become invasive; (4) normal embryonic cells migrate widely before homing toward and remaining in their correct domain, whereas cancer cells demonstrate hypertrophy and metastases; (5) in normal development, cells are programmed to achieve a terminal differentiation, whereas in cancer, cells are driven toward dedifferentiation or undifferentiation; and (6) normal development eventually results in senescence (i.e., cells "know" when to age and die, even in in-vitro cell culture), whereas in cancer, cells are "immortal" and most malignant cell lines can be cultured limitlessly.

5. During preparation of these proceedings, an important paper by Van Beneden et al. (1993) was published that described the isolation of DNA from clam tumors that is able to transform mouse fibroblasts in-vitro, thus suggesting that a transforming gene (oncogene) is present in these tumor cells, from which the isolations were made. Attempts to identify the oncogene is in progress.

6. A comprehensive review of this subject as it pertains to bivalve mollusks was published as these proceedings were in preparation. See Elston et al. (1992).

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DISTRIBUTION OF SARCOMA IN THE COCKLE, *Cerastoderma edule*, AROUND THE IRISH COAST, 1982-91: IMPLICATIONS FOR POLLUTION ETIOLOGY[7]

E. Twomey[8]

INTRODUCTION

The discovery of a high incidence of disseminated sarcoma in the cockle, *Cerastoderma edule*, was made in Ireland in October 1982 (Twomey and Mulcahy 1984). A similar condition was also found in cockles in Britanny at the same time (Poder and Auffret 1986). Investigations of the sarcoma in Ireland concentrated on: (1) histopathology (Twomey 1987); (2) epizootiology in relation to such factors as locality, season, age, size, and sex (Twomey and Mulcahy 1988a); and (3) disease transmission (Twomey and Mulcahy 1988b). A second condition, a probable gonadal neoplasm (or arrested gametogenetic condition?), was also encountered. However, the rarity of the latter prevented any kind of epizootiological investigation.

Disseminated sarcoma consists of populations of very large, mitotically active, pleomorphic, highly invasive cells. It has a seasonal pattern of occurrence, with greatest prevalences in early summer and in autumn, and lowest prevalences in winter. It appears more common among females than males, and also in younger, smaller cockles. The sarcoma was demonstrated to be

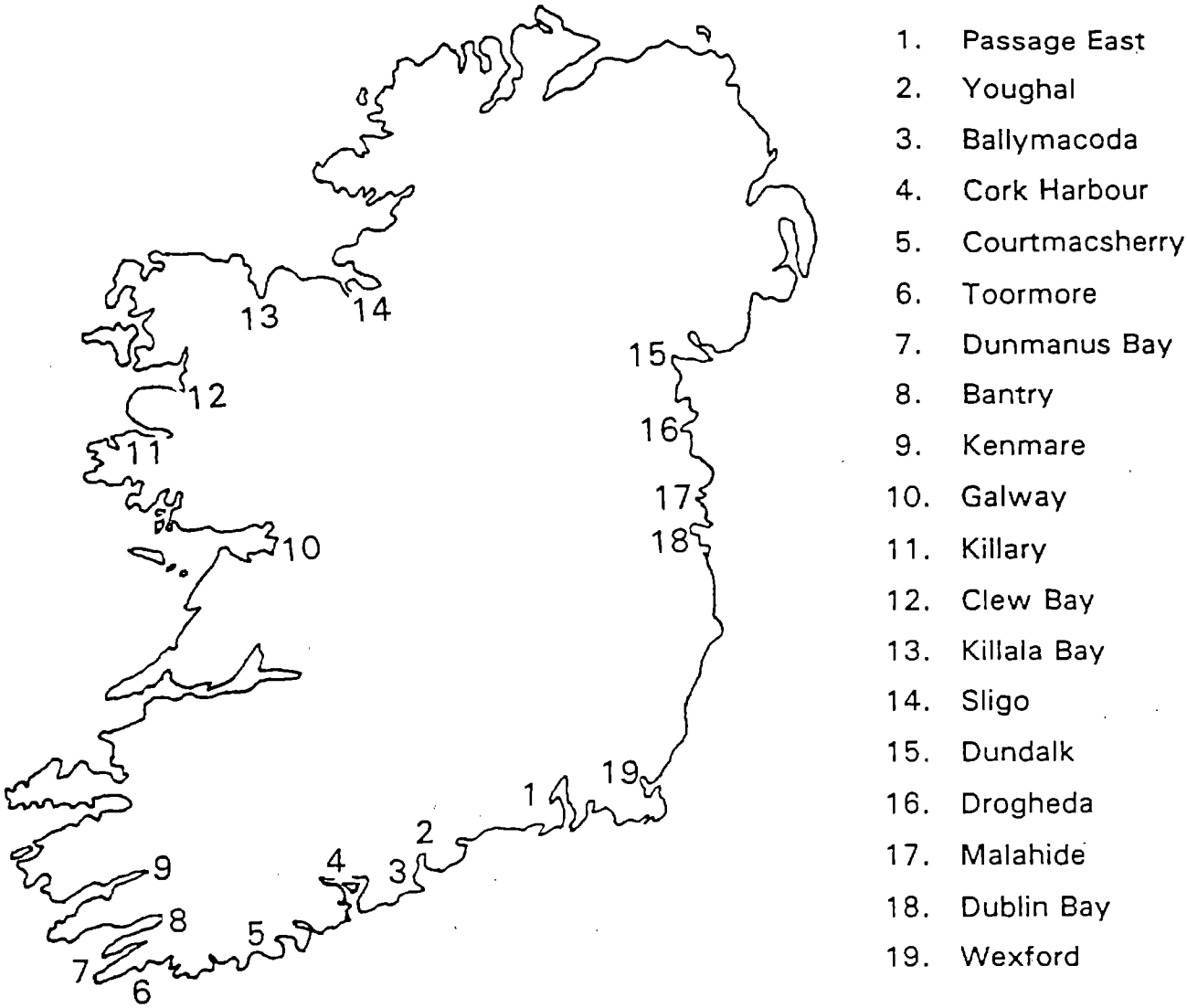

Figure 1. Map of Ireland indicating the cockle, *Cerastoderma edule*, collection sites along the south, west, and east coasts, July-August 1990

transmissible using whole-cell inocula, although preliminary attempts at transmission with cell-free filtrates failed.

Initially, over the 1982-86 period, the geographical localities surveyed were confined to the south coast of Ireland, centering on Cork Harbour, with sites to the east and west. (Figure 1). Apart from one record from a shore to the east (at Youghal), the disease was confined to shores within and west of the harbor, with prevalence in the harbor double that of other sites on the coast (31% vs. 14%). This pattern of distribution fit neatly with the prevailing coastal current which flows westward, and suggested that the harbor was a focus for the disease.

Cork Harbour is a busy international port and Ireland's most intensely industrialized estuary, with pharmaceutical, petrochemical, and steel milling concerns. Based on disease distribution, a pollution/stress etiology for epizootic neoplasia in bivalves was circumstantially supported: contaminants and/or infected larvae were being carried from the harbor to shores to the west. However, trace elements and polychlorinated biphenyls (PCBs) were either undetectable or below baseline levels in cockle samples from both within the harbor and from a disease-free site to the east. It was suggested that an undetected contaminant, or stress due to seasonal eutrophication, might be responsible for the disease.

To test the pollution etiology hypothesis, a national survey of cockle populations was undertaken in summer 1990. Distribution and prevalence of sarcoma around the Irish coast were investigated in localities selected to reflect various levels of anthropogenic effects.

MATERIALS AND METHODS

Over a 5-wk period, from 27 July to 29 August 1990, samples of cockle populations were collected from four sites within Cork Harbour and from 18 sites around the west, south, and east coasts of Ireland (Figure 1). Sarcoma was diagnosed either by histology, hemolymph smear, or both. All samples were fixed using 1% glutaraldehyde and 4% formaldehyde (1G4F), and processed for histology using standard methods (Howard and Smith 1983). For 15 samples, hemolymph smears were also prepared according to the histocytological technique described by Farley et al. (1986).

RESULTS AND DISCUSSION

During the 1990 survey, sarcoma was found at all sites within Cork Harbour and also on the south coast west of the harbor at Courtmacsherry. Apart from the repeat occurrence of one neoplastic specimen at Youghal, no sarcomas were observed at sites east of the harbor (Ballymacoda and Passage East). The original distribution pattern established for the south coast in 1985 thus appeared to hold. However, sarcoma was present at three of the eight west coast populations sampled (Kenmare, Bantry, and Clew Bays), with Bantry having a dramatically high prevalence of 94% (32 out of 34). In contrast, sarcoma was not detected among the five east coast samples.

Table 1. Prevalence of sarcomas in the cockle, *Cerastoderma edule*, at Ballymacoda, County Cork, Ireland.

Date	Sample Size	Prevalence (%)
May 1983 - March 1986 (17 samples)	386 (total)	0
August 1990	30	0
July 1991	48	17

A year later, July 1991, a sample of cockles collected in Ballymacoda had a 17% prevalence of sarcoma (8 of 48). Occurrence at this site is worthy of note because cockle populations had been repeatedly sampled from 1983 to 1986, and again in 1990 (Table 1), and sarcoma was never detected.

The distribution pattern for sarcoma around the coast of Ireland as revealed in this most recent survey bears no relationship to quality of environment. As a generalization, if the pollution stress hypothesis is correct, one would expect to find sarcoma in centers subject to major industrialization/human effect (e.g., Dublin Bay), and absent from clean, undeveloped sites on the west coast (i.e., Kenmare Bay, Bantry Bay, and Clew Bay). Moreover, if environmental stress factors are involved in epizootic neoplasia, one would expect that other species known to develop similar, high incidence disseminated sarcomas in other countries might also be affected. Yet, blue mussels, *Mytilus edulis*, in Bantry Bay (Wallace 1989) had a sarcoma prevalence of 0.1% (3 of 2925), in contrast to the 94% recorded there for the cockle.

Thus, the observed pattern of distribution, confined mainly to the southwest coast of the country, suggests an infectious etiology independent of environmental quality. The relative isolation of Ireland as a small island provides an unusual opportunity to monitor the distribution and further spread of an epizootic neoplasm in a marine bivalve.

ENDNOTES

7. This work was funded in part by the Irish Science and Technology Agency and by Angus Fine Chemicals as project HEIC/90/266, under the Higher Education - Industry Cooperation Scheme.

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XENOBIOTICS AND IMMUNE MECHANISMS OF THE EASTERN OYSTER, *Crassostrea virginica*: EFFECTS OF BENZO[a]PYRENE ON ACTIVITY, PHENOTYPE, AND MACROMOLECULAR SYNTHESIS OF HEMOCYTES

M. Faisal[9] and F.M. Hetrick[10]

BACKGROUND

Polycyclic aromatic hydrocarbons (PAHs) are common lipophilic contaminants in Chesapeake Bay, and an apparent relationship exists between exposure to these compounds and several ulcerative and neoplastic diseases of aquatic organisms (Huggett et al. 1987). Concentrations of PAHs in sediments of a subestuary of the bay, the Elizabeth River, Virginia, are among the highest anywhere in the United States (Bieri et al. 1986). Benzo[a]pyrene (B[a]P), a high-molecular-weight PAH compound with documented carcinogenicity and mutagenicity, has been found in great concentrations (up to 10,000 μg/kg) in mantle fluid of eastern oysters, *Crassostrea virginica*, and sediments of the Elizabeth River. B[a]P-metabolizing enzymes that transform the parent inert compound into both water-soluble non-toxic and DNA-reactive metabolites have been identified in several species of bivalve mollusks, including eastern oysters (Livingstone 1985). Bioavailability and direct effects of B[a]P on oyster cells remain, however, to be defined.

Like other bivalve mollusks, eastern oyster hemocytes play a major role in the host defense (Fisher 1988). To assess hemocyte activity, many endpoints have been proposed that measure alterations in morphological parameters, potency of processes governing phagocytosis, and viability. Most of these measurements, however, do not reflect the early aberration that a chemical carcinogen induces at the molecular level.

Most recently, investigations in our laboratory using radiolabeled precursors have demonstrated that oyster hemocytes are capable of synthesizing major cellular macromolecules (DNA, RNA, and protein) for up to 6 mo under in-vitro conditions. It was also demonstrated that RNA synthesis is 25-50X higher than DNA (Sami et al. 1991). In the course of a program aimed at developing sensitive biomarkers for exposure/effects of PAH, we compared the effects of B[a]P and its metabolites on eastern oyster hemocyte activity. Three parameters were used: macromolecular synthesis, the expression of concanavalin A (ConA) binding sites, and phagocytosis of yeast particles.

RESULTS

Effects of B[a]P

Hemocytes cultured for 10 hr in the presence of B[a]P resulted in a dose-dependent decrease of phagocytosis and of the percentage of hemocytes expressing ConA binding sites. Significant differences were observed at concentrations which were as low as 2×10^{-12} M B[a]P. The suppression in ConA expression was 3X higher than that of phagocytosis. Cell viability was, however, unaffected.

Both DNA and RNA synthesis showed a 7-9X augmentation at concentrations between 2×10^{-12} and 2×10^{-7} M B[a]P, and suppression at higher concentrations. Mitosis was observed in 5-12.5% of the cells exposed to concentrations of 2×10^{-11} to 2×10^{-9} M B[a]P.

Effects of B[a]P Metabolites

A similar trend to the above was obtained with B[a]P-7,8-diol; however, both stimulation at high dilutions and suppression at higher concentrations were more potent than those observed with the parent compound.

Phagocytosis, but not ConA-binding-site expression of hemocytes exposed to 3-hydroxy-B[a]P at concentrations of 2×10^{-4} and 2×10^{-7} M, was not significantly decreased. Greater concentrations of the 3-hydroxy-B[a]P (2×10^{-4} and 2×10^{-6} M), however, significantly decreased both phagocytosis and ConA-binding-site expression. Again, DNA and RNA synthesis increased with high dilutions (10^{-11} M), and was inhibited with higher concentrations. The inhibition of RNA was 2X higher than DNA.

Slight but statistically significant decreases in phagocytosis were observed with addition of B[a]P-4,5-epoxide and B[a]P-6,12-dione at 2×10^{-8} and 2×10^{-7} M. The 6,12-dione metabolite produced a slightly greater decrease (30-35%) than the epoxide metabolite. At these concentrations, metabolites did not affect cell viability. At a concentration of 2×10^{-6} M, however, both metabolites suppressed phagocytosis and ConA expression by 50% and 90%, respectively. At a concentration of 2×10^{-4} M, the epoxide and quinone produced a 100% and 85% decrease, respectively, in all parameters measured. In contrast, addition of B[a]P-9,10-diol and B[a]P-4,5-diol to hemocytes induced a major increase in DNA synthesis, but not in RNA synthesis. Mitosis was observed in 50% of the cells.

Is Cytochrome P-450 Responsible for B[a]P-induced Modulation of Hemocyte Activity?

Addition of an exogenous source of cytochrome P-450 (S9 fraction of rat or fish hepatocytes) did not induce any significant changes in the results obtained above.

In another set of experiments, [a]-naphthaflavone (ANF), a classical cytochrome P-450 blocker, was coincubated with hemocytes and B[a]P. However, concentrations of 10^{-7} - 10^{-5} M ANF blocked both augmentation and suppression induced by the parent compounds or the B[a]P-7,8 by 40-60%. ANF could not reverse the effects of other B[a]P metabolites.

CONCLUSIONS

1. Our data suggest that B[a]P has a direct effect on hemocyte activity, and that this effect was not due to extensive cytotoxic damage. These findings are in full agreement with our previous report (Faisal and Huggett, in press) that B[a]P has direct immunomodulatory effects on fish leukocytes.

2. As B[a]P is an inert compound, the effects it induced in hemocyte activities imply that hemocytes are capable of metabolizing B[a]P into its active metabolites.

3. Breakdown of B[a]P by hemocytes appears to be optimal/maximal since addition of exogenous sources of B[a]P-metabolizing enzymes (S9 fraction) did not augment modulation.

4. On the other hand, B[a]P breakdown by hemocytes could be partially attributed to the cytochrome P-450 enzyme system, because a partial reversal was achieved upon treating cells with ANF.

5. B[a]P at higher dilutions (similar to environmental levels) is capable of stimulating DNA and RNA synthesis and of inducing a transient phase of mitosis which was interrupted upon removal of B[a]P.

6. Among all parameters tested, RNA synthesis appears the most sensitive indicator for exposure to B[a]P or its metabolites.

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MOLLUSCAN TUMOR PATHOLOGY: ENVIRONMENTAL POLLUTANTS AND CARCINOGEN EXPOSURES

G.R. Gardner[11]

The appearance of spontaneous growths and malformations among mollusk populations attracted early investigators to study carcinogenesis in aquatic invertebrates. Their epidemiological observations led to experimental studies under controlled conditions. Beginning with studies in the 1930s, the first evidence linking progressive cellular phenomena to treatment with specific mammalian carcinogens was described by Labbe (1934) and Goldsmith (1937). Mollusks, coelenterates, platyhelminths, and aquatic arthropods have since been used in attempts to identify and understand fundamental processes involved with chemical exposures and subsequent effects (Krieg 1973; Sparks 1985).

These toxicologic characterizations of effects have led to the use of several model species that demonstrate susceptibility to chemical carcinogens. For example, platyhelminths exposed to coal tar implants (Goldsmith 1937), DDT, Sevin, Scarlet Red dye solutions (An der Lan 1962), B[a]P, and 3-methylcholanthrene (3MC) (Foster 1969) provide good validation of the relationship of chemical exposures and tumorigenesis. In one study, malignant reticulomas developed in planaria exposed to cadmium in solution as an initiator followed by phorbol ester (12-O-tetradecanoylphorbol-13-acetate) as a promoter (Hall et al. 1986). However, mollusks are the most widely used aquatic invertebrate species in studies designed to determine the toxic effects of chemicals on target organs and to characterize those chemicals' carcinogenic and mutagenic potential (Gardner 1993).

Mollusks for use in carcinogenetic research mostly include gastropods and marine pelecypods. Research has shown invasive epidermal neoplasms to develop on snails with the application of B[a]P and 3MC. However, bivalve mollusks are more sensitive and useful for a variety of investigations. Examples include invasive hemocytic and renal neoplasms produced in freshwater mussels in water-column studies using diethylnitrosamine and dimethylnitrosamine (Khudoley and Syrenko 1978). In more recent studies, neoplasms were induced in the eastern oyster exposed to chemical carcinogens present in contaminated sediment. In controlled experimental feeding studies, the eastern oyster provided good evidence of carcinogenic activity from diets of suspended sediments containing: (1) PAHs such as 3,4-benzopyrene and 1,2-benzanthracene; (2) an aromatic amine; (3) PCBs; (4) nitrosamine; and (5) heavy metals by mixture (Gardner, Pruell, and Malcolm 1992) and chemical class (unpublished data[12]). The results validated earlier test systems (including in-situ deployment) developed in previous studies to demonstrate carcinogenicity of several PAHs, quinones, carbazoles, and some metals in sediments from Black Rock Harbor (BRH), Bridgeport, Connecticut (Gardner, Yevich, Harshbarger, et al. 1991). Together, these oyster model experiments and supporting short-term tests using fractionated BRH sediment provide good evidence for presence of carcinogens and tumor promotors in nearshore and harbor aquatic environments (Malcolm et al. 1989; Gardner, Yevich, Harshbarger, et al. 1991).

Aquatic invertebrates living under natural conditions have been shown to accumulate potent carcinogens. In a study by Shimkin et al. (1951), PAH accumulations were demonstrated in apparently healthy barnacles attached to creosote-treated wooden pilings in a Corona Del Mar, California, marina. They tested the carcinogenic potency of benzopyrene by injecting extracted homologs from the barnacles under the epidermal surfaces of mice. Thirty-three percent of the treated animals developed subcutaneous tumors. These studies suggested that carcinogenic contaminants may be passed through the food chain via resistant species such as the barnacle, to species less resistant to induced toxic change. For example, analyses have shown that chemical carcinogens accumulate in blue mussels filter feeding on contaminated sediments. While the mussels showed resistance to accumulated toxic contaminants, winter flounder--when fed contaminated mussels--developed renal and pancreatic neoplasms and hepatotoxic precursor lesions (Gardner, Yevich, Harshbarger, et al. 1991).

A recent literature search (Gardner 1993) supports Hueper's (1963) belief that mollusk species will develop tumors through exposure to environmental agents. Among the systematic observations in natural conditions are those providing evidence of neoplastic responses from exposure to herbicide- and PAH-contaminated environments. In Maine and Florida, softshell populations are identified as being at risk of developing epithelial papillomas, germinomas, and pericardial mesotheliomas, from environmental exposure to herbicides (Gardner, Yevich, Hurst, et al. 1991); in the Yaquina Bay, Oregon, blue mussel population, a significant proliferative disorder is associated with B[a]P pollution (Mix et al. 1979). In-situ exposures are useful approaches for assessing effects of chemical and biological agents on populations in estuarine and coastal systems. In BRH and Quincy Bay (Boston Harbor), in-situ exposures helped to identify the importance of sediment with elevated PAH levels as an etiological source of neoplastic diseases of the eastern oyster.

Current studies focus on improved exposure and population effects assessments. In addition to use of traditional techniques, investigators now incorporate biochemical and molecular methods to study mechanisms through which chemicals interact to produce morphological responses. In biochemical studies, some annelid, crustacean, and mollusk species transform PAHs and other chemical carcinogens occurring in the environment, and initiate neoplastic responses (Lee 1981; Anderson 1985; Kuralec and Krea 1987; Stegeman and Lech 1991). At the molecular level, PAHs are mutagenic (Pittinger et al. 1987;

Malcolm et al. 1989), cause sister chromatid exchange (Mueller et al. 1991), and are damaging to DNA (Nacci et al. 1991). Further, transfection assays suggest chemical carcinogens induce malignant transformation through activation of cellular oncogenes. Because DNA from clam germinomas can transform mouse fibroblasts, results suggest a transforming gene is present in these tumor cells (Gardner, Van Beneden, and Blake 1992; Van Beneden et al. 1993). Investigations continue into the role of suppressor genes and activated oncogenes in these clam gonadal tumors, and into the possible link with the transcriptional activation activity of the aryl hydrocarbon receptor (Van Beneden 1994).

The complementary information derived by these observations under natural conditions and scientific experimentation suggests a role for mollusks and some other aquatic invertebrates in cancer causation research and environmental risk assessment.

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VIRAL TRANSMISSION AND TUMOR PROMOTION OF SARCOMA IN THE SOFTSHELL, *Mya arenaria*[13]

I. Sunila[14]

INTRODUCTION

Disseminated sarcoma of the softshell, *Mya arenaria*, occurs at epidemic levels at several sites on the East Coast of the United States. During disease progression, normal hemocytes are replaced by neoplastic cells. Neoplastic cells have pleomorphic nuclei and often abnormal mitotic figures. The disease leads eventually to death of the animal (Appeldoorn et al. 1984). Monoclonal antibodies have been developed against the sarcoma cell, and a hemocytic origin is suggested (Smolowitz et al. 1989).

INITIATION AND PROMOTION MECHANISMS

Initiation

Experimental studies suggest that icosahedral 100-nm viruses may be present in ova of sarcomatous softshells. Homogenated samples were clarified by centrifugation, supernatant passed through 0.45-ìm filters, and the filtrate fractionated by sucrose-gradient centrifugations. The light-scattering band was washed with buffer for negative-stained electron microscopy preparations.

A visible band could not be detected after centrifugation of control samples. Ultraviolet absorbance of ova preparations from diseased animals showed a peak at 260 nm which could not be detected in control samples or preparations of sarcoma cells (this study). Formation of a visible band and an absorption maximum at 260 nm of tissues from diseased animals are in accordance with results of Oprandy et al. (1981).

Protein analysis of the virus fraction with sodium dodecylsulfate - polyacrylamide gel eletrophoresis and western blotting and nucleic acid analysis with DNase, RNase, and restriction enzyme digestions are in progress.

Transportation

Transmission of sarcoma by injecting hemolymph from affected softshells in healthy recipients was first demonstrated by Appeldoorn et al. (1984). In this study, sarcoma was transmitted by injecting healthy animals with preparations of ova from diseased animals. Ripe gonads were stripped in 15‰ salinity sterile artificial seawater and washed on 44-ìm filters which retained ova (70-80 ìm), but passed sarcoma cells (10 ìm) through. The preparation was kept at -20°C for a year before the experiment.

Injection with the preparation from the filters resulted in sarcomas in 70% of recipients. The first positive samples were detected at 6 wk post injection. A control sample was prepared of male gonads passed through 5-ìm filters which retained sarcoma cells, but let sperm through. Injection with the preparation passing through the filter did not result in sarcomas in recipients. Efforts to demonstrate viral transmission or transfection with hemolymph preparations have failed.

I conclude that softshell sarcoma can be transmitted in two different ways: cell transplantation after injecting a hemolymph sample, and viral transmission after injecting the ova. Transportation via ova is the likely mechanism for infection in the field (Figure 2).

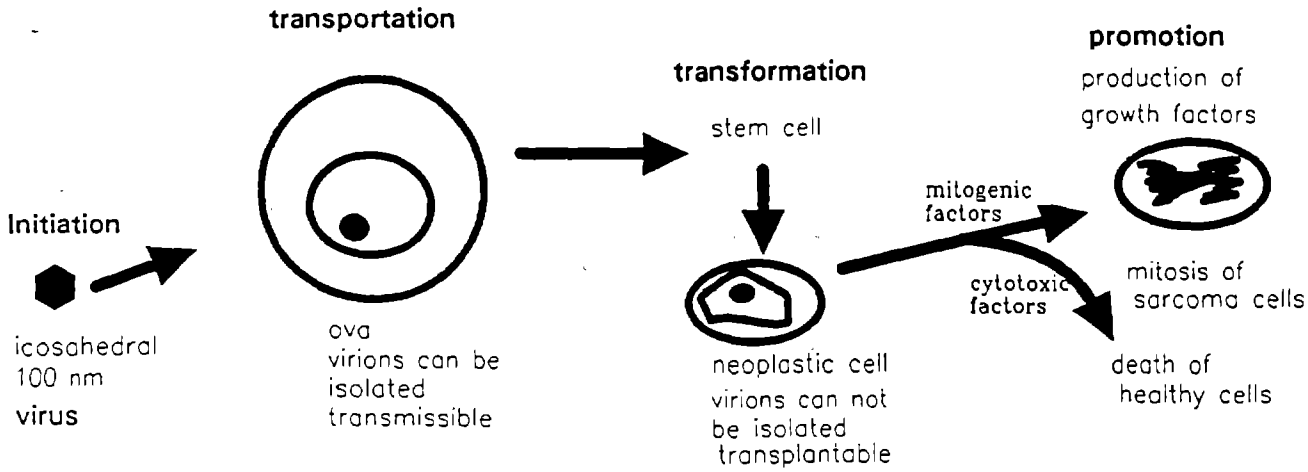

Figure 2. Diagrammatic presentation of initiation and promotion mechanisms in sarcoma of the softshell, *Mya arenaria*

Transformation

Viruses can use a cell (ova) for maturation and transportation other than the cell which they transform (cell of origin of the neoplastic cell, presumably a hemocytoblast). This mechanism has been demonstrated also in avian leukosis/sarcoma group (Purchase and Payne 1984).

Promotion

According to Sunila (1992), there are tumor-promoting proteins in hemolymph sera of sarcomatous softshells. Sarcoma cells were isolated and administered to recipients at the same cell density in different sarcoma-protein-free diluents: seawater, serum from normal animals, heat-treated sarcoma serum, or protease-digested sarcoma serum. Transmission in these groups was significantly slower than in the group where sarcoma cells were administered in intact sarcoma serum.

Tumor-promoting factors were heat-sensitive proteins. These tumor-promoting factors are analogous to growth factors demonstrated in malignant cells of higher organisms.

Basically, there are two possible mechanisms for oncogenic transformation when a virus is involved. Virus brings its own active oncogene into the cell, or it activates host-cell oncogenes while incorporating in the genome. Growth factors are coded by oncogenes (Burck et al. 1988).

Tumor-promoting factors in softshell sarcoma might be mitogenic to sarcoma cells. Mitotic index in the beginning of infection is very high (10-99), declining to 1-2 at the terminal stages of sarcoma (Sunila 1991). Injection with normal hemocytes administered in sarcoma serum caused mortality, but not sarcoma transmission, suggesting presence of cytotoxic factors in sarcoma serum (Sunila 1992).

According to Sunila and Dungan (1992), there are quantitative and qualitative differences in hemolymph serum proteins between sarcomatous and healthy softshells. Sarcomatous animals had unique 23-, 45-, and 54-kDa serum proteins, whereas healthy animals had unique 24, 103, and 104 kDal serum proteins. During disease progression, sarcoma-specific proteins appeared while normal proteins disappeared. Some sarcoma-associated proteins may have tumor-promoting or cytotoxic functions described above.

EVENTS LEADING TO DEATH OF A SARCOMATOUS SOFTSHELL

At the terminal stage of softshell sarcoma, hemolymph is strongly hypoxic. In the beginning of infection, partial pressure of oxygen in the hemolymph does not differ from control animals, but declines as the sarcoma advances and falls finally below the detection limit (Sunila 1991). Hypoxia is generally

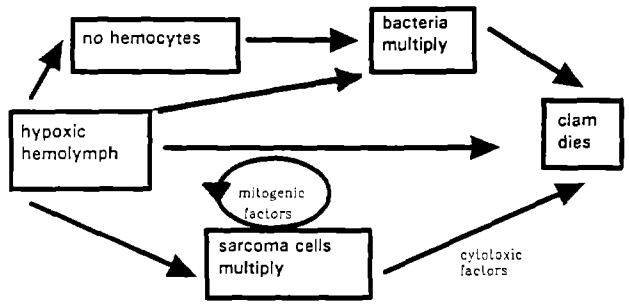

Figure 3. Summary of events leading to death of a sarcomatous softshell, *Mya arenaria*

believed to be a characteristic state of tumors in rapid growth (Chance et al. 1988). Low partial pressure of oxygen is tolerated by malignant cells due to their enchanced ability to utilize fermentation. During continuous hypoxia of the hemolymph, sarcoma cells are able to replace normal hemocytes.

Sarcoma cells might produce mitogenic growth factors which stimulate other sarcoma cells to multiply (Sunila 1992). When normal hemocytes are replaced by sarcoma cells, both cellular and humoral defense mechanisms are impaired. Beckmann et al. (1992) demonstrated that sarcoma cells, which they call diseased hemocytes, are unable to adhere and ingest yeast. Hypoxia in the hemolymph facilitates multiplication of bacteria which sarcoma cells are not able to phagocytize.

Also, humoral defense is impaired. Hemocytes produce lysosomal enzymes, cytotoxic molecules, and agglutinins, which are released in the hemolymph. According to Sunila and Dungan (1992), some proteins associated only with serum from healthy softshells may be involved in the humoral defense system. Cytotoxic factors in the hemolymph sera from sarcomatous softshells, as demonstrated by Sunila (1992), might originate from bacterial endotoxins and exotoxins. They might also be secreted by sarcoma cells in the way some malignant cells secrete proteolytic enzymes, thereby contributing to their invasive or metastatic nature.

A summary of events leading to death of a sarcomatous softshell is given in Figure 3.

ENDNOTES

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CAUSAL INVOLVEMENT OF DIFFERENTIATION GENES IN INITIATION OF CANCER IN *Drosophila melanogaster*

E. Gateff[15]

Twenty tumor suppressor genes, when mutated, cause in one step the malignant and/or benign transformation of specific cell types in *Drosophila* embryos and larvae (Gateff 1978; Gateff and Mechler, 1989). The wild-type alleles of these genes are instrumental in development and cell differentiation. Loss of gene function arrests differentiation, thus promoting uncontrolled malignant or benign growth resembling in all aspects that of vertebrate malignant and benign tumors.

Only one embryonic mutation is presently known to induce tumorous growth. This is the temperature-sensitive shibire gene mutation. Temperature shifts to 29°C from 4th to 14th hour of embryonic life transform the entire embryo into a tumorous mass (Williams 1981). Molecular analysis shows shibire to encode multiple forms of dynamin, a guanosine-triphosphate-dependent protein which has been implicated in endocytic protein sorting (Poodry 1990; Chen et al. 1991; van der Bliek and Meyerowitz 1991).

Five of the 20 *Drosophila* tumor suppressor genes transform the female germ-line alone, and another two mutants render both the female and male germ cells tumorous (Gateff and Mechler 1989; McKearin and Spradling 1990). The mutations interfere with early germ-line development, resulting in the case of the above five mutants in tumorous egg chambers filled with numerous cystocytes. In the mutants affecting both sexes, development is interrupted at an even earlier stage, showing ovaries and testes filled with oogonia and spermatogonia-like cells. All gonial cell tumors are benign and cause sterility. The *otu* gene has been cloned and shown to be required in germ-cell differentiation (Steinhauer and Kalfayan 1992).

Seven mutations induce tumors in the larvae. They affect adult primordial cells such as the imaginal disks and the neuroblasts in the presumptive adult optic centers in the larval brain. Five further mutations transform the phagocytic blood cell type, the so-called plasmatocytes. Two of these genes have been cloned and their functions analyzed. The disk-large tumor suppressor gene encodes a guanylate kinase homolog localized at septate junctions (Woods and Bryant 1991).

The function of the lethal (2) giant larvae gene is less clear, since it shows no homology to any functionally defined protein. It is located on the inner surface of the cell membrane (D. Stand and B.M. Mechler, personal communication[16]).

Four additional genes are being cloned in my laboratory: (a) the imaginal-disk tumor suppressor gene 1 (2) tumorous imaginal disks (Kurzik-Dumke et al. 1992); (b) the benign (2) gonial-cell neoplasm gene (Gateff 1982); (c) the lethal (3) malignant blood-cell neoplasm gene (Shrestha and Gateff 1986); and (d) the lethal (3) malignant brain tumor gene (l(3)mbt) (Gateff et al., in press). All genes show an early embryonic expression which is responsible for normal development of the respective cells. Shift-up experiments of animals belonging to the temperature-sensitive allele of the l(3)mbt mutant (i.e., *ts* 1(3)mbt) show likewise an early embryonic, temperature-sensitive period responsible for tumor suppression. The gene is constitutively expressed, having multiple function for development of the nervous system and female germ line (Gateff et al., in press).

A further interesting group of genes, which causes imaginal-disk overgrowth without affecting differentiation capacity of cells, is also intensely studied. One member of this family, the 1(2)fat gene, codes for a cadherin-like protein (Mahoney et al. 1991).

The functional analysis of tumor suppressor genes of *Drosophila* is well underway and certainly will yield basic information on primary causal events leading to tumori-genesis, not only in *Drosophila*, but also in vertebrates.

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INVERTEBRATE *c-myc* AND EVOLUTION OF GROWTH (CELL-CYCLE) REGULATION

A.G. Marsh[17]

Although invertebrates comprise 99% of animal species, we know far less about how their cell division rates are regulated than we do for vertebrates. In single-cell eukaryotes, cell division is tightly correlated with nutrient availability. At the other end of the evolutionary spectrum, in higher vertebrates, homeostasis achieved by the mammalian body plan has replaced that primary nutritional control with numerous hormonal and growth-factor signals to limit mitotic proliferative events to particular developmental stages, seasonal periods of growth, or specific target tissues. So where do invertebrates lie between these two evolutionary extremes? We don't know. It is evident that despite the complexity of biochemical and genetic controls present in mammals, some cell types can inappropriately become mitotically proliferative. An interesting question in considering how invertebrates regulate cell division rates is whether or not more or fewer cell-cycle control mechanisms are important in tumor etiology.

Myc is the most prevalent oncoprotein present in proliferating cells. As such, the expression of the *c-myc* gene provides a sensitive assay for mitotic activity under experimental conditions. In general, I am interested in describing the nutritional and growth-factor components that regulate expression of invertebrate cell-cycle control genes to determine the relative level of "complexity" regulating mitotic proliferative events. To date, only one of the 186 Myc sequences listed in GeneBank is from an invertebrate (starfish; Walker et al., in press). Currently, I have putative *c-myc* polymerase chain-reaction (PCR) fragments that have been subcloned and sequenced from oyster, zebra mussel, two polychaetes, and a nematode. An oyster cDNA library has been screened with the oyster PCR product, and a 2.3-kb clone has been isolated and is currently being sequenced. A polychaete cDNA library is being synthesized for Myc screening as well. The nucleotide sequence of full-length cDNA clones should verify whether or not the PCR fragments are authentic vertebrate-Myc homologs. Insulin-like growth factors (IGFs) and insulin PCR products have been successfully amplified in oyster (M.J. Shamblott, in progress[18]) and polychaete (A.G. Marsh), and will be used to screen the libraries. Whole-animal growth studies with oyster, zebra mussel, and polychaetes are currently being conducted to establish "normal" levels of *c-myc* expression.

A proliferative cell line from a polychaete has been isolated in tissue culture to be used as an experimental system for investigating mitogenic potential of different chemical compounds via expression of *c-myc* and growth factors (insulin, IGFs). A fusion transgene consisting of the human *c-ets* gene promoter and [b]-galactosidase reporter (courtesy of National Cancer Institute) has been successfully expressed in the polychaete cell line and suggests that polychaetes may have a vertebrate *c-ets* homolog functional during cell division. Use of transgene constructs may facilitate future gene expression studies. Dr. Chen's laboratory, at the Center of Marine Biotechnology, is currently developing a transgene cassette with the bacterial LuxAB reporter (luciferase). With this construct, expression of a target gene could be measured by quantifying light emission. A practical application of this research would be to screen potential mitogens quickly by identifying chemical agents capable of promoting cell proliferation (via increased Myc or growth-factor synthesis) without having to assay tumor development in lengthy animal culture experiments. Overall, our gene expression studies should provide information about regulatory mechanisms that exist in different invertebrates to control cellular proliferation. This information is vital to understanding etiology of invertebrate neoplasias.

ENDNOTES

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ROLE OF SPECIFIC GENE PRODUCTS IN NEOPLASIA CAUSED BY LOSS OF FUNCTION OF *Drosophila* TUMOR SUPPRESSOR GENES

E. Woodhouse[19], E. Hersperger[19], W. Stetler-Stevenson[20], P. Steeg[20], L. Liotta[20], and A. Shearn[19]

There are several recessive lethal mutations in *Drosophila* leading to overgrowth of neural and/or imaginal cells (Gateff and Mechler 1989). The overgrowth phenotype caused by such mutations has been described as tumorous, or neoplastic, in those cases where the overgrowth is accompanied by loss of normal structure and of capacity for normal differentiation (Gateff and Mechler 1989). Since the absence or reduced level of function of the genes identified by these mutations leads to growth of tumors, the genes have been called "tumor suppressor genes."

One of the best studied tumor suppressor genes of *Drosophila* is lethal giant larvae (*lgl*). Null mutations of *lgl* cause a mutant phenotype in brains, imaginal disks, and ovaries, i.e., in mitotic, post-embryonic cells (Gateff and Mechler 1989). The *lgl* gene has been cloned by chromosome walking (Mechler et al. 1985). It encodes both 78 and 127 kDa proteins (Gateff and Mechler 1989).

The brains in *lgl* mutant larvae are overgrown and highly disorganized, but no invasion occurs in situ (Gateff and Schneiderman 1974). When fragments of mutant brains are transplanted into wild-type adult hosts, however, they give rise to invasive tumors which ultimately kill the hosts (Gateff and Schneiderman 1974). In this respect, they are analogous to mammalian brain tumors which rarely metastasize in situ, but give rise to highly metastatic tumors when transplanted (Katz and Liotta 1986).

To illustrate the invasiveness of cells from these mutant brains, we have taken advantage of the availability of an enhancer trap insertion (a gift from M. Meise and W. Janning[21]) that causes [b]-galactosidase to be expressed in brains. Like normal brains when transplanted into adult hosts, brains from larvae carrying this insertion neither grow appreciably nor do they dissociate. After culture, they can be removed from the hosts as intact masses which can be identified as donor tissue by their expression of [b]-galactosidase activity. However, when this insertion is carried in an *lgl* mutant background, the results can be dramatically different. As illustrated by the widespread distribution of cells expressing [b]-galactosidase, the *lgl* mutant brains grow extensively, do not remain as intact tissues, and, as first reported by Gateff and Schneiderman (1974), invade host tissues. The only endogenous [b]-galactosidase in the adult host is in the digestive system.

We have begun to investigate whether the invasive phenotype caused by mutations of the *lgl* gene is mediated by biochemical changes that are similar to those that have been observed in metastatic mammalian tumors. Using antibodies directed against mammalian type IV collagenase and tissue inhibitor of metalloproteinase-2 (TIMP-2), we have observed, first, that *Drosophila* larvae have cross-reacting proteins and, second, that in *lgl* mutant larvae the level of putative type IV collagenase protein of *Drosophila* is increased compared to normal larvae, and the level of putative TIMP-2 protein is decreased. If the cross-reacting proteins are indeed *Drosophila* homologs of type IV collagenase and TIMP-2, then the net effect of this pair of changes would be to increase dramatically the type IV collagenase activity in mutant larvae. The direction of these changes is identical to that observed in mammalian tumors (Liotta et al. 1990).

In *Drosophila* larvae that are homozygous for a mutation which causes invasive neoplasms, we have documented biochemical changes that are similar to biochemical changes occurring in metastatic mammalian cells. This raises the possibility that at the molecular level as well as at the cellular level, invasive *Drosophila* cells are similar to metastatic mammalian cells (Biggs et al. 1990).

There are other identified genes in which mutations cause a phenotype similar to *lgl*, including lethal (1) disks large, lethal (2) brain tumor, and lethal (3) malignant brain tumor (Gateff and Mechler 1989). It is important to examine larvae that are homozygous for these mutations to see if they have the same biochemical changes as those we have observed in *lgl* mutant larvae. These biochemical changes that we have documented are correlated with the *lgl* mutant phenotype. To show that the relationship goes beyond the observed correlation, the gene product of the putative *Drosophila* type IV collagenase and TIMP-2 could be mutated and assayed in an *lgl* mutant background for suppression of the invasive phenotype, or could be ectopically expressed in an *lgl* mutant background and assayed for enhancement or suppression of the invasive phenotype.

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DIVERSITY OF TYPES OF NEOPLASMS AND DIVERSITY OF AFFECTED SPECIES AMONG INVERTEBRATES: EXAMPLES FROM THE COLLECTION IN THE REGISTRY OF TUMORS IN LOWER ANIMALS

E.C. Peters[22]

The Registry of Tumors in Lower Animals (RTLA)[23] was established in 1965 as a cooperative project between the National Cancer Institute (Contract No. N01-CB-33874) and the Smithsonian Institution to study tumors in invertebrate and poikilothermic vertebrate animals. The RTLA's primary mission is collection, identification, and preservation of specimens with neoplasms or related disorders obtained from natural habitats, zoos, aquaria, and laboratory experiments. The RTLA staff collaborates on experimental studies and surveys of the nature, incidence, and etiology of these diseases as performed by investigators at federal and state agencies, universities, and private industrial or other research facilities. Materials submitted with each case, in addition to further diagnosis and description, are archived for easy retrieval. The RTLA also maintains a complete library of reprints and books relating to neoplasms in these organisms. RTLA staff analyze, correlate, and disseminate information on the cases in the RTLA, mainly either as peer-reviewed publications and/or as presentations to professional or general scientific meetings and workshops.

Records compiled by the RTLA are utilized to: (1) improve our understanding of biology and etiology of cancer as it occurs at various phylogenetic levels; (2) discover organisms that may be useful in cancer research; (3) locate possible vectors or reservoirs of oncogenic viruses; (4) identify sentinels of carcinogens in the environment; and (5) explore other factors relating to susceptibility of certain organisms to neoplasia. Materials archived in the RTLA are available to qualified investigators for comparative study upon written request to the Director, Dr. John C. Harshbarger (Registry of Tumors in Lower Animals, Department of Pathology, George Washington Medical Center, Washington, DC 20037; telephone = 202-949-1056; fax = 202-294-2618). Overall, the RTLA serves as a clearinghouse for information on lower animal neoplasia, and has provided a valuable service to the scientific community by linking researchers around the world to broaden our understanding of cellular proliferative disorders.

Two decades ago at a symposium on "Neoplasms and Related Disorders of Invertebrate and Lower Vertebrate Animals," Dr. Clyde Dawe observed that "There exists a gap, phylogenetically viewed, in our knowledge of neoplasms and related disorders among the animals proximal to fishes in time of evolutionary origin" (Dawe 1969). He outlined criteria for study of invertebrate tumors, including anatomical and biological characteristics, and reviewed the difficulties of recognizing neoplastic diseases in organisms which possess different tissue systems and cells. Many tumor-like lesions and cell proliferations in invertebrates result from injuries or wound repair processes, so there are often problems in assessing neoplasia in these organisms. Other papers at that symposium discussed the occurrence of a variety of cellular proliferative disorders in brittlestars, insects and other arthropods, annelids, planarians, peanut worms, bivalve mollusks, hydrozoans, and protozoans. A number of definite neoplastic growths have now been recognized in invertebrates.

Over the years, the RTLA collection has grown to over 5500 accessions (including acquisition of the Registry of Marine Pathology from the National Marine Fisheries Service), with representatives of most major phyla and a global distribution. Approximately one-fourth of the cases are from invertebrates. In numbers of animals, however, invertebrates represent closer to one-third or one-half of the specimens, since many accessions, particularly the early ones, have multiple animals per case. Approximately one-half of all cases are neoplasms, but only one-

Table 2. Invertebrate accessions up to No. 5550 in the Registry of Tumors in Lower Animals

Phylum	No. of Cases	Neoplasms	Developmental Anomalies	Other	Problematic Lesions
Porifera	3	0	0	2	1
Cnidaria	107	17 (6?)	2 (3?)	78	1
Platyhelminthes	12	2	0	8	2
Nematoda	2	0	1	1	0
Acanthocephala	1	0	0	1	0
Mollusca	859	418 (13?)	13 (2?)	411	2
Annelida	33	2	2 (1?)	28	0
Arthropoda	233	16	3 (1?)	209	4
Echinodermata	44	24	1 (1?)	10	8
Prevertebrates	11	0	0	11	0
TOTAL	1305	498	30	759	18

third of invertebrate cases are neoplasms (Table 2). The remaining cases include examples of infectious diseases, parasites, injury and wound repair, developmental anomalies, nutritional disorders, and normal tissues. Those cases in the "Problematic Lesions" category were either in too poor condition for diagnosis (as a result of improper fixation, artifacts, or other factors) or require further study. Numbers of cases with question marks (in parentheses) indicate uncertainty in the nature of some lesions; their diagnosis may change as our understanding of invertebrate cells and tissues evolves.

Many cases represent published independent studies and were contributed by the investigators, including participants in this workshop. Invertebrates used in these disease studies have been largely limited to species associated with commerce, agriculture, or aquaculture, and those adapted for laboratory investigations. Studies of neoplasia have focused on the etiologies of disseminated sarcomas and germinomas of marine bivalve mollusks and the genetic basis of neuroblastoma, hemic sarcoma, imaginal disk, and ovarian neoplasms in the fruit fly *D. melanogaster*.

While research has been directed to the most prevalent neoplastic disorders in these groups, it is important to note, however, that unusual neoplasms also occur in these phyla (Mollusca and Arthropoda) and in members of other phyla. For example, adenomas, epithelial papillomas, fibrosarcomas, gonadoblastomas, neurofibromas, and pericardial mesotheliomas are among the types of neoplasms that have been found in various species of bivalves; and embryonal carcinoma, hematopoietic neoplasia, and papillomas occur in shrimp. Some examples of neoplasms observed in other phyla include calicoblastic epitheliomas in scleractinian corals (Cnidaria), a ganglioneuroblastoma in a parasitic flatworm (Platyhelminthes) from a shark, and a neoplasm of sclerocytes (cells that form the test) of sand dollars (Echinodermata). Causes of these neoplastic diseases are still under investigation, but may be related to various pollutants, viruses, or genetics.

Despite gaps in the RTLA collection, the existence of various types of neoplasms suggests that further study may provide additional information on oncogenesis and the etiologies of neoplasms in diverse cell types. The challenge will be to adapt new methods and techniques in cellular and molecular biology and chemistry to interpret these cases properly, as well as to determine why neoplasms have not been found in some phyla. Future insights on the controlling mechanisms of cellular proliferation in these and other invertebrates may prove to be necessary to understand the neoplastic disease process.

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22. Tetra Tech, Inc., Fairfax, VA.

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APPENDIX: 18 March 1995 update to "Diversity of Types of Neoplasms and Diversity of Affected Species among Invertebrates: Examples from the Collection in the Registry of Tumors in Lower Animals"

The RTLA has added 87 new invertebrate cases to its collection, bringing the total to 1392. Forty-six cases were contributed in the phylum Cnidaria, including five calicoblastic epitheliomas in *Acropora clathrata* from the Gulf of Oman, and 41 gorgonian and scleractinian coral cases having a variety of bacterial, algal, protozoan, or other types of lesions. In the phylum Mollusca, 25 cases with neoplasms and 15 cases with parasites or other lesions were submitted for diagnosis. One sea star, phylum Echinodermata, was submitted, with a possible neoplasm, and is currently being studied.

In summary, as of mid-March 1995, the RTLA has a total of 6130 cases archived, 529 invertebrate neoplasm cases, 30 developmental anomalies cases, 815 other disease cases, and 18 cases with problematic lesions.

TRANSMISSION STUDIES OF SARCOMA IN THE SOFTSHELL, *Mya arenaria*

S.M. McLaughlin[24]

Etiology of softshell, *Mya arenaria*, sarcomas has been attributed to a number of factors including: (1) viral, environmental, and physiologic stressors; (2) genetic factors; and (3) transplantation of cells. Softshell sarcomas have been transmitted in the laboratory by holding healthy animals under head tanks containing neoplastic animals, and through inoculation of neoplastic cells (Brown 1980; Appeldoorn et al. 1984; Farley 1989; McLaughlin et al. 1992). Studies recently conducted at the Oxford Cooperative Laboratory were designed to examine further the transmission of softshell sarcomas.

The purpose of the first such study was to determine whether temperature affected development of sarcomas in softshells. Sarcoma cells were collected by withdrawing hemolymph from animals with advanced sarcomas, and healthy animals were injected with the neoplastic cells. Animals were held at two temperatures in recirculating aquaria for 3 wk, and examined for sarcomas by histocytology and histology. The difference observed (Table 3) in sarcoma prevalences between the two groups suggests that temperature may affect development of sarcomas in softshells. This apparent effect may be due to physiological and metabolic activity of the clam being reduced at lower temperatures. Perhaps temperature also affects the infectious agent, if one is present.

In a second study, the viral etiology of softshell sarcomas reported by Oprandy et al. (1981) was further examined using a cell-free ultrafiltrate (McLaughlin et al. 1992). One group of healthy animals was inoculated with untreated neoplastic hemolymph collected from animals with advanced cases of sarcomas. A second group was inoculated with a cell-free ultrafiltrate which was prepared by sonicating a subsample of the neoplastic hemolymph. The sonicant was centrifuged and then filtered through a 0.22-µm membrane filter. A control group was inoculated with hemolymph collected from non-neoplastic animals. Experimental and control animals were held separately for 17 wk in flow-through aquaria at ambient salinity (10-12‰) and temperature (2-17°C). Histocytology and histology results (Figure 4) indicate sarcomas were transmitted to animals injected with untreated neoplastic hemolymph, with prevalences increasing from 6% at 6 wk to 41% at 17 wk. Sarcomas were not observed in animals injected with cell-free ultrafiltrate or in controls. The lack of disease transmission with the ultrafiltrate supports a non-viral etiology of disease transmission.

In a third study, transplantation of softshell sarcomas as suggested by Farley (1989) and Sunila and Farley (1989) was further explored. To determine whether animals actually release sarcoma cells into the water column in the natural environment, sarcoma and normal animals were placed in individual beakers containing 350-750 ml of ambient seawater. A 3-ml aliquot of water was sampled periodically in a chamber on a poly-L-lysine-coated slide (Farley et al. 1986). Fixed and stained slides were examined for presence of hemocytes and sarcoma cells. Results indicate that sarcoma cells are released by softshells into the water column and will, therefore, be available for filtration by softshells in the natural environment.

Table 3. Prevalence (both as proportion and--within parentheses--as percent) of sarcomas in softshells, *Mya arenaria*, inoculated with neoplastic cells and held at two temperatures.

	Holding Temperature			
Treatment	**12°C**		**18°C**	
Inoculation	10/30	(33%)	17/30	(57%)
	6/30	(20%)	14/30	(47%)
	6/30	(20%)	21/30	(70%)
TOTAL (avg.)	22/90	(24%)	52/90	(58%)
Control	0/30	(0%)	0/30	(0%)
	0/30	(0%)	0/30	(0%)
TOTAL (avg.)	0/60	(0%)	0/60	(0%)

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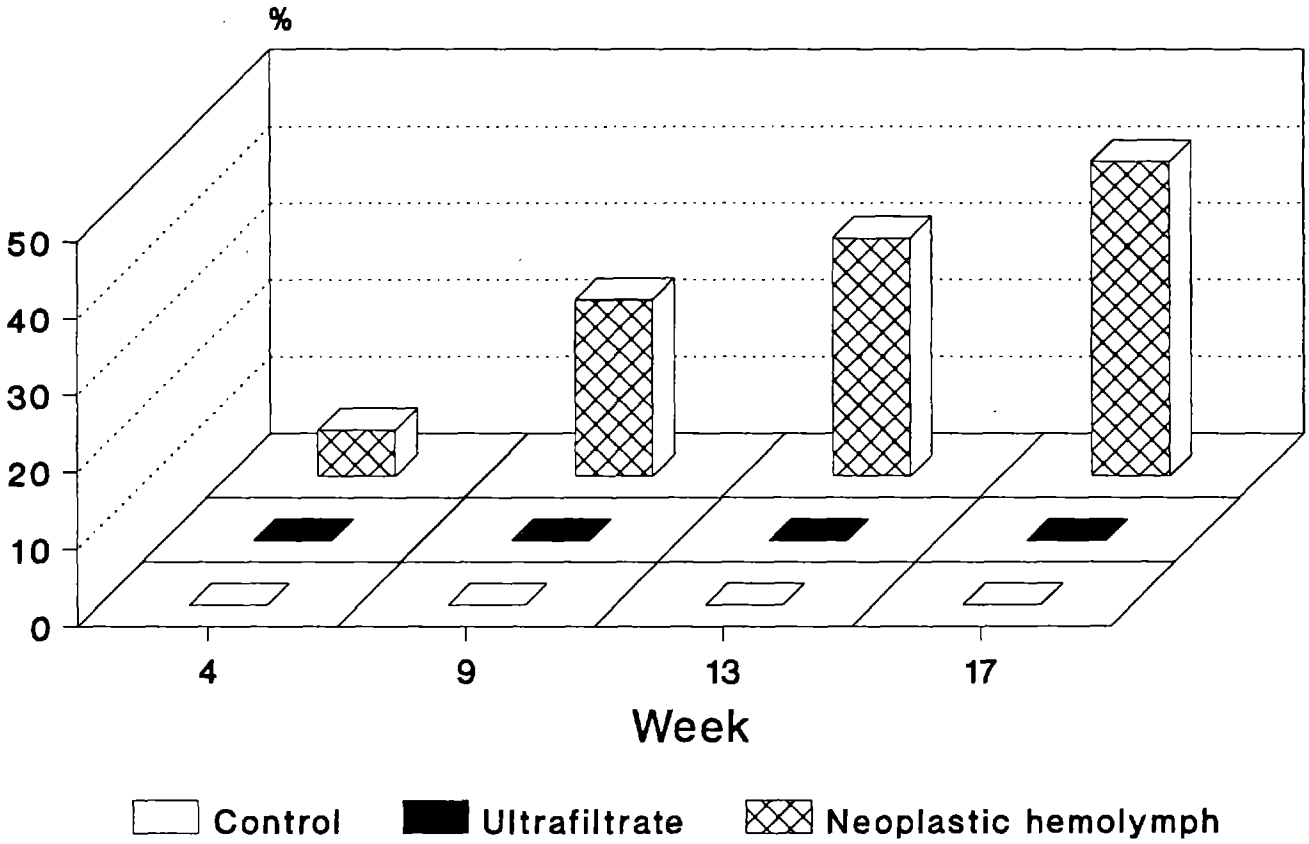

Figure 4. Prevalences over time of sarcomas in the softshell, *Mya arenaria*, inoculated with whole neoplastic hemolymph and an ultrafiltrate

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INITIATION AND PROMOTION OF HEMATOPOIETIC NEOPLASIA IN SOFTSHELLS, *Mya arenaria*, EXPOSED TO NATURAL SEDIMENT

D.F. Leavitt[25], D. Miosky Dragos[26], B.A. Lancaster[25], A.C. Craig[26], C.L. Reinisch[26], and J.M. Capuzzo[25]

Prevalence of hematopoietic neoplasia has been shown to be significantly higher in populations of softshells, *Mya arenaria*, collected from New Bedford Harbor (Fairhaven - New Bedford, MA) than in softshells collected from a control site in Little Buttermilk Bay (Bourne, MA) (Leavitt et al. 1990). New Bedford Harbor has a well documented history of contaminant inputs, including PCBs, petroleum hydrocarbons, and heavy metals (Summerhayes et al. 1977; Pruell et al. 1990). Yet researchers have had limited success in correlating contaminant history of a specific site to prevalence of hematopoietic neoplasia in softshells (Walker et al. 1981; Craig et al. 1989). In an attempt to study the relationship of contaminated sediment to processes influencing development of hematopoietic neoplasia, we conducted a laboratory experiment where softshells were exposed to either contaminated (from New Bedford Harbor) or non-contaminated (from Little Buttermilk Bay) sediment, and where the disease status of the animals was closely monitored.

Using a protocol modified from Goldsworthy and Pitot (1985), we collected softshells from Little Buttermilk Bay and diagnosed them for presence of hematopoietic neoplasia using an immunoperoxidase procedure (Smolowitz and Reinisch 1986; Leavitt et al. 1990). Those softshells diagnosed as free of disease were divided into two groups. One group was injected with 0.1 ml of 0.22-µm filtered seawater, whereas the second group was inoculated with 10^6 neoplastic cells (harvested from an animal with >99% neoplastic cells) in 0.1 ml of hemolymph. Hematopoietic neoplasia has been shown to be transmissible by injecting diseased cells into hemolymph of non-diseased bivalves (Twomey and Mulcahy 1988; Kent et al. 1991; Sunila 1992). In addition, softshells that had been initially diagnosed as neoplastic stage 1 (i.e., <15% of circulating hemocytes being neoplastic) were collected and placed into the experiment as a third treatment. Each of these treatment groups was randomly split into two subgroups and deployed into 2-gal plastic pots containing sediment collected from either New Bedford Harbor or Little Buttermilk Bay. Softshells were deployed at 10 individuals per pot to duplicate densities observed in the field. Pots were randomly placed in a concrete raceway where ambient seawater flowed as an open once-through system.

Six pots were retrieved per treatment (a total of 60 softshells per treatment) at six time intervals over 4 mo, with the exception of stage 1 neoplastic animals where only enough animals were collected to allow four monthly collections of three pots per treatment (30 animals). Animals were removed from the sediment, mortalities were recorded, and a hemolymph sample was collected for hematopoietic neoplasia diagnosis using the immunoperoxidase staining procedure.

Following experimental transmission of neoplasia, there was a distinct increase in both mortality and disease prevalence in those softshells which were exposed to neoplastic cells (Figure 5). This observation supports similar observations by Kent et al. (1991) in studying transmission of hematopoietic neoplasia in the bay mussel, *Mytilus trossulus*, and by Sunila (1992) in reporting on transmission of hematopoietic neoplasia in softshells. By the third month, the level of mortality (24.2% overall) had increased in all treatments, complicating interpretation of any treatment effects that might be attributable to disease processes (Figure 5). This experiment was initiated in early December and conducted during winter months when experimental animals were exposed to very cold ambient conditions. These conditions routinely cause die-offs in local softshell populations. Mean prevalence of hematopoietic neoplasia in the transmission treatment was 16.7% (±8.8% standard deviation) compared to 2.4% (±3.2%) disease prevalence in animals deployed following seawater injection.

There seemed to be little effect of contaminants from sediment on progression of hematopoietic neoplasia in those softshells initially diagnosed as neoplastic (Figure 6). Very few disease cases progressed to higher stages of neoplasia (i.e., >15% circulating neoplastic cells) in either sediment exposure. In addition, there was no indication of a sediment treatment effect in those animals that did show advances in neoplasia. The interesting observation one can make from these data is the number of animals showing remission of hematopoietic neoplasia. Cooper et al. (1982) reported that softshells with early low levels of hematopoietic neoplasia can undergo remission, where all diagnosable signs of the disease disappear.

In the present study, 32.1% of those softshells initially diagnosed with neoplasia showed no sign of disease following a 1-4 mo interval. This level of remission is similar to the level reported by Elston et al. (1988) for hemic neoplasia in blue mussels, *M. edulis*, in the state of Washington. In the present study, sediment exposure may have influenced remission during the first month, where a significantly higher number of softshells initially diagnosed with hematopoietic neoplasia showed no sign of disease following deployment in Little Buttermilk Bay sediment compared to those softshells deployed in New Bedford Harbor sediment. This difference is not evident from the second through fourth months, and this may be due to the cold environmental temperatures during the experimental period.

Overall, these results do not reflect the field observation of an increase in prevalence of hematopoietic neoplasia in softshells collected from New Bedford Harbor. If one were to divide the experiment into two time intervals, 0-4 wk and >4 wk, then one can observe some differences in the effect of sediment source during the early part of the experiment. At 1 mo, exposure to sediment from New Bedford Harbor resulted in a greater degree of disease transmission in softshells, whereas, at the same time

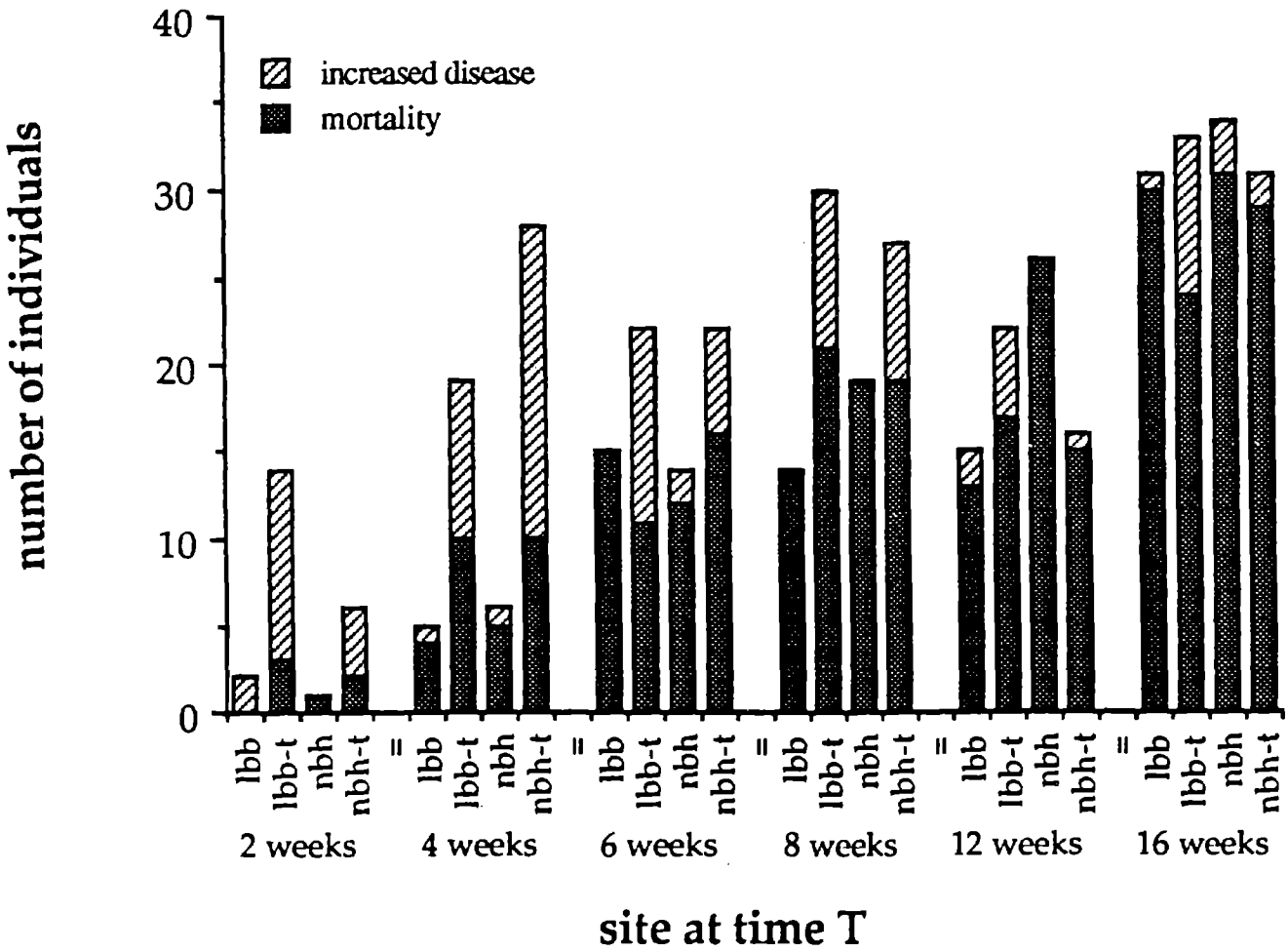

Figure 5. Change in health (i.e., death and/or increased disease) of softshells, *Mya arenaria*, inoculated with neoplastic cells or injected with filtered seawater and deployed in sediment from Little Buttermilk Bay or New Bedford Harbor (llb = softshells injected with filtered seawater and deployed in sediment from Little Buttermilk Bay; llb-t = softshells inoculated with neoplastic cells and deployed in sediments from Little Buttermilk Bay; nbh = softshells injected with filtered seawater and deployed in sediment from New Bedford Harbor; and nbh-t = softshells inoculated with neoplastic cells and deployed in sediments from New Bedford Harbor)

interval, exposure to sediment from Little Buttermilk Bay resulted in higher levels of disease remission. After the initial 4-wk experimental period, mortality rates increased with decreasing water temperature; these two factors may overshadow the disease effect in this experiment. To develop a more complete understanding of the role that environmental contaminants might play in development of hematopoietic neoplasia in populations of softshells, a series of experiments must be designed which encompasses the seasonal variation in environmental conditions that these populations experience.

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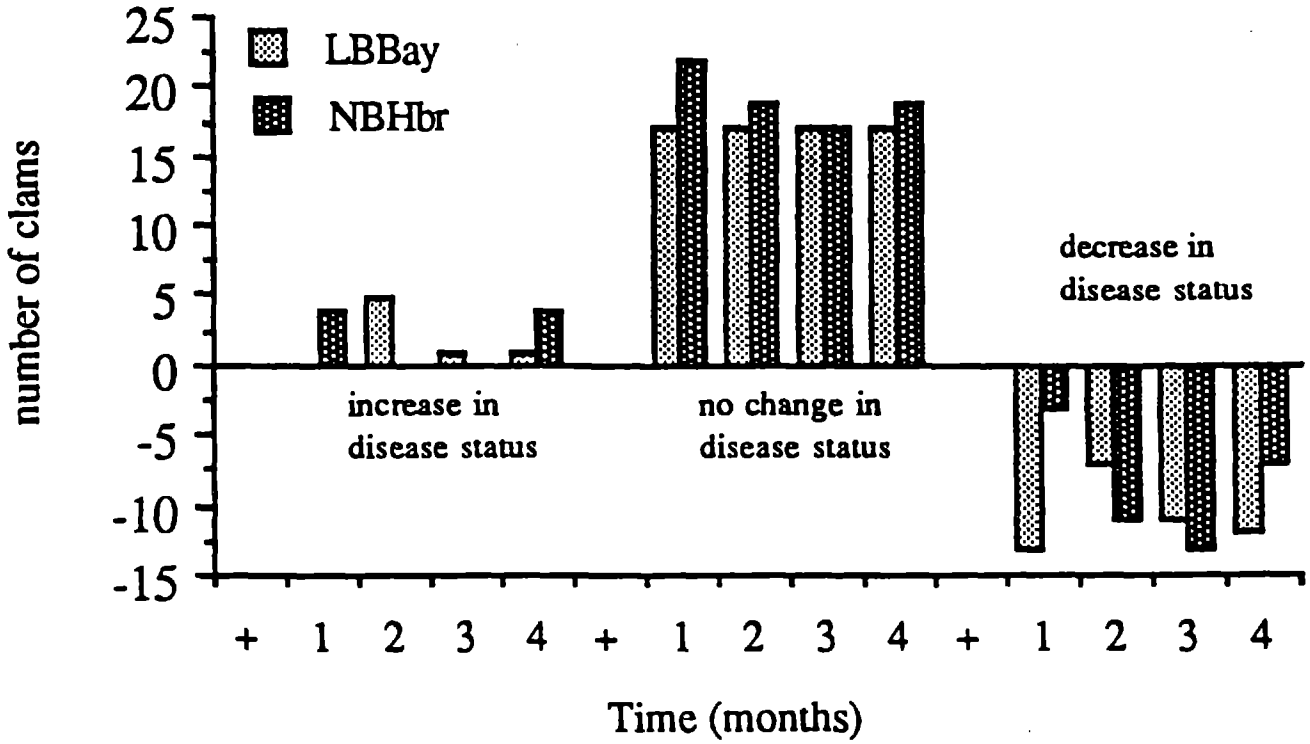

Figure 6. Change in disease status of softshells, *Mya arenaria*, initially diagnosed with hematopoietic neoplasia and deployed in sediment from Little Buttermilk Bay (LBBay) or New Bedford Harbor (NBHbr)

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EXOGENOUS VIRUSES AS POSSIBLE INITIATORS OF NEOPLASMS IN AQUATIC INVERTEBRATES

C.A. Farley[27]

The first virus (Iridoviridae) from a marine invertebrate was described from the octopus in 1971 (Rungger et al. 1980). Since that time, viruses have been found in at least five invertebrate phyla, with up to 14 families of viruses represented (Farley 1981; Adams and Bonami 1991). Generally, five families of viruses (Papovaviridae, Adenoviridae, Herpetoviridae, Poxviridae, and Retroviridae) have been implicated in oncogenic activity in animals. All are characterized by genomes with double-stranded DNA, with the exception of the Retroviridae which has a single-stranded RNA genome that is enzymatically convertible to single-stranded DNA via reverse transcriptase. Gross (1983) published the first volume on oncogenic viruses of vertebrates in 1961, which was later expanded into a two-volume work.

The first virus to be implicated as an oncogenic virus was isolated from chicken tumors by Ellerman and Bang (1908), and was used to transmit the neoplasm. Earlier, however, a virus was implicated in rabbit myxomatosis in 1898 by G. Sonarelli (in Gross 1983). Much of the earlier work involved chickens and mice, and was primarily concerned with retrovirus-induced leukemias and sarcomas. Other viruses implicated in oncogenesis were papova (rabbit papilloma, warts, polyoma tumors in mice, and SV40 in hamsters), experimental adenovirus neoplasms in hamsters, and herpesvirus neoplasms, e.g., Lucké virus in frogs, Marek's disease in chickens, and Burkitt's lymphoma in humans. Numerous isolations of various retroviruses have been made from tumors in such domestic animals as cats, dogs, cows, and pigs, and even from leukemias in humans (Gross 1983). The most recent isolation is, of course, HIV and Kaposi's sarcoma.

In marine invertebrates, four groups of viruses with oncogenic potential have been identified. Adenovirus-type infections were described in *Hydra* (Bonnefoy et al. 1972) and flatworms (Reuter 1975); neither was implicated in neoplastic processes. Papova-type infections have been described from eastern oysters, *Crassostrea virginica*, (Farley 1976) and softshells, *Mya arenaria* (Koepp 1984). The former caused viral gametocyte hypertrophy, and the latter was possibly associated with gill dysplasia (Farley 1978). Herpesvirus-like virions were described as lytic hemocyte infections in eastern oysters (Farley et al. 1972) and European oysters (Alderman from Farley 1978). Virus-like particles resembling retrovirus were seen budding from plasma membranes of digestive gland epithelial cells of an eastern oyster (Farley 1978). None of the above-mentioned viruses were clearly implicated in a neoplastic process; however, the papovavirus associated with proliferative gill epithelia in softshells could be oncogenic in its manifestation. Oprandy et al. (1981) claimed isolation of a viral agent from sarcomatous softshells, but the characterization was inconclusive from an ultrastructural standpoint, and no one has been able to repeat these results.

Numerous experimental studies have demonstrated that softshell sarcomas can be transplanted into healthy softshells using viable sarcoma cells (Appeldoorn et al. 1984; Farley 1989; McLaughlin et al. 1992). McLaughlin et al. attempted cell-free transmission in the same experiment, but were unable to transmit the disease. Sunila and Farley (1989) showed that sarcoma cells could survive at least 6 hr in a variety of conditions in seawater, and suggested the possibility that disease is transmitted from animal to animal by natural transplantation of sarcoma cells.

At least six species and a total of more than 10 major sites worldwide have now been implicated in epizootic bivalve neoplasia, including at least four different types of neoplasms (Peters 1988). There are two reports that indicate there may be a statistical relationship between neoplasia and exposure to organic carcinogenic compounds in blue mussels, *Mytilus edulis*, and softshells from polluted environments (Mix et al. 1979; Farley et al. 1991). However, none of these compounds were tested or experimentally shown to produce neoplasms. To date, only Gardner and Yevich (1986) have been successful in producing a malignant neoplasm in eastern oysters. This was done under laboratory conditions using oysters exposed to heavily contaminated sediment from Black Rock Harbor, Connecticut. Unfortunately, while this exposure material contained many known carcinogens, chemical-specific, cause-and-effect relationships were not determined.

Most epizootic neoplasms in bivalve mollusks have several common features: (1) increased genome size due to polyploidy/aneuploidy, and enlarged hyperchromatic nuclei in neoplastic cells; (2) anaplastic-neoplastic cells being undifferentiated and usually not easily identified or related back to tissue of origin; (3) diffuse infiltration of tissues with neoplastic cells, especially hemolymph spaces (sinuses); and (4) seasonal peaks of neoplasia prevalence generally from 10 to <70%.

There is some evidence that neoplastic disease has been transferred geographically (possibly by introduction of affected animals). This transfer is demonstrated by recent observation of this disease in Chesapeake Bay softshells which historically were free of the disease prior to 1979 (Farley et al. 1986). All other cases of epizootic neoplasia were present when first examined.

In summary, most mollusk neoplasias cannot be related to environmental carcinogens; however, some neoplasias appear to be the result of infectious processes, suggesting that either an infectious agent such as one of the oncogenic viruses is responsible, or that a heritable change has occurred in the genome of

neoplastic cells followed by a natural transplantation phenomenon. This mechanism could explain transmission of neoplasia with high prevalences. Perhaps either or both mechanisms are operating. I hope that we have new insights about this question as a result of this workshop.

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SCREENING TUMORS FROM BIVALVES FOR HOMOLOGS TO HUMAN CANCER GENES

C.W. Walker[28]

INTRODUCTION

Malignant neoplasms (i.e., cancers) have been recognized in bivalves since the 1960s, and their prevalence at >1% has been recorded for commercially valuable shellfish from both coasts of the United States (Farley and Sparks 1970). On the East Coast, deterioration and, ultimately, death of populations of the softshell, *Mya arenaria*, result in costly problems for aquaculturists and shellfishermen. Because one softshell can infect another, introduced softshells may have a large effect on local commercial fisheries. Also, since softshells filter water and their tissues undoubtedly sequester mutagenic toxins from the environment, human consumption of bivalves from environmentally compromised populations would deliver highly concentrated doses of potentially dangerous mutagens to consumers.

We will attempt to demonstrate that similar genes [i.e., the oncogenes *c-fos*, ornithine decarboxylase (ODC), and *c-myc*] are involved in carcinogenesis in humans and softshells (and possibly other shellfish). If this is so, such genes may be similarly affected by mutagens present in the local environment. It seems desirable to be in a position to inform aquaculturists so that they can choose to avoid using softshells from compromised populations as seed stocks. Based on the molecular probes that we construct, we should be able to discriminate between individual softshells containing proto-oncogenic and oncogenic forms of these important cell-cycle genes.

RATIONALE

Increasingly, publications such as *Diseases of Aquatic Organisms, Journal of Invertebrate Pathology*, and *Marine Pollution Bulletin* document results of burgeoning oceanic pollution on the health of marine organisms. Disease is especially devastating both locally and nationally when marine species we use as food are compromised (Sparks 1972; Ahmed 1991). In an article on blood and gonadal "tumors" in marine bivalve mollusks, Peters (1988) suggests that proto-oncogenes may be similar between vertebrates and commercially valuable marine bivalves. When mutated to form oncogenes, some proto-oncogenes are directly responsible for certain kinds of cancer in vertebrates. For example, proto-oncogenes such as *c-fos*, ODC, and *c-myc* produce gene products essential to normal cell division; mutated or dysfunctional forms of these genes, called oncogenes, produce abnormally high levels of, or abnormally acting, gene products that lead to uncontrolled cell division. Peters further suggests that hypothetical invertebrate proto-oncogenes may be modified to form oncogenes following exposure to mutagens, carcinogens, or viral oncogenes found in coastal effluents. While these are challenging ideas, no molecular data exist to demonstrate unequivocally that any invertebrates experience vertebrate-like cancers based on expression of similar genes.

The softshell is harvested commercially along the northeastern United States. Annually, the fishery is worth tens-of-millions of dollars. Blood (i.e., leukemic) and gonadal tumors have been reported throughout the range of the softshell, principally in populations that have been compromised by PCBs or other hydrocarbons (Yevich and Barszcz 1976, 1977). In affected individuals, increased numbers of morphologically altered cells circulate in the hemolymph. These neoplastic cells are round and undergo constant cell division; unlike normal cells, they will not attach in vitro. Theses cells also contain granules and have unique antigenic properties (Miosky et al. 1989; Smolowitz et al. 1989). As normal tissues are invaded by neoplastic cells, stunted growth of individuals and, ultimately, reduced reproductive output and fatality of entire populations of softshells result (Brousseau and Baglivo 1991).

Because virtually no nucleotide sequence data are available for cell-cycle genes in any marine invertebrate, it has previously been impossible to employ modern molecular techniques in screening normal or tumorous tissues of any invertebrate to determine if its cancers are indeed similar to those of vertebrates. Nonetheless, detailed comparisons of the nucleotide sequences of genes responsible for so-called invertebrate "tumor/cancers" with their vertebrate counterparts should be possible by using appropriately designed molecular probes to highly conserved regions of human genes. We have already successfully employed such a strategy in cloning and characterizing the first non-vertebrate *c-myc* proto-oncogene from testes of the sea star *Asterias vulgaris*, a marine invertebrate common in the Gulf of Maine (Walker et al. 1992). Practical use of these probes (or related peptides to similar regions of their protein products) should ultimately permit development of kits for screening tissues of softshells and other commercially valuable marine species for proto-oncogenes and oncogenes.

Our present studies are aimed at: (1) demonstrating the likely existence of homologs to authentic vertebrate proto-oncogenes and related oncogenes in normal and presumed "cancer/tumors" of blood and gonads of the softshell; and (2) using probes we develop to consider the construction of kits for screening softshell populations for normal or modified versions of these genes. The information that we gather about these genes and the probes that we develop will be useful for softshells and many other species of commercially valuable shellfish which are challenged by oceanic pollution and similarly develop genetically related diseases [e.g., eastern oyster, *Crassostrea virginica*

(Harshbarger et al. 1977), northern quahog, *Mercenaria mercenaria* (Barry and Yevich 1972; Hesselman et al. 1988), and blue mussel, *Mytilus edulis* (Lowe and Moore 1978)].

REVIEW OF PREVIOUS WORK

In somatic cells, advancement into and through successive events of the mitotic cell cycle are highly regulated by the cytoplasmic environment in which nuclei find themselves. For example, upon incorporation into S-phase cytoplasm of the same species, donor G1 nuclei promptly duplicate DNA and histone proteins mimicking host nuclei. Furthermore, G1 nuclei from one species can be introduced into S-phase cytoplasm of quite different species (e.g., chicken mouse and mouse frog) with the same result. MPF is one cytoplasmic constituent active early in the cell cycle (Lee and Nurse 1987). MPF will cause mitotic cell cycling either when microinjected or introduced by cell fusion across vastly divergent species lines (Figure 7). Together, the serine/threonine kinase, p34, and cyclin produce the MPF heterodimer. In all eukaryotes from yeasts to humans, p34 contains a stretch of 16 amino acids (**EGVPSTAIREISLLKE**) (abbreviated **PSTAIR**). Microinjection of the **PSTAIR** peptide is sufficient to induce meiotic maturation in softshell oocytes, and accelerates the action of microinjected MPF in *Xenopus*.

Other genes involved in production of unique cell-cycle-specific cytoplasmic constituents should also contain highly conserved nucleotide sequences across a broad phylogenetic range. Our preliminary observations show that the *c-fos*, ODC, and *c-myc* gene products do contain long stretches of perfectly conserved amino acid sequences (i.e., *c-fos* – 16 amino acids, ODC – 20 amino acids, and *c-myc* – 28 amino acids) across a broad phylogenetic range which can be used in constructing probes which detect homologs for these genes in softshell RNA and DNA.

In somatic cells, sequential expression of both *c-fos* and *c-myc* is correlated with initiation of the mitotic cell cycle; *c-myc* expression follows that of *c-fos*. Expression of ODC leads to production of polyamines which induce expression of *c-myc*. The assumption behind this study is that all eukaryotes possess a similar group of genes involved in promoting one of the most basic and highly conserved processes characteristic of eukaryotic cellular life–mitotic cell division. Currently, with the exception of *A. vulgaris c-myc* (Walker et al. 1992), these genes have not been detected in any invertebrates. Based on our results with *A. vulgaris c-myc*, we believe this is primarily because no one has yet looked for them using: (1) probes to highly conserved functional sites in each gene, and (2) tissues in which mRNAs for the appropriate genes should be maximally present.

We have already successfully used a complementary oligonucleotide probe in identifying the *c-myc* proto-oncogene in *A. vulgaris*. Sequencing this gene has demonstrated approximately 30% amino acid identity and 46% overall conservation to human *c-myc* (Figure 8). Regions of substantially higher conservation (57-95%) correspond to transcriptional activation (boxes A, B, and C of Figure 8), casein kinase II phosphorylation, basic DNA-binding, nuclear targeting, and oligomerization domains in the second and third exons of human *c-myc*. *A. vulgaris c-myc* cDNA detects a 2.7-kb transcript in northern blots of monthly samples of testicular tissue from field populations, indicating peak expression during active spermatogenesis.

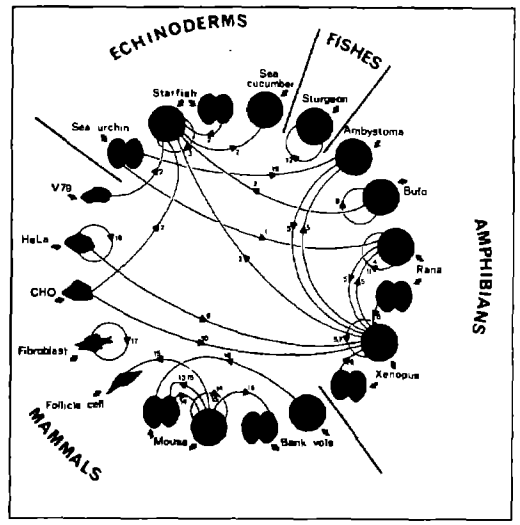

Figure 7. Effective stimulation of the cell cycle across wide species boundaries when maturation-promoting factor is delivered by microinjection or cellular fusion

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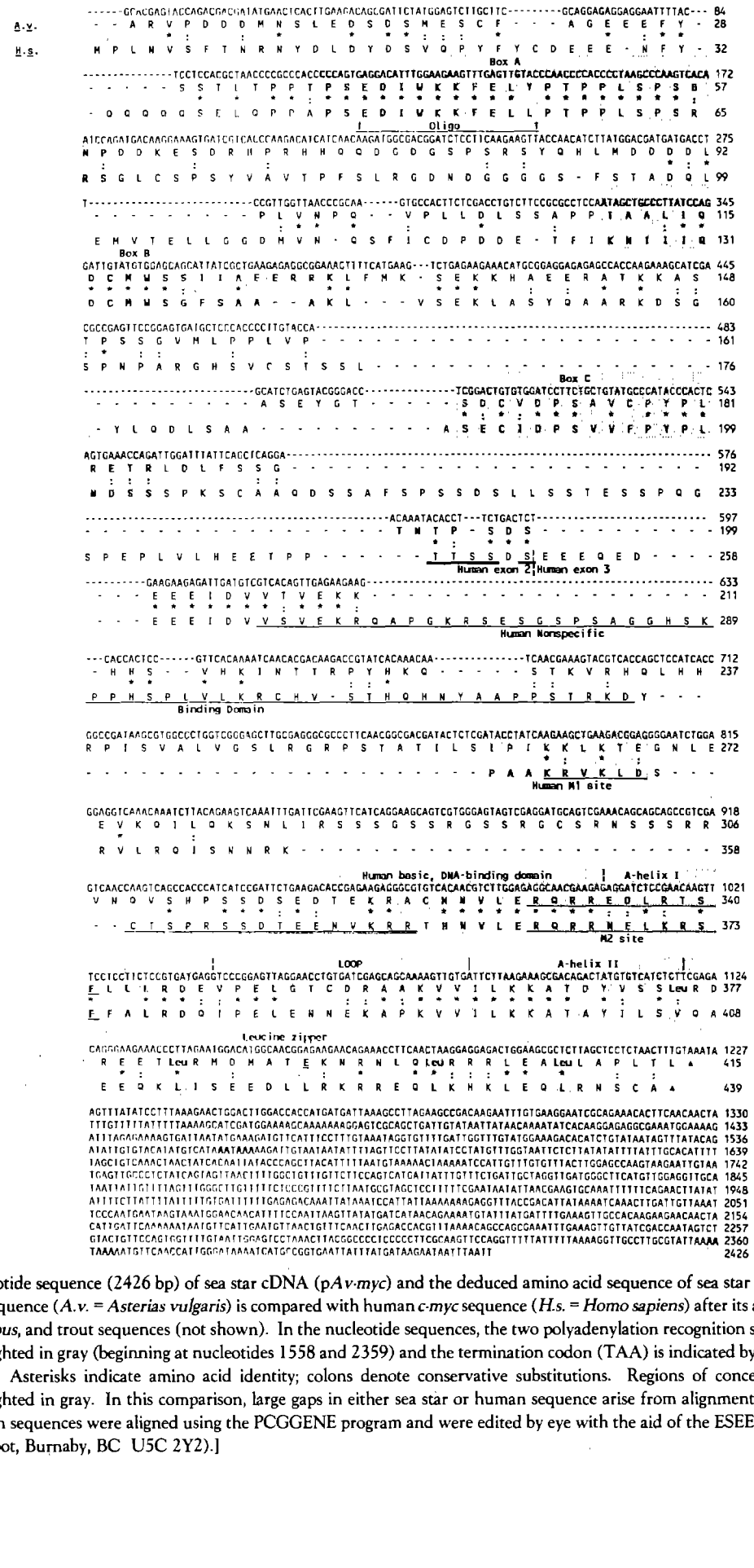

Figure 8. Nucleotide sequence (2426 bp) of sea star cDNA (p*Av-myc*) and the deduced amino acid sequence of sea star *c-myc* protein [The sea star sequence (*A.v.* = *Asterias vulgaris*) is compared with human *c-myc* sequence (*H.s.* = *Homo sapiens*) after its alignment with chicken, *Xenopus*, and trout sequences (not shown). In the nucleotide sequences, the two polyadenylation recognition sites (5'-AATAA-3') are highlighted in gray (beginning at nucleotides 1558 and 2359) and the termination codon (TAA) is indicated by a triangle at nucleotide 1221. Asterisks indicate amino acid identity; colons denote conservative substitutions. Regions of concentration homology are highlighted in gray. In this comparison, large gaps in either sea star or human sequence arise from alignments with other organisms. Protein sequences were aligned using the PCGGENE program and were edited by eye with the aid of the ESEE program (version 1.05, E. Cabot, Burnaby, BC U5C 2Y2).]

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